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MADQASQANG NQDVIEGELI VSVEPKNGKP DVSISQASQSV TGQEDVLKSK GFTIVDTLHV
EDSSVKPLVS KNFNKKVVEK MGLVYLVQYS PDQFKSSNIA KKELRKELMD LGEKVRYISE
NYKMAIEGT ATLQAVHPNQ NWHYNMIKAP EAWAITSGSS SVKVAVLDTG IDHNHPSLAN
FVNTNLGRSF VGGNTMDVQG HGTHVAGTIA SYGSVSGVMQ SATLIPVKVL DNSGSGSLYG
ITQGILYAAD IDADVINMSL GGGGYNQSMQ EAVQTAVAQG TIVVAATGND GASSISYPAA
YSSVIAVGSV TSNRTRSSFS NYGSGLEVMA PGSNIYSTYP NSRYTSLSGT SMATPHVAGV
AGLIRAANPD ISVAQARQIL RDTAQEAGSF TQYGYGIVDA HAAVVAASGG SGGGTTTPPP
SSTDVTTVTS TNSYYYRGE TVYMSTVKD SNGVAISNAT VNFKITRPNG TSVTATAATN
SSGVATYSIG TNYSTATGTY RVDATASKSG YTTSSASTTF RVRGQTGHHH HHH

FIG. 1

(57) Abstract: The present disclosure provides engineered protease polypeptides, recombinant polynucleotides encoding the engineered protease polypeptides, and uses of the engineered protease polypeptides in therapeutic applications.



ENGINEERED PROTEASE POLYPEPTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application 63/505,055, filed May 30, 2023, which is incorporated by reference herein.

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0002] The Sequence Listing concurrently submitted herewith as file name CX7-253WO2_ST26.xml, created on May 30, 2024, with a file size of 4,736,576 bytes, is hereby incorporated by reference herein in its entirety.

TECHNICAL FIELD

[0003] The present disclosure relates to engineered protease polypeptides, compositions thereof, polynucleotides encoding the engineered protease polypeptides, and uses of the engineered polypeptides and recombinant polynucleotides in therapeutic and other applications.

BACKGROUND

[0004] Pancreatic exocrine insufficiency (PEI) or exocrine pancreatic insufficiency (EPI) is a condition in which the pancreas does not supply a sufficient amount of digestive enzymes needed to digest food efficiently. Following passage of food through the stomach, ingested food is converted into acidic chyme that flows in the duodenum of the small intestine, which receives, among others, pancreatic enzymes (e.g., lipase, amylase, and protease) that break down the food for absorption in the small intestine. Poor digestion of food in PEI/EPI can lead to malabsorption of fats, proteins, carbohydrates, and vitamins by the intestines, which can lead to malnutrition, changes in bone density, and increased risk of mortality. The reduction in pancreatic enzymes may arise from inadequate pancreatic stimulation of pancreatic secretion, insufficient secretion of pancreatic digestive enzymes by the pancreatic acinar cells, or outflow obstruction of the pancreatic duct, and inadequate mixing of the pancreatic enzymes with food.

[0005] PEI/EPI is often associated with pancreatitis, cystic fibrosis, celiac disease, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, and pancreatic cancer, all of which can lead to decreased secretion of pancreatic enzymes into the duodenum. An approved treatment for PEI/EPI is pancreatic replacement therapy (PERT), which is an orally administered cocktail of digestive enzymes amylase, lipase, and protease, mostly derived from porcine origin (e.g., Creon™, Zenpep™, Pertzye™, and Pancreaz™). However, PERT treatment may not alleviate the condition in some people due to insufficient activity of the PERT enzymes in the gastrointestinal tract and/or insufficient patient compliance with the therapy due to the significant pill burden associated with current treatment protocols. In some cases, the coefficient of fat absorption (CFA) and/or coefficient of nitrogen

absorption (CNA) is inferior to that of healthy patients, resulting in weight loss and other health concerns. Thus, a need remains in the art for improved PERT treatments.

SUMMARY

[0006] The present disclosure provides engineered protease polypeptides, recombinant polynucleotides encoding the engineered protease polypeptides, and uses of the engineered protease polypeptides for degrading target proteins and polypeptides. As provided in detail herein, in some embodiments, the protease polypeptides have been engineered to exhibit an improved property compared to the naturally occurring protease, including among others, enhanced expression, increased proteolytic activity of the active protease, increased thermostability, increased resistance against gastric proteases, increased activity at acidic pH, and increased stability at acidic pH.

[0007] In some embodiments, the present disclosure provides an engineered protease polypeptide, or a biologically active fragment thereof, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or to a reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0008] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or to the reference sequence corresponding to SEQ ID NO: 4 or 628, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0009] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0010] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%,

93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0011] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 11, 31, 42, 45, 50, 53, 84, 99, 100, 126, 128, 129, 130, 131, 132, 134, 135, 136, 137, 138, 139, 140, 141, 143, 145, 151, 154, 156, 157, 159, 160, 161, 162, 163, 169, 172, 173, 174, 179, 180, 184, 185, 186, 187, 188, 190, 191, 192, 193, 194, 198, 199, 212, 214, 220, 221, 222, 223, 225, 231, 232, 233, 235, 237, 238, 239, 240, 242, 243, 245, 246, 249, 250, 251, 252, 253, 254, 256, 258, 262, 263, 264, 265, 266, 267, 268, 269, 271, 273, 274, 275, 277, 278, 279, 280, 281, 283, 285, 290, 292, 293, 294, 296, 297, 300, 302, 303, 311, 312, 313, 314, 315, 316, 318, 324, 328, 336, 339, 341, 342, 343, 345, 346, 355, 358, 360, 364, 367, 368, 369, 370, 371, 372, 373, 374, 375, 377, 381, 382, 384, 386, 389, 391, 392, 401, 402, 405, 406, 409, 410, 411, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0012] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 11K, 31G, 42W, 45Y, 50R, 53A, 84M, 99V, 100V, 126T, 128G/I/K/L/P/R/S/T/V, 129E/F/H/I/K/L/R/S/T/V, 130A/F/G/N/V, 131E/P/R/T/V/Y, 132A/C/D/E/G/P/R/V/Y, 134A/C/D/E/G/I/L/M/N/P/S/T/V/W/Y, 135C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 136C/G/I/M, 137A/D/N/S, 138Q, 139C/D/E/F/H/I/K/L/M/R/S, 140L, 141A/C/D/E/F/G/H/I/L/M/Q/R/S/T/V/W/Y, 143A/C/D/N/Q/S/T, 145A/C/D/E/F/G/H/I/K/L/P/Q/R/S/T/V/W, 151D/Q, 154C/D/L/R, 156C/V, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W, 159G, 160A/C/D/E/F/K/L/M/N/P/R/Q/T/V/W/Y, 161D/E/G/L/R, 162I, 163H/L, 169S, 172Q, 173F/S, 174L, 179K/S, 180H/L/M, 184A/D/G/L/M/Q/R, 185A/D/E/F/G/L/M/P/Q/R/S/T/V, 186A/R/S/T/Y, 187A, 188A/C/D/F/G/L/M/S/T/W, 190S, 191R, 192C/D/M/N, 193T, 194A/D/L/T, 198G, 199C/K/L, 212S, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 220K/L/R, 221A/C/D/E/F/G/H/I/K/L/M/P/Q/R/T/V/W/Y, 222G, 223S, 225V, 231H/V, 232S, 233G/I/L, 235Q/R/V, 237A/G, 238Q, 239L/M, 240A/L, 242E/S, 243E/L/M/R/S/T, 245L/V, 246I/V, 249G/M/S, 250A/C/F/L/N/T, 251D/S, 252P, 253C/I/V, 254C/E, 256L/M, 258W, 262A/S, 263E/H/P/Q/R/S, 264A/C/F/I/L/N/P/R/T/V, 265C/G/R, 266H/T/Y, 267A/G/H/I/L/M/R/S/T/V/W, 268A/F/G/H/I/N/P/Q/T/V/Y, 269Q/T, 271A, 273A/C/F/L/M/S/T, 274A/G/K/L/T/V/W, 275A/V, 277D/G, 278L/N/S/V/Y, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 280D/K/S/T, 281C/V, 283M, 285S,

290E/G/S, 292V, 293A, 294V/W, 296M/R, 297F, 300R/V, 302G/P, 303A/V, 311A/E/D/G/K/M/Q/S, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 313A/Q/S/T, 314G, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/V/W/Y, 316K, 318N/P/R, 324A/D/E/I/R/V/W/Y, 328L/M, 336F, 339S/W, 341G, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/T/V/W/Y, 343S, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/T/V/W/Y, 355A, 358S, 360S, 364A/V, 367V, 368G/T, 369I/V/W, 370C/E/F/G/I/K/L/P/Q/R/S/V, 371L, 372A/C/F/L/R/V/Y, 373A/C/E/F/M/S/Y, 374E/G/L/R/S/W/Y, 375A/E/I/L/M/S/T/V, 377H, 381N, 382G/R/S/T, 384C, 386P/W, 389C/P, 391L/S, 392Y, 401L, 402G/*, 405L/Q, 406C/M/R/W, 409E/R/*, 410C/I/W/*, 411L/R/T/V, 412P/T/*, or 413A/C/D/S/*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0013] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 157, 160, 214, 268, 273, 279, 311, 312, 315, 328, 342, 345, 346, or 372, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0014] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 137A/D/N/S, 139C/D/E/F/H/I/K/L/M/R/S, 141A/C/D/E/F/G/H/I/L/M/Q/R/S/T/V/W/Y, 143A/C/D/N/Q/S/T, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W, 160A/C/D/E/F/K/L/M/N/P/R/Q/T/V/W/Y, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 268A/F/G/H/I/N/P/Q/T/V/Y, 273A/C/F/L/M/S/T, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 311A/E/D/G/K/M/Q/S, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/V/W/Y, 328L/M, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/T/V/W/Y, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/T/V/W/Y, or 372A/C/F/L/R/V/Y, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0015] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set at amino acid positions 135/141/160/311/315/372, 143/328/342/345, 139/157/268/273/312/346, or 137/139/214/279, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0016] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 185, 134, 129, 135, 184, 132, 186, 193, 263, 370, 45/134, 199, 368, 161, 141, 267, 179, 264, 160, 138, 131, 372, 151, 274, 128, 339,

313, 374, 314, 191, 324, 315, 375, 136, 220, 194, 231, 277, 369, 251, 180, 163, 343, 264/279, 279, 232, 141/300, 367, 266, 188, 130, 318, 265, 341, 190, 145, 126/192, 11/220, 192, 370/392, 99/278, 265/311, 84/159/265/279/311/370, 311/316, 342/370, 265/311/370, 192/311/316, 141/154/192, 265/311/316/342, 279/311/316, 141/265/279/311/342, 141/192/311/316/370, 141/265/311, 198/279, 392, 342/370/392, 141/198/265, 265/392, 184/267, 342, 312, 100/251, 141/220, 311/316/370, 99, 278, 405, 311/342/370, 141/198, 311/342, 141/311, 279/311/377/392, 186/198/311/342/370/392, 141/392, 311/370/392, 141/311/392, 311/370, 311/316/392, 265/311/392, 141/192, 311, 141/265/311/392, 192/311/370/392, 198/265/311/316/370, 141/186/265/311, or 141/198/265/311/370, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0017] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 145, 145, 157, 160, 214, 221, 268, 273, 279, 311, 312, 315, 315, 342, 345, 346, 402, 409, 410, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0018] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 242, 157, 250, 373, 243, 336, 187, 240, 280, 271, 237, 386, 382, 328, 42, 391, 381, 275, 249, 239, 384, 139, 364, 346, 389, 254, 246, 345, 360, 303, 300, 269, 135/141/372, 311/315/372, 136/141/311, 141/188, 135/136, 135/141/315, 372, 135/141/160/267/372, 135/136/141/160/185/188/267/311/315, 160/185, 135/141/188/279/311, 135/136/141, 135/136/141/372, 135/141/160/185/267/279, 135/141/160/267, 141/188/311/372, 160/185/188/279/311, 136/141/279, 135/136/141/160/185/188, 141/372, 135/136/141/311, 185/311/315/372, 135/141/188, 136/185, 135/141, 135/136/141/279/315/372, 135/311/315, 141, 311/372, 188/311, 135/141/188/372, 141/160/279, 313/392, 342/392, 279/392, 128, 198/342, 313, 128/312, 50, 145/263, 313/342, 279/312, 312/392, 279/342, 128/342, 342, 263, 143, 262, 156, 169, 143/237, 136/160/185/267/311/372, 135/160/311/372, 135/141/311/315, 141/311/315, 136/141/160/185/188/311/315/372, 135/141/311/315/372, 135/141/160/185/311/315, 135/141/267/311/315/372, 135/136/141/160/311/315, 135/136/141/279, 135/141/267/279/311/315, 135/141/160, 135/141/160/311/315/372, 135/141/160/311/315, 135/136/141/188/311, 141/160/311, 135/141/160/279/311/315/372, 141/160/185/279/311/372, 135/136/141/160/315/372, 135/136/160/279/311/372, 128/279/312/342, 128/198/312/342, 263/342, 145/263/279/312/342/392, or 128/145/198/312/313/392, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0019] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution as set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0020] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set of an engineered set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0021] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to an amino acid sequence comprising at least a substitution or substitution set of an engineered protease polypeptide variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0022] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0023] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242.

[0024] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence

corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0025] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0026] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 11, 31, 42, 45, 50, 53, 84, 99, 100, 126, 128, 129, 130, 131, 132, 134, 135, 136, 137, 138, 139, 140, 141, 143, 145, 151, 154, 156, 157, 159, 160, 161, 162, 163, 169, 172, 173, 174, 179, 180, 184, 185, 186, 187, 188, 190, 191, 192, 193, 194, 198, 199, 212, 214, 220, 221, 222, 223, 225, 231, 232, 233, 235, 237, 238, 239, 240, 242, 243, 245, 246, 249, 250, 251, 252, 253, 254, 256, 258, 262, 263, 264, 265, 266, 267, 268, 269, 271, 273, 274, 275, 277, 278, 279, 280, 281, 283, 285, 290, 292, 293, 294, 296, 297, 300, 302, 303, 311, 312, 313, 314, 315, 316, 318, 324, 328, 336, 339, 341, 342, 343, 345, 346, 355, 358, 360, 364, 367, 368, 369, 370, 371, 372, 373, 374, 375, 377, 381, 382, 384, 386, 389, 391, 392, 401, 402, 405, 406, 409, 410, 411, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0027] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 11K, 31G, 42W, 45Y, 50R, 53A, 84M, 99V, 100V, 126T, 128G/I/K/L/P/R/S/T/V, 129E/F/H/I/K/L/R/S/T/V, 130A/F/G/N/V, 131E/P/R/T/V/Y, 132A/C/D/E/G/P/R/V/Y, 134A/C/D/E/G/I/L/M/N/P/S/T/V/W/Y, 135A/C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 136C/G/I/M, 137A/D/N/S, 138Q, 139C/D/E/F/H/I/K/L/M/N/R/S, 140L, 141A/C/D/E/F/G/H/I/L/M/N/Q/R/S/T/V/W/Y, 143A/C/D/H/N/Q/S/T, 145A/C/D/E/F/G/H/I/K/L/P/Q/R/S/T/V/W, 151D/Q, 154C/D/L/R, 156C/V, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/S/T/V/W, 159G, 160A/C/D/E/F/K/L/M/N/P/R/Q/S/T/V/W/Y, 161D/E/G/L/R, 162I, 163H/L, 169S, 172Q, 173F/S, 174L, 179K/S, 180H/L/M, 184A/D/G/L/M/Q/R, 185A/D/E/F/G/L/M/P/Q/R/S/T/V, 186A/R/S/T/Y, 187A, 188A/C/D/F/G/L/M/S/T/W, 190S, 191R, 192C/D/M/N, 193T, 194A/D/L/T, 198G, 199C/K/L, 212S, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 220K/L/R, 221A/C/D/E/F/G/H/I/K/L/M/P/Q/R/T/V/W/Y, 222G, 223S, 225V, 231H/V, 232S, 233G/I/L, 235Q/R/V, 237A/G, 238Q, 239L/M, 240A/L, 242E/S, 243E/L/M/R/S/T, 245L/V, 246I/V, 249G/M/S.

250A/C/F/L/N/T, 251D/S/T, 252P, 253C/I/V, 254C/E, 256L/M, 258W, 262A/S, 263E/H/P/Q/R/S, 264A/C/F/I/L/N/P/R/T/V, 265C/G/R, 266H/T/Y, 267A/G/H/I/L/M/R/S/T/V/W, 268A/F/G/H/I/N/P/Q/S/T/V/Y, 269Q/T, 271A, 273A/C/F/L/M/S/T/V, 274A/G/K/L/T/V/W, 275A/V, 277D/G, 278L/N/S/V/Y, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 280D/K/S/T, 281C/V, 283M, 285S, 290E/G/S, 292V, 293A, 294V/W, 296M/R, 297F, 300R/V, 302G/P, 303A/V, 311A/E/D/G/K/M/Q/S/T, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/S/T/V/W/Y, 313A/Q/S/T, 314G, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/T/V/W/Y, 316K, 318N/P/R, 324A/D/E/I/R/V/W/Y, 328L/M/V, 336F, 339S/W, 341G, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/S/T/V/W/Y, 343S, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/S/T/V/W/Y, 355A, 358S, 360S, 364A/V, 367V, 368G/T, 369I/V/W, 370C/E/F/G/I/K/L/P/Q/R/S/V, 371L, 372A/C/F/L/R/S/V/Y, 373A/C/E/F/M/S/Y, 374E/G/L/R/S/W/Y, 375A/E/I/L/M/S/T/V, 377H, 381N, 382G/R/S/T, 384C, 386P/W, 389C/P, 391L/S, 392Y, 401L, 402G/*, 405L/Q, 406C/M/R/W, 409E/R/*, 410C/I/W/*, 411L/R/T/V, 412P/T/*, or 413A/C/D/S/*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0028] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 157, 160, 214, 268, 273, 279, 311, 312, 315, 328, 342, 345, 346, or 372, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0029] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135A/C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 137A/D/N/S, 139C/D/E/F/H/I/K/L/M/N/R/S, 141A/C/D/E/F/G/H/I/L/M/N/Q/R/S/T/V/W/Y, 143A/C/D/H/N/Q/S/T, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/S/T/V/W, 160A/C/D/E/F/K/L/M/N/P/R/Q/S/T/V/W/Y, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 268A/F/G/H/I/N/P/Q/S/T/V/Y, 273A/C/F/L/M/S/T/V, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 311A/E/D/G/K/M/Q/S/T, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/S/T/V/W/Y, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/T/V/W/Y, 328L/M/V, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/S/T/V/W/Y, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/S/T/V/W/Y, or 372A/C/F/L/R/S/V/Y, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0030] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%,

93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or to the reference sequence corresponding to SEQ ID NO: 948, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0031] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 950-1154, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 950-1154, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0032] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 411, 402, 285, 245, 266, 355, 258, 222, 140, 268, 225, 283, 406, 410, 143/145/243/312, 139/143/145/157/312, 139/157/345, 139/269, 156/157/342/346, 139/143, 269, 139/243/328, 269/328, 143/145/169, 328, 143/145/262, 145/262/312/328, 139/156/157, 139/145/312, 312, 139, 139/312, 139/156, 139/143/145/243, 145/157, 145/346, 145/262/312/328/345/346, 145/262, 312/342, 143/243, 139/345, 342, 143/145/262/342, 139/143/169, 139/143/145/312, 169, 139/145/262/312/328/342/345/346, 139/328, 139/243, 139/143/328, 139/143/243, 139/145, 145/312, 145/169, 139/143/157/312, 84/139/143, 145/269, 143/145/157/269/312/328, 143/145/269, 157, 139/143/312, 256, 273, 409, 172, 401, 281, 253, 143/145/243/328, 145, 139/143/145/328/342/345, 143/328/342/345, 145/342/345, 143, 139/145/328/342/345, 143/145/169/312/328/345/346, 143/243/328/342/345/346, 139/143/157/169/328/346, 143/145/156/312/328, 139/145/157/312/328, 143/328/342/345/346, 143/145, 143/145/312/342/345, or 143/145/328, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0033] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or to the reference sequence corresponding to SEQ ID NO: 1126, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0034] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1156-1422, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1156-1422, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0035] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 279, 250, 154, 214, 249, 275, 137, 161, 180, 174, 139, 254, 145, 278, 136, 154/413, 294, 237, 274, 264, 185, 277, 293, 233, 173, 312, 302, 238, 135, 221, 290, 263, 267, 239, 163, 292, 246, 243, 235, 156, 223, 278/413, 297, 194, 251, 253/411, 145/157/253/268/273/281/312/346/411, 139/346, 253, 346/411, 253/346, 312/346, 273/312, 253/281, 157/253/273/312/346/411, 139/157/253/268/273/281/312/346, 253/273/411, 139/253/268/273/281, 139/157/411, 157, 273, 139/253/268/273/281/312/411, 157/253/411, 139/145/253/346, 139/157/253/273/312, 139/157/268/273/312/346, 157/273/312/346, 139/411, 139/253/268/273/281/312/346/411, 157/273/346/411, 139/145/157/162/253/273/281/312, 139/253/273/281/312, 157/253/268/273/281/312, 139/253/268, 139/157/312, 253/273/281/346, 157/253/312/346/411, 157/273/312/346/411, 139/145/157/253/268/281/312, 139/273/312/346, 157/253/268/273/312/346, 139/268/346, 268/273/312/346, 139/157/253/268/273/312, 139/157/253, 139/253/281, 139/157/253/268/273, 253/312/411, or 139/268/273, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0036] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or to the reference sequence corresponding to SEQ ID NO: 1368, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0037] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1424-1608, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1424-1608, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence

corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0038] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 31, 318, 296, 252, 303, 253, 413, 386, 312, 235, 412, 342, 302, 371, 405, 389, 391, 358, 139/273/311/328/372, 311, 372, 139/143/157/160/268/273/311/315, 143, 139/143, 139/160/312/372, 143/273/328, 346, 135/139/160/268/312/342/346, 139/141/273, 135/141/143/268/273/312/372, 139/141/143/311, 139/157/268/328/346/372, 53/139/141/143/273/372, 139, 139/141/143/273/312, 137/139/221/233/413, 233, 221/279, 137/139/233/279, 221, 139/214, 137/139/156, 139/214/221, 214/233, 137/139/221/233/279, 137/139/221, 137/139, 137/139/279, 137, 137/139/214/279, 266, 139/221, 137/221, 137/156/214/312, 137/221/233, 137/139/156/221, 137/139/214, 279, 137/139/233, 137/139/214/233, 221/413, 137/214/233, 137/156, 137/139/221/233, 214/221, 137/221/413, 214, 137/233, 137/413, 137/221/279, 137/139/156/214/233/413, or 137/139/156/214, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0039] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or to the reference sequence corresponding to SEQ ID NO: 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0040] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1610-2242, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1610-2242, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0041] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 145/273/372, 145/256/273, 221/243/273/328/372, 372, 145/221/279/372/406, 273/328, 145/169/273/346/406, 256/273, 145/221/273/328/346/406, 243/273, 145/214/256/273/279/328/372, 169/221/328/372/406, 372/406, 169/328/372/406, 221/372, 145/221/273/328/372, 214/243/273/328, 145/221, 214/256/273/346/372, 221/406, 169/372, 145/214/221/273, 145/221/346/372, 243/273/328/372/406, 145/169/273/328/346, 328,

169/273/372, 145/221/328, 169/214/273, 221/273/328, 221, 145/328, 214/346, 312, 212, 279, 212/312, 145, 179/346, 214, 346, 315/372, 375, 264, 179, 185, 220/372, or 324, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0042] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0043] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set of an engineered protease polypeptide variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0044] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to an amino acid sequence comprising at least a substitution or substitution set of an engineered protease polypeptide variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0045] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence comprising residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or an amino acid sequence comprising an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242.

[0046] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence comprising residues 135-413 of SEQ ID NO: 628, 948, 1126, 1368, 1548, 1640, or 1710, or an amino acid sequence comprising SEQ ID NO: 628, 948, 1126, 1368, 1548, 1640, or 1710.

[0047] In some embodiments, the engineered protease polypeptide is capable of converting to a proteolytically active polypeptide or is an active protease. In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises amino acid residues 135-413 or amino acid residues 128-413, wherein the engineered protease polypeptide is proteolytically active or is an active protease.

[0048] In some embodiments, the proteolytic active polypeptide or active protease of an engineered protease polypeptide is characterized by an improved property selected from: i) increased protease activity, ii) increased resistance to pepsin, iii) increased stability and/or activity at acidic pH, iv) increased stability and/or activity at neutral pH, or v) increased thermostability, or any combination of i), ii), iii), iv), and v) as compared to a reference protease. In some embodiments, the reference protease has an amino acid sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or an amino acid sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548. In some embodiments, the reference protease has an amino acid sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or an amino acid sequence corresponding to SEQ ID NO: 4, 628.

[0049] In some embodiments, the engineered protease polypeptide comprises at least a carboxy terminal deletion of SEQ ID NO: 2, wherein the deletion maintains protease activity of the mature form of SEQ ID NO: 2 with the carboxy terminal deletion. In some embodiments, the carboxy terminal deletion comprise deletion of the Big1 domain. In some embodiments, the carboxy terminal deletion is up to and including amino acid residue 426, or up to and including amino acid residue 414 of SEQ ID NO: 2. In some embodiments, the engineered protease polypeptide further comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 amino acid deletions of the carboxy terminus at amino acid residue 413 of SEQ ID NO: 2, wherein the further amino acid deletion(s) maintains proteolytic activity of the mature form of SEQ ID NO: 2 having the further amino acid deletions. In some embodiments, the engineered protease polypeptide is the mature form having an amino terminus at amino acid residue 128 or 135 of SEQ ID NO: 2.

[0050] In another aspect, the present disclosure provides a recombinant polynucleotide comprising a polynucleotide sequence encoding an engineered protease polypeptide described herein.

[0051] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 403 to 1239 of SEQ ID NO: 3, 627, 947, 1125, 1367, or 1547, or to a reference polynucleotide corresponding to SEQ ID NO: 3, 627, 947, 1125, 1367, or 1547, wherein the recombinant polynucleotide encodes an engineered protease polypeptide.

[0052] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 403 to 1239 of an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-2241, or to a reference polynucleotide corresponding to an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-2241, wherein the recombinant polynucleotide encodes an engineered protease polypeptide.

[0053] In some embodiments, the polynucleotide sequence of the recombinant polynucleotide encoding an engineered protease polypeptide is codon optimized. In some embodiments, the polynucleotide sequence is codon optimized for expression in a bacterial cell, fungal cell, insect cell, or mammalian cell.

[0054] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence comprising nucleotide residues 403-1239 of an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-1709, or comprising an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-1709.

[0055] In another aspect, the present disclosure further provides an expression vector comprising a recombinant polynucleotide encoding an engineered protease polypeptide described herein. In some embodiments, the expression vector further comprises a control sequence operably linked to the recombinant polynucleotide. In some embodiments, the control sequence comprises at least a promoter, particularly a heterologous promoter.

[0056] In another aspect, the present disclosure provides a host cell comprising an expression vector comprising a recombinant polynucleotide encoding an engineered protease polypeptide. In some embodiments, the host cell is a bacterial cell, fungal cell, insect cell, or mammalian cell.

[0057] In a further aspect, also provided is a method of producing an engineered protease polypeptide, comprising culturing a host cell described herein under suitable conditions such that the encoded engineered protease is expressed or produced. In some embodiments, the method further comprises isolating the expressed or produced engineered protease polypeptide from culture medium and/or cells. In some embodiments, the method further comprises purifying the expressed or produced engineered protease polypeptide.

[0058] In some embodiments, provided herein is a method of preparing a proteolytically active protease polypeptide, comprising incubating an engineered protease polypeptide described herein under suitable conditions such that the proteolytically active protease polypeptide or active protease is produced. In some embodiments, the proteolytically active protease polypeptide or active protease has an amino terminus at amino acid residue 128 or 135, wherein the amino acid positions are numbered with respect to SEQ ID NO: 4, or equivalent positions thereof for any engineered protease polypeptide variant.

[0059] In some embodiments, the suitable conditions for preparing a proteolytically active protease polypeptide is sufficient for activation of the engineered protease polypeptide. In some embodiments, the method for preparing a proteolytically active protease comprises incubating the engineered protease polypeptide under suitable conditions for autoproteolysis. In some embodiments, the method for preparing a proteolytically active protease comprises contacting the engineered protease polypeptide with a proteolytically active polypeptide or an active protease of an engineered protease polypeptide described herein.

[0060] In another aspect, the engineered protease polypeptide is provided as a composition. In some embodiments, the composition comprises an engineered protease polypeptide and a protein-containing

food or drink. In some embodiments, the composition comprises an engineered protease polypeptide admixed with a protein-containing food or drink.

[0061] In some embodiments, the engineered protease polypeptide is provided as a pharmaceutical composition. In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable excipient and/or carrier. In some embodiments, the pharmaceutical composition comprises an effective amount of the engineered protease for treating exocrine pancreatic insufficiency.

[0062] In some embodiments, the engineered protease polypeptide is used in the treatment of a condition or disease associated with a deficiency in pancreatic digestive enzymes. In some embodiments, a method of treating a disease or condition associated with a deficiency in pancreatic enzymes, comprises administering to a subject in need thereof an effective amount of an engineered protease polypeptide described herein or a pharmaceutical composition thereof. In some embodiments, the disease or condition associated with a deficiency in pancreatic digestive enzymes is exocrine pancreatic insufficiency.

[0063] In some embodiments, the engineered protease polypeptide or pharmaceutical composition thereof is administered immediately prior to, concurrently with, or subsequent to consumption of a protein-containing food or drink.

[0064] In some embodiments, the subject for treatment with an engineered protease polypeptide is a human infant or child. In some embodiments, the subject for treatment with an engineered protease polypeptide is a human adult.

[0065] In some embodiments, an engineered protease polypeptide is used for treating exocrine pancreatic insufficiency.

[0066] In some embodiments, an engineered protease polypeptide is used in the preparation of a medicament for treating exocrine pancreatic insufficiency.

BRIEF DESCRIPTION OF THE DRAWINGS

[0067] **FIG. 1** shows the full length naturally occurring amino acid sequence of the protease pro-polypeptide from *Bacillus sinesaloumensis* Marseille P3516 with a His-tag sequence (*italicized*) at the carboxy terminus. The polypeptide is composed of the pro-domain, which is within residues 1 to 127; a protease domain, which is within residues 128 to 413; and a bacterial Ig-like domain 1 (Big1-1), which is within residues 440 to 522.

[0068] **FIG. 2** shows the naturally occurring amino acid sequence of the pro-polypeptide of the protease from *Bacillus sinesaloumensis* Marseille P3516 (SEQ ID NO: 4). The pro-polypeptide is converted to an active protease by cleavage between amino acid residues 127/128 and/or 134/135 in the illustrated sequence.

DETAILED DESCRIPTION

[0069] The present disclosure provides engineered protease polypeptide, a proteolytically active engineered protease polypeptide, recombinant polynucleotides encoding the engineered protease polypeptides, and use of the engineered protease polypeptides. In some embodiments, the protease polypeptide is engineered to have advantageous properties compared to the naturally occurring protease, including among others, enhanced expression, increased proteolytic activity of the active protease, increased thermostability, increased resistance against other proteases, increased activity at acidic pH, and increased stability at acidic pH. In some embodiments, the enhanced properties of the engineered protease make is useful as a therapeutic in the treatment of exocrine pancreatic insufficiency, e.g., as enzyme replacement therapy (ERT).

Abbreviations and Definitions

[0070] In reference to the present disclosure, the technical and scientific terms used in the descriptions herein will have the meanings commonly understood by one of ordinary skill in the art, unless specifically defined otherwise.

[0071] Furthermore, the headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the application as a whole.

[0072] It is to be understood that the invention herein is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art. Accordingly, the terms defined immediately below are more fully described by reference to the application as a whole.

[0073] As used herein, the singular “a”, “an,” and “the” include the plural references, unless the context clearly indicates otherwise.

[0074] As used herein, the term “comprising” and its cognates are used in their inclusive sense (i.e., equivalent to the term “including” and its corresponding cognates).

[0075] It is to be further understood that where description of embodiments use the term “comprising” and its cognates, the embodiments can also be described using language “consisting essentially of” or “consisting of.”

[0076] Numeric ranges are inclusive of the numbers defining the range. Thus, every numerical range disclosed herein is intended to encompass every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein. It is also intended that every maximum (or minimum) numerical limitation disclosed herein includes every lower (or higher) numerical limitation, as if such lower (or higher) numerical limitations were expressly written herein.

[0077] “About” as used herein means an acceptable error for a particular value. In some instances, “about” means within 0.05%, 0.5%, 1.0%, or 2.0%, of a given value range. In some instances, “about” means within 1, 2, 3, or 4 standard deviations of a given value.

[0078] “EC” number refers to the Enzyme Nomenclature of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). The IUBMB biochemical classification is a numerical classification system for enzymes based on the chemical reactions they catalyze.

[0079] “ATCC” refers to the American Type Culture Collection whose biorepository collection includes genes and strains.

[0080] “NCBI” refers to National Center for Biological Information and the sequence databases provided therein.

[0081] “Polynucleotide” is used herein to denote a polymer comprising at least two nucleotides where the nucleotides are either deoxyribonucleotides or ribonucleotides. The abbreviations used for the genetically encoding nucleosides are conventional and are as follows: adenosine (A); guanosine (G); cytidine (C); thymidine (T); and uridine (U). Unless specifically delineated, the abbreviated nucleosides may be either ribonucleosides or 2'-deoxyribonucleosides. The nucleosides may be specified as being either ribonucleosides or 2'-deoxyribonucleosides on an individual basis or on an aggregate basis. When nucleic acid sequences are presented as a string of one-letter abbreviations, the sequences are presented in the 5' to 3' direction in accordance with common convention, and the phosphates are not indicated.

[0082] “Protein,” “polypeptide,” and “peptide” are used interchangeably to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). Unless indicated otherwise, amino acid sequences are written left to right in amino to carboxy orientation.

[0083] “Amino acids” are referred to herein by either their commonly known three-letter symbols or by the one-letter symbols recommended by IUPAC-IUB Biochemical Nomenclature Commission. The abbreviations used for the genetically encoded amino acids are conventional and are as follows: alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartate (Asp or D), cysteine (Cys or C), glutamate (Glu or E), glutamine (Gln or Q), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and valine (Val or V). When the three-letter abbreviations are used, unless specifically preceded by an “L” or a “D” or clear from the context in which the abbreviation is used, the amino acid may be in either the L- or D-configuration about α -carbon ($C\alpha$). For example, whereas “Ala” designates alanine without specifying the configuration about the α carbon, “D-Ala” and “L-Ala” designate D-alanine and L-alanine, respectively. When the one-letter abbreviations are used, upper case letters designate amino acids in the

L-configuration about the α -carbon and lower case letters designate amino acids in the D-configuration about the α -carbon. For example, “A” designates L-alanine and “a” designates D-alanine. When polypeptide sequences are presented as a string of one-letter or three-letter abbreviations (or mixtures thereof), the sequences are presented in the amino (N) to carboxy (C) direction in accordance with common convention.

[0084] “Fusion protein” or “fusion polypeptide” refer to hybrid proteins created through the joining of two or more genes or polynucleotides that originally encoded separate proteins. In some embodiments, fusion proteins and fusion polypeptides are created by recombinant technology (e.g., molecular biology techniques known in the art).

[0085] “Protease,” “proteinase,” and “peptidase” refer to enzymes that hydrolyze proteins, polypeptides, and/or oligopeptides. Proteases, proteinases, and peptidases breakdown proteins, polypeptides, or oligonucleotides into smaller peptides or single amino acids.

[0086] “Proteolysis” or “proteolytic activity” refers to the breakdown (e.g., through hydrolysis) of proteins and/or polypeptides into smaller peptides and/or amino acids.

[0087] “Auto-proteolysis” refers to the self breakdown of the subject protein, for example the breakdown of proteases through their own action on their structures.

[0088] “Lipase” refers to any enzyme commonly referred to as “lipase” that catalyzes the hydrolysis of fats by hydrolyzing the ester bonds of triglycerides. Pancreatic lipases are important in the breakdown of fats to fatty acids, glycerol, and other alcohols. Lipases are essential in the digestion, transport, and processing of dietary lipids in most organisms.

[0089] “Lipid” refers to a class of water-insoluble macromolecules that include fatty acids and their esters, sterols, prenols, certain poorly soluble vitamins, and other related compounds. “Fats” are a subset of lipids composed of fatty acid esters (e.g., triglycerides, which are made from glycerol and three fatty acids). It is not intended that the present invention be limited to any specific lipid and/or fat. Taking the context into consideration, the terms “fat” and “lipid” are used interchangeably herein.

[0090] “Amylase” refers to an enzyme that is capable of hydrolyzing glycosidic bonds in starch to converting it to smaller polysaccharides, such as disaccharides (e.g., maltose) and trisaccharides, or simple sugars, such as glucose.

[0091] “Mature protein” or “mature polypeptide” refers to the final processed biological protein or polypeptide or product.

[0092] “Pro-protein,” “pro-polypeptide,” or “pro-peptide” refers to a precursor protein, polypeptide, or peptide that is processed by post-translational modification, to form a biologically active protein, polypeptide, or peptide. In some embodiments, the post translational modification is a cleavage reaction

to form the protein, polypeptide, or peptide. “Pro-enzyme” refers to a precursor polypeptide that is processed by post-translational modification, in particular a cleavage reaction, to form an active enzyme.

[0093] “Pre-pro-protein,” “pre-pro-polypeptide,” or “pre-pro-peptide” refers to a precursor protein, polypeptide, or peptide that includes a signal sequence and which can be processed by posttranslational modification, in particular a cleavage reaction, to generate a pro-protein, pro-polypeptide, or pro-peptide. Generally, a cleavage reaction removes a signal sequence to generate a pro-protein, pro-polypeptide, or pro-peptide. “Pre-pro-enzyme” refers to a precursor protein, polypeptide, or peptide that is processed by post-translational modification, in particular a cleavage reaction that removes a signal sequence, to form a pro-enzyme.

[0094] “Full-length” in context of a protein or polypeptide refers to the protein or polypeptide which is not processed to alter the amino acid sequence of the entire protein or polypeptide. For example, a full-length protein is the entire protein encoded in the corresponding mRNA.

[0095] “Engineered,” “recombinant,” “non-naturally occurring,” and “variant,” when used with reference to a cell, a polynucleotide or a polypeptide refers to a material or a material corresponding to the natural or native form of the material that has been modified in a manner that would not otherwise exist in nature or is identical thereto but produced or derived from synthetic materials and/or by manipulation using recombinant techniques.

[0096] “Wild-type” and “naturally-occurring” refer to the form found in nature. For example, a wild-type polypeptide or polynucleotide sequence is a sequence present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation.

[0097] “Coding sequence” refers to that part of a nucleic acid (e.g., a gene) that encodes an amino acid sequence of a protein.

[0098] “Percent (%) sequence identity” is used herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence for optimal alignment of the two sequences. The percentage may be calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by determining the number of positions at which either the identical nucleic acid base or amino acid residue occurs in both sequences or a nucleic acid base or amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art

appreciate that there are many established algorithms available to align two sequences. Optimal alignment of sequences for comparison can be conducted (e.g., by the local homology algorithm of Smith and Waterman; Smith and Waterman, *Adv. Appl. Math.*, 1981, 2:482), by the homology alignment algorithm of Needleman and Wunsch (Needleman and Wunsch, *J. Mol. Biol.*, 1970, 48:443), by the search for similarity method of Pearson and Lipman (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA.*, 1988, 85:2444), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection, as known in the art. Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity include, but are not limited to the BLAST and BLAST 2.0 algorithms (see, e.g., Altschul et al., *J. Mol. Biol.*, 1990, 215:403-410; and Altschul et al., *Nucleic Acids Res.*, 1977, 25:3389-3402). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length “W” in the query sequence, which either match or satisfy some positive-valued threshold score “T,” when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (See, Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters “M” (reward score for a pair of matching residues; always >0) and “N” (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity “X” from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see, e.g., Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA*, 1989, 89:10915). Exemplary determination of sequence alignment and % sequence identity can employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using default parameters provided.

[0099] “Reference sequence” refers to a defined sequence used as a basis for a sequence comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, at least 100 residues in length or the full-length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (i.e., a portion of the complete sequence) that is similar between the two sequences,

and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptide are typically performed by comparing sequences of the two polynucleotides or polypeptides over a “comparison window” to identify and compare local regions of sequence similarity. In some embodiments, a “reference sequence” can be based on a primary amino acid sequence, where the reference sequence is a sequence that can have one or more changes in the primary sequence.

[0100] “Comparison window” refers to a conceptual segment of contiguous nucleotide positions or amino acids residues wherein a sequence may be compared to a reference sequence. In some embodiments, the comparison window is at least 15 to 20 contiguous nucleotides or amino acids and wherein the portion of the sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. In some embodiments, the comparison window can be longer than 15-20 contiguous residues, and includes, optionally 30, 40, 50, 100, or longer windows.

[0101] “Corresponding to,” “reference to,” and “relative to” when used in the context of the numbering of a given amino acid or polynucleotide sequence refer to the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence. In other words, the residue number or residue position of a given polymer is designated with respect to the reference sequence rather than by the actual numerical position of the residue within the given amino acid or polynucleotide sequence. For example, a given amino acid sequence, such as that of an engineered protease polypeptide, can be aligned to a reference sequence by introducing gaps to optimize residue matches between the two sequences. In these cases, although the gaps are present, the numbering of the residue in the given amino acid or polynucleotide sequence is made with respect to the reference sequence to which it has been aligned.

[0102] “Mutation” refers to any change in a polypeptide or polynucleotide sequence. It is intended to encompass any number (i.e., one or more) of substitutions, insertions, deletions, and/or rearrangements present in a sequence (i.e., as compared to the starting or reference sequence). Thus, mutations in polynucleotide sequences can result in the production of variant polypeptides (e.g., variant or engineered proteases), as provided herein. In some embodiments, where the reference sequence is a subsequence of a longer sequence, amino acid positions and corresponding mutations (e.g., substitutions) or mutation sets within the subsequence are selected.

[0103] “Amino acid difference” and “residue difference” refer to a difference in the amino acid residue at a position of a polypeptide sequence relative to the amino acid residue at a corresponding position in a reference sequence. The positions of amino acid differences generally are referred to herein as “X_n,” where n refers to the corresponding position in the reference sequence upon which the residue difference is based. For example, a “residue difference at position X135 as compared to SEQ ID NO: 4” (or a

“residue difference at position 135 as compared to SEQ ID NO: 4”) refers to a difference of the amino acid residue at the polypeptide position corresponding to position 135 of SEQ ID NO: 4. Thus, if the reference polypeptide of SEQ ID NO: 4 has an alanine at position 135, then a “residue difference at position X135 as compared to SEQ ID NO: 4” refers to an amino acid substitution with any residue other than alanine at the position of the polypeptide corresponding to position 135 of SEQ ID NO: 4. In some instances herein, the specific amino acid residue difference at a position is indicated as “XnY” where “Xn” specifies the corresponding residue and position of the reference polypeptide (as described above), and “Y” is the single letter identifier of the amino acid found in the engineered or recombinant polypeptide (i.e., the different residue than in the reference polypeptide). In some embodiments, the amino acid difference, e.g., a substitution, is denoted by the abbreviation “nY,” without the identifier for the residue in the reference sequence. In some embodiments, the phrase “an amino acid residue nY” denotes the presence of the amino residue in the engineered or recombinant polypeptide, which may or may not be a substitution in context of a reference sequence. In some embodiments, the “substitution” comprises the deletion of an amino acid, which can be denoted by “-”, or a replacement with a termination codon, which can be denoted by “*.”

[0104] In some instances, a polypeptide of the present disclosure can include one or more amino acid residue differences relative to a reference sequence, which is indicated by a list of the specified positions where residue differences are present relative to the reference sequence. In some embodiments, where more than one amino acid can be used in a specific residue position of a polypeptide, the various amino acid residues that can be used are separated by a “/” (e.g., X151D/X151Q, X151D/Q, or 151D/Q).

[0105] “Amino acid substitution set” and “substitution set” refers to a group of amino acid substitutions within a polypeptide sequence. In some embodiments, substitution sets comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions. In some embodiments, a substitution set refers to the set of amino acid substitutions that is present in any of the variant protease polypeptides listed in any of the Tables in the Examples. In these substitution sets, the individual substitutions are separated by a semicolon (“;”; e.g., A126T;G192C) or slash (“/”; e.g., A126T/G192C or 126T/192C). In some embodiments, the phrase “mutation set” can be used.

[0106] “Conservative amino acid substitution” refers to a substitution of a residue with a different residue having a similar side chain, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. By way of example and not limitation, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid (e.g., alanine, valine, leucine, and isoleucine); an amino acid with hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain (e.g., serine and threonine); an amino acids having aromatic side chains is substituted with another amino acid having an aromatic side chain (e.g., phenylalanine, tyrosine, tryptophan, and histidine); an amino acid with a basic side chain is substituted with another amino acid with a basis side chain (e.g., lysine and arginine); an amino acid with

an acidic side chain is substituted with another amino acid with an acidic side chain (e.g., aspartic acid or glutamic acid); and/or a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively.

[0107] “Non-conservative substitution” refers to substitution of an amino acid in the polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affects (a) the structure of the peptide backbone in the area of the substitution (e.g., proline for glycine) (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example and not limitation, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[0108] “Deletion” refers to modification to the polypeptide by removal of one or more amino acids from the reference polypeptide. Deletions can comprise removal of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, or up to 20% of the total number of amino acids making up the reference enzyme while retaining enzymatic activity and/or retaining the properties of a protease polypeptide. Deletions can be directed to the internal portions and/or terminal portions of the polypeptide. In various embodiments, the deletion can comprise a continuous segment or can be discontinuous.

[0109] “Insertion” refers to modification to the polypeptide by addition of one or more amino acids from the reference polypeptide. Insertions can be in the internal portions of the polypeptide, or to the carboxy or amino terminus. Insertions as used herein include fusion proteins as is known in the art. The insertion can be a contiguous segment of amino acids or separated by one or more of the amino acids in the naturally occurring polypeptide.

[0110] “Functional fragment” and “biologically active fragment” are used interchangeably herein, to refer to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion(s) and/or internal deletions, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence to which it is being compared (e.g., a full-length engineered protease polypeptide) and that retains substantially all of the activity of the full-length polypeptide.

[0111] “Isolated polypeptide” refers to a polypeptide which is substantially separated from other contaminants that naturally accompany it (e.g., protein, lipids, and polynucleotides). The term embraces polypeptides which have been removed or purified from their naturally-occurring environment or expression system (e.g., host cell or in vitro synthesis). The engineered protease polypeptides may be present within a cell, present in the cellular medium, or prepared in various forms, such as lysates or

isolated preparations. As such, in some embodiments, the engineered protease polypeptides provided herein are isolated polypeptides.

[0112] “Substantially pure polypeptide” or “purified polypeptide” refers to a composition in which the polypeptide species is the predominant species present (i.e., on a molar or weight basis it is more abundant than any other individual macromolecular species in the composition), and is generally a substantially purified composition when the object species comprises at least about 50 percent of the macromolecular species present by mole or % weight. Generally, a substantially pure protease polypeptide composition will comprise about 60% or more, about 70% or more, about 80% or more, about 90% or more, about 95% or more, and about 98% or more of all macromolecular species by mole or % weight present in the composition. In some embodiments, the object species is purified to essential homogeneity (i.e., contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species. In some embodiments, the isolated engineered protease polypeptides are substantially pure polypeptide compositions.

[0113] “Improved enzyme property” and “improved property” refers to a property of an engineered protease polypeptide which comprises an improvement in any enzyme property as compared to a reference protease polypeptide and/or as a wild-type protease polypeptide or another engineered protease polypeptide. Improved properties include but are not limited to such properties as increased protein expression, increased thermostability, increased pH activity, increased stability, increased enzymatic activity, increased substrate specificity or affinity, increased chemical stability, improved solvent stability, increased tolerance to acidic or basic pH, increased tolerance to protease activity (i.e., reduced sensitivity to proteolysis), reduced aggregation, increased solubility, and altered temperature profile.

[0114] “Increased enzymatic activity” or “enhanced catalytic activity” refers to an improved property of the engineered protease polypeptides, that can be represented by an increase in specific activity (e.g., product produced/time/weight protein) or an increase in percent conversion of the substrate to the product (e.g., percent conversion of starting amount of substrate to product in a specified time period using a specified amount of protease) as compared to the reference protease enzyme. Exemplary methods to determine enzyme activity are provided in the Examples. Any property relating to enzyme activity may be affected, including the classical enzyme properties of K_m , V_{max} or k_{cat} , changes of which can lead to increased enzymatic activity. Improvements in enzyme activity can be from about 1.1 fold the enzymatic activity of the corresponding wild-type enzyme, to as much as 2-fold, 5-fold, 10-fold, 20-fold, 25-fold, 50-fold, 75-fold, 100-fold, 150-fold, 200-fold or more enzymatic activity than the naturally occurring protease or another engineered protease from which the protease polypeptides were derived.

[0115] “Protease stable” and “stability to proteolysis” refer to the ability of a protein (e.g., an engineered protease of the present invention) to function and withstand proteolysis mediated by any proteolytic

enzyme or other proteolytic compound or factor and retain its function following treatment with the protease. It is not intended that the term be limited to the use of any particular protease to assess the stability of a protein. In some embodiments, the engineered proteases are stable in the presence of a gastric protease.

[0116] “pH stability” refers to the ability of a protein (e.g., an engineered protease of the present invention) to function after incubation at a particular pH. In some embodiments, the present disclosure provides engineered proteases that are stable at a range of pHs, including, acid, neutral, and/or basic pH. In some embodiments, the engineered proteases are stable at different pH ranges, as indicated in the Examples provided herein.

[0117] “Physiological pH” refers to the pH range generally found in a subject’s (e.g., human) blood (e.g., pH 7.2 – 7.4).

[0118] “Basic pH” (e.g., used with reference to improved stability to basic pH conditions or increased tolerance to basic pH) means a pH range of >7 , for example $>\text{pH } 7$ to 11 , or in some embodiments, greater than pH 11 .

[0119] “Acidic pH” (e.g., used with reference to improved stability to acidic pH conditions or increased tolerance to acidic pH) means a pH range that encompasses any pH values < 7 . In some embodiments, the acid pH is less than 7 , while in some other embodiments, the pH is less than about 6 , 5 , 4 , 3 , 2 , or lower. In some alternative embodiments, the engineered proteases of the present disclosure are stable at pH levels of 2 to 4 .

[0120] “Improved tolerance to acidic pH” means that an engineered protease according to the invention will have increased stability (higher retained activity at $< \text{pH } 7$, e.g., 6 , 5 , 4 , 3 , 2 , or even lower, after exposure to the acidic pH for a specified period of time (e.g., 1 hour, up to 24 hours, etc.) as compared to a reference protease or another enzyme.

[0121] “Improved tolerance to basic pH” means that an engineered protease according to the invention will have increased stability (higher retained activity at about $\text{pH } >7$, e.g., 8 , or 9 , or even higher, after exposure to basic pH for a specified period of time, e.g., 1 hour, up to 24 hours, etc., as compared to a reference protease or another enzyme.

[0122] “Gastric challenge” refers to the exposure of the engineered proteases of the present invention to a low pH environment and the presence of at least one gastric enzyme, such as a protease (e.g., pepsin), such that the recombinant protease is exposed to the conditions that may be encountered in the stomach (e.g., the human stomach).

[0123] “Thermal stability” and “thermostability” refer to the ability of a protein (e.g., an engineered protease of the present invention) to function at a particular temperature. In some embodiments, the term refers to the ability of a protein to function following incubation at a particular temperature. In some

embodiments, the engineered proteases of the present invention are “thermotolerant” (i.e., the enzymes maintain their catalytic activity at elevated temperatures). In some embodiments, the engineered proteases resist inactivation at elevated temperatures and in some embodiments, maintain catalytic activity at elevated temperatures for prolonged exposure times. In some embodiments, thermal stability is measured following incubation of a protein (e.g., an engineered protease of the present invention) at a particular temperature.

[0124] “Suitable reaction conditions” refers to those conditions in the enzymatic conversion reaction solution (e.g., ranges of enzyme loading, substrate loading, temperature, pH, buffers, co-solvents, etc.) under which a protease polypeptide of the present application is capable of converting a substrate to the desired product compound. Exemplary “suitable reaction conditions” are provided in the present application and illustrated by the Examples.

[0125] “Codon optimized” refers to changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is more efficiently expressed in that organism. Although the genetic code is degenerate, in that most amino acids are represented by several codons, called “synonyms” or “synonymous” codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding regions of an organism's genome. In some embodiments, the polynucleotides encoding the protease polypeptide are codon optimized for optimal production from the host organism selected for expression.

[0126] “Control sequence” refers herein to include all components that are necessary or advantageous for the expression of a polynucleotide and/or polypeptide of the present disclosure. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, leaders, polyadenylation sequences, pro-peptide sequences, promoter sequences, signal peptide sequences, initiation sequences, and transcription terminators. In some embodiments, at a minimum, the control sequences include a promoter, and transcriptional and translational stop signals.

[0127] “Operably linked” is defined herein as a configuration in which a control sequence is appropriately placed (i.e., in a functional relationship) at a position relative to a polynucleotide of interest such that the control sequence directs or regulates the expression of the polynucleotide and/or encoded polypeptide of interest.

[0128] “Heterologous” or “recombinant” refers to the relationship between two or more nucleic acid or polypeptide sequences (e.g., a promoter sequence, signal peptide, terminator sequence, etc.) that are derived from different sources and are not associated in nature.

[0129] “Promoter sequence” refers to a nucleic acid sequence that is recognized by a host cell for expression of a polynucleotide of interest, such as a coding sequence. The promoter sequence contains transcriptional control sequences, which mediate the expression of a polynucleotide of interest. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0130] “Vector” refers to a polynucleotide construct for introducing a polynucleotide sequence into a cell. In some embodiments, the vector is an expression vector that is operably linked to a suitable control sequence capable of effecting the expression in a suitable host of the polypeptide encoded in the polynucleotide sequence. In some embodiments, an “expression vector” has a promoter sequence operably linked to the polynucleotide sequence (e.g., transgene) to drive expression in a host cell, and in some embodiments, also comprises a transcription terminator sequence.

[0131] “Culturing” refers to the growing of a population of cells, such as host cells, under suitable conditions using any suitable medium (e.g., liquid, gel, or solid). In some embodiments, the cells are microbial cells (e.g., bacteria), while in some other embodiments, the cells are mammalian cells, insect cells, or cells obtained from another animal. It is not intended that the present invention be limited to culturing of any particular cells or cell types or any specific method of culturing.

[0132] “Expression” includes any step involved in the expression of a polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, and post-translational modification. In some embodiments, the term also encompasses secretion of the polypeptide from a cell. “Produces” refers to the expression of proteins and/or other compounds by cells.

[0133] “Host cell” and “host strain” refer to suitable hosts for expression vectors comprising polynucleotides provided herein (e.g., a polynucleotide sequences encoding at least one protease polypeptide). In some embodiments, the host cells are prokaryotic or eukaryotic cells that have been transformed or transfected with vectors constructed using recombinant techniques as known in the art.

[0134] “Hybridization stringency” relates to hybridization conditions, such as washing conditions, in the hybridization of nucleic acids. Generally, hybridization reactions are performed under conditions of lower stringency, followed by washes of varying but higher stringency. The term “moderately stringent hybridization” refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, about 85% identity to the target DNA, with greater than about 90% identity to target-polynucleotide. Exemplary moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42 °C, followed by washing in 0.2×SSPE, 0.2% SDS, at 42 °C. “High stringency hybridization” refers generally to conditions that are about 10 °C or less from the thermal melting temperature T_m as determined under the solution condition for a defined polynucleotide sequence. In some embodiments, a

high stringency condition refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65 °C (i.e., if a hybrid is not stable in 0.018M NaCl at 65 °C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in conditions equivalent to 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42 °C, followed by washing in 0.1×SSPE, and 0.1% SDS at 65 °C. Another high stringency condition is hybridizing in conditions equivalent to hybridizing in 5X SSC containing 0.1% (w:v) SDS at 65 °C and washing in 0.1x SSC containing 0.1% SDS at 65 °C. Other high stringency hybridization conditions, as well as moderately stringent conditions, are described in the references cited above.

[0135] “Composition” and “formulation” encompass products comprising at least one engineered protease of the present invention, intended for any suitable use (e.g., pharmaceutical compositions, dietary and/or nutritional supplements, etc.).

[0136] “Pharmaceutical composition” refers to a composition suitable for pharmaceutical use in a subject (e.g., human).

[0137] “Pharmaceutically acceptable” means a material that can be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the components in which it is contained and that possesses the desired biological activity.

[0138] “Excipient” refers to any pharmaceutically acceptable additive, carrier, diluent, adjuvant, or other ingredient, other than the active pharmaceutical ingredient. Excipients are typically included for formulation and/or administration purposes.

[0139] “Carrier” when used in reference to a pharmaceutical composition means any of the standard pharmaceutical carrier, buffers, and excipients, such as stabilizers, preservatives, and adjuvants.

[0140] “Administration” and “administering” a composition mean providing a composition of the present invention to a subject, such as a patient.

[0141] “Concurrent administration,” or “co-treatment,” as used herein includes administration of the agents together, or before or after each other.

[0142] “Effective amount” means an amount sufficient to produce the desired result. One of general skill in the art may determine what the effective amount by using experimentation.

[0143] “Therapeutically effective amount” when used in reference to symptoms of disease/condition refers to the amount and/or concentration of a compound (e.g., engineered protease polypeptides) that ameliorates, attenuates, or eliminates one or more symptom of a disease/condition or prevents or delays the onset of symptom(s). A “therapeutically effective amount” when used in reference to a disease/condition refers to the amount and/or concentration of a composition (e.g., engineered protease polypeptides) that ameliorates, attenuates, or eliminates the disease/condition. In some embodiments, the

term is used in reference to the amount of a composition that elicits the biological (e.g., medical) response by a tissue, system, or animal subject that is sought by the researcher, physician, veterinarian, or other clinician.

[0144] “Treating” or “treatment” of a disease, disorder, or syndrome, as used herein, includes (i) preventing the disease, disorder, or syndrome from occurring in a subject, i.e., causing the clinical symptoms of the disease, disorder, or syndrome not to develop in an animal that may be exposed to or predisposed to the disease, disorder, or syndrome but does not yet experience or display symptoms of the disease, disorder, or syndrome; (ii) inhibiting the disease, disorder, or syndrome, i.e., arresting its development; and (iii) relieving the disease, disorder, or syndrome, i.e., causing regression of the disease, disorder, or syndrome. As such, the terms “treating,” “treat” and “treatment” encompass preventative (e.g., prophylactic), as well as palliative treatment. As is known in the art, adjustments for systemic versus localized delivery, age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable by one of ordinary skill in the art.

[0145] “Modulate,” “attenuate” or “ameliorate” means any treatment of a disease or disorder in a subject, such as a mammal, including: preventing or protecting against the disease or disorder, e.g., causing the abnormal biological reaction or symptoms not to develop; inhibiting the disease or disorder, arresting or suppressing the development of abnormal biological reactions and/or clinical symptoms; and/or relieving the disease or disorder, e.g., causing the regression of abnormal biological reactions and/or symptoms.

[0146] “Preventing” or “inhibiting” refers to the prophylactic treatment of a subject in need thereof. The prophylactic treatment can be accomplished by providing an appropriate dose of a therapeutic agent to a subject at risk of suffering from an ailment, thereby substantially averting onset of the ailment.

[0147] “Subject” encompasses mammals such as humans, non-human primates, livestock, companion animals, and laboratory animals (e.g., rodents and lagamorphs). It is intended that the term encompass females as well as males. In some embodiments, a “patient” means any subject that is being assessed for, treated for, or is experiencing disease.

[0148] “Infant” refers to a child in the period of the first month after birth to approximately one (1) year of age. As used herein, the term “newborn” refers to child in the period from birth to the 28th day of life.

[0149] “Child” refers to a person who has not attained the legal age for consent to treatment or research procedures. In some embodiments, the term refers to a person between the time of birth and adolescence. In some embodiments, “child” can be further subdivided into children older than 12 months and younger than 4 years, and children 4 years and older up to 18 years of age.

[0150] “Adult” refers to a person who has attained legal age for the relevant jurisdiction (e.g., 18 years of age in the United States). In some embodiments, the term refers to any fully grown, mature organism.

Engineered Protease Polypeptides

[0151] The engineered protease polypeptides described herein is based on the naturally occurring protease of *Bacillus sinesaloumensis* Marseille P3516. The naturally occurring protease is composed of a pro-region, a protease domain, and a Big-1 domain (see **FIG. 1** and SEQ ID NO: 2). The Big-1 domain is a region of about 95 amino acids present in bacterial adhesion molecules of the intimin/invasin family. In the amino acid sequence of SEQ ID NO: 2, the Big-1 domain is within amino acid residues about 440 to 522. The present disclosure shows that protease activity is maintained in engineered proteases deleted of the Big -1 domain, as well as deletion of a peptide region linking the Big-1 domain to the protease domain. Deletion of the carboxy terminal region up to and including amino acid residue 426, or up to and including amino acid residue 414 maintains the protease activity.

[0152] Furthermore, the pro-polypeptide or pro-enzyme form of the naturally occurring protease can transform into or covert to an active protease. Without being bound by any theory of operation, the pro-domain appears to promote formation of the active protease, which is formed by cleavage of the pro-polypeptide. For the pro-polypeptide of SEQ ID NO: 4 (see **FIG. 2**), the cleavage can occur between amino acid residues 127/128 and/or 134/135 to form the proteolytically active polypeptide or active protease. This cleavage can occur through auto-proteolysis, a self catalyzed reaction that occurs under suitable activation conditions. In some instances, the proteolytically active polypeptide may be formed by action of another protease, including the active protease forms of the engineered protease polypeptide described herein.

[0153] In one aspect, the present disclosure provides engineered protease polypeptides, with or without the Big-1 domain, as well as engineered protease polypeptides having one or more amino acid deletions of the peptide region linking the Big-1 domain to the protease domain. In some embodiments, the engineered protease polypeptides include the pro-polypeptide and corresponding proteolytically active polypeptide form, e.g., an active protease or the mature form of the protease.

[0154] In some embodiments, the present disclosure provides engineered protease polypeptides which exhibit an improved property, including, among others, enhanced expression, increased proteolytic activity of the active protease, increased thermostability, increased resistance against gastric proteases, increased activity at acidic pH, and/or increased stability at acidic pH.

[0155] In some embodiments, an engineered protease polypeptide, or a biologically active fragment thereof, comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or to a reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference

sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0156] In some embodiments, the engineered protease polypeptide, or a biologically active fragment thereof, comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or to the reference sequence corresponding to SEQ ID NO: 4 or 628, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0157] In some embodiments, the engineered protease polypeptide, or a biologically active fragment thereof, comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0158] In some embodiments, the engineered protease polypeptide, or a biologically active fragment thereof, comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0159] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 11, 31, 42, 45, 50, 53, 84, 99, 100, 126, 128, 129, 130, 131, 132, 134, 135, 136, 137, 138, 139, 140, 141, 143, 145, 151, 154, 156, 157, 159, 160, 161, 162, 163, 169, 172, 173, 174, 179, 180, 184, 185, 186, 187, 188, 190, 191, 192, 193, 194, 198, 199, 212, 214, 220, 221, 222, 223, 225, 231, 232, 233, 235, 237, 238, 239, 240, 242, 243, 245, 246, 249, 250, 251, 252, 253, 254, 256, 258, 262, 263, 264, 265, 266, 267, 268, 269, 271, 273, 274, 275, 277, 278, 279, 280, 281, 283, 285, 290, 292, 293, 294, 296, 297, 300, 302, 303, 311, 312, 313, 314, 315, 316, 318, 324, 328, 336, 339, 341, 342, 343, 345, 346, 355, 358, 360, 364, 367, 368, 369, 370, 371, 372, 373, 374, 375, 377, 381, 382, 384, 386, 389, 391, 392, 401, 402, 405, 406, 409, 410, 411, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-

413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0160] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 11K, 31G, 42W, 45Y, 50R, 53A, 84M, 99V, 100V, 126T, 128G/I/K/L/P/R/S/T/V, 129E/F/H/I/K/L/R/S/T/V, 130A/F/G/N/V, 131E/P/R/T/V/Y, 132A/C/D/E/G/P/R/V/Y, 134A/C/D/E/G/I/L/M/N/P/S/T/V/W/Y, 135C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 136C/G/I/M, 137A/D/N/S, 138Q, 139C/D/E/F/H/I/K/L/M/R/S, 140L, 141A/C/D/E/F/G/H/I/L/M/Q/R/S/T/V/W/Y, 143A/C/D/N/Q/S/T, 145A/C/D/E/F/G/H/I/K/L/P/Q/R/S/T/V/W, 151D/Q, 154C/D/L/R, 156C/V, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W, 159G, 160A/C/D/E/F/K/L/M/N/P/R/Q/T/V/W/Y, 161D/E/G/L/R, 162I, 163H/L, 169S, 172Q, 173F/S, 174L, 179K/S, 180H/L/M, 184A/D/G/L/M/Q/R, 185A/D/E/F/G/L/M/P/Q/R/S/T/V, 186A/R/S/T/Y, 187A, 188A/C/D/F/G/L/M/S/T/W, 190S, 191R, 192C/D/M/N, 193T, 194A/D/L/T, 198G, 199C/K/L, 212S, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 220K/L/R, 221A/C/D/E/F/G/H/I/K/L/M/P/Q/R/T/V/W/Y, 222G, 223S, 225V, 231H/V, 232S, 233G/I/L, 235Q/R/V, 237A/G, 238Q, 239L/M, 240A/L, 242E/S, 243E/L/M/R/S/T, 245L/V, 246I/V, 249G/M/S, 250A/C/F/L/N/T, 251D/S, 252P, 253C/I/V, 254C/E, 256L/M, 258W, 262A/S, 263E/H/P/Q/R/S, 264A/C/F/I/L/N/P/R/T/V, 265C/G/R, 266H/T/Y, 267A/G/H/I/L/M/R/S/T/V/W, 268A/F/G/H/I/N/P/Q/T/V/Y, 269Q/T, 271A, 273A/C/F/L/M/S/T, 274A/G/K/L/T/V/W, 275A/V, 277D/G, 278L/N/S/V/Y, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 280D/K/S/T, 281C/V, 283M, 285S, 290E/G/S, 292V, 293A, 294V/W, 296M/R, 297F, 300R/V, 302G/P, 303A/V, 311A/E/D/G/K/M/Q/S, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 313A/Q/S/T, 314G, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/V/W/Y, 316K, 318N/P/R, 324A/D/E/I/R/V/W/Y, 328L/M, 336F, 339S/W, 341G, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/T/V/W/Y, 343S, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/T/V/W/Y, 355A, 358S, 360S, 364A/V, 367V, 368G/T, 369I/V/W, 370C/E/F/G/I/K/L/P/Q/R/S/V, 371L, 372A/C/F/L/R/V/Y, 373A/C/E/F/M/S/Y, 374E/G/L/R/S/W/Y, 375A/E/I/L/M/S/T/V, 377H, 381N, 382G/R/S/T, 384C, 386P/W, 389C/P, 391L/S, 392Y, 401L, 402G/*, 405L/Q, 406C/M/R/W, 409E/R/*, 410C/I/W/*, 411L/R/T/V, 412P/T/*, or 413A/C/D/S/*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0161] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution N11K, D31G, G42W, D45Y, K50R, T53A, V84M, I99V, A100V, A126T, E128G/I/K/L/P/R/S/T/V, G129E/F/H/I/K/L/R/S/T/V, R130A/F/G/N/V, A131E/P/R/T/V/Y, T132A/C/D/E/G/P/R/V/Y, Q134A/C/D/E/G/I/L/M/N/P/S/T/V/W/Y, A135C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, V136C/G/I/M, H137A/D/N/S, P138Q,

N139C/D/E/F/H/I/K/L/M/R/S, Q140L, N141A/C/D/E/F/G/H/I/L/M/Q/R/S/T/V/W/Y, H143A/C/D/N/Q/S/T, N145A/C/D/E/F/G/H/I/K/L/P/Q/R/S/T/V/W, E151D/Q, A154C/D/L/R, T156C/V, S157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W, S159G, S160A/C/D/E/F/K/L/M/N/P/R/Q/T/V/W/Y, S161D/E/G/L/R, V162I, K163H/L, T169S, D172Q, H173F/S, N174L, A179K/S, N180H/L/M, T184A/D/G/L/M/Q/R, N185A/D/E/F/G/L/M/P/Q/R/S/T/V, L186A/R/S/T/Y, G187A, R188A/C/D/F/G/L/M/S/T/W, F190S, V191R, G192C/D/M/N, G193T, N194A/D/L/T, V198G, Q199C/K/L, Y212S, S214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, Q220K/L/R, S221A/C/D/E/F/G/H/I/K/L/M/P/Q/R/T/V/W/Y, A222G, T223S, I225V, D231H/V, N232S, S233G/I/L, S235Q/R/V, S237A/G, L238Q, Y239L/M, G240A/L, T242E/S, Q243E/L/M/R/S/T, I245L/V, L246I/V, A249G/M/S, D250A/C/F/L/N/T, T251D/S, D252P, A253C/I/V, D254C/E, I256L/M, M258W, G262A/S, G263E/H/P/Q/R/S, G264A/C/F/I/L/N/P/R/T/V, Y265C/G/R, N266H/T/Y, Q267A/G/H/I/L/M/R/S/T/V/W, S268A/F/G/H/I/N/P/Q/T/V/Y, M269Q/T, E271A, V273A/C/F/L/M/S/T, Q274A/G/K/L/T/V/W, T275A/V, V277D/G, A278L/N/S/V/Y, Q279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, G280D/K/S/T, T281C/V, V283M, A285S, D290E/G/S, A292V, S293A, S294V/W, S296M/R, Y297F, A300R/V, S302G/P, S303A/V, T311A/E/D/G/K/M/Q/S, S312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, N313A/Q/S/T, R314G, T315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/V/W/Y, R316K, S318N/P/R, S324A/D/E/I/R/V/W/Y, V328L/M, Y336F, Y339S/W, N341G, S342A/C/D/E/F/G/I/K/M/N/P/R/Q/T/V/W/Y, R343S, T345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, S346A/C/D/F/G/K/L/M/N/P/Q/R/T/V/W/Y, P355A, A358S, V360S, I364A/V, A367V, N368G/T, P369I/V/W, 370C/E/F/G/I/K/L/P/Q/R/S/V, I371L, S372A/C/F/L/R/V/Y, V373A/C/E/F/M/S/Y, A374E/G/L/R/S/W/Y, Q375A/E/I/L/M/S/T/V, R377H, R381N, D382G/R/S/T, A384C, E386P/W, S389C/P, T391L/S, Q392Y, H401L, A402G/*, V405L/Q, A406C/M/R/W, G409E/R/*, G410C/I/W/*, S411L/R/T/V, G412P/T/*, or G413A/C/D/S/*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0162] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 157, 160, 214, 268, 273, 279, 311, 312, 315, 328, 342, 345, 346, or 372, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0163] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 137A/D/N/S, 139C/D/E/F/H/I/K/L/M/R/S, 141A/C/D/E/F/G/H/I/L/M/Q/R/S/T/V/W/Y, 143A/C/D/N/Q/S/T, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W, 160A/C/D/E/F/K/L/M/N/P/R/Q/T/V/W/Y, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y,

268A/F/G/H/I/N/P/Q/T/V/Y, 273A/C/F/L/M/S/T, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W,
 311A/E/D/G/K/M/Q/S, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y,
 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/V/W/Y, 328L/M, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/T/V/W/Y,
 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/T/V/W/Y, or
 372A/C/F/L/R/V/Y, or combinations thereof, wherein the amino acid positions are relative to the
 reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the
 reference sequence corresponding to SEQ ID NO: 4 or 628.

[0164] In some embodiments, the amino acid sequence of the engineered protease polypeptide
 comprises at least a substitution A135C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, H137A/D/N/S,
 N139C/D/E/F/H/I/K/L/M/R/S, N141A/C/D/E/F/G/H/I/L/M/Q/R/S/T/V/W/Y, H143A/C/D/N/Q/S/T,
 S157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W, S160A/C/D/E/F/K/L/M/N/P/R/Q/T/V/W/Y,
 S214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, S268A/F/G/H/I/N/P/Q/T/V/Y, V273A/C/F/L/M/S/T,
 Q279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, T311A/E/D/G/K/M/Q/S,
 S312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, T315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/V/W/Y,
 V328L/M, S342A/C/D/E/F/G/I/K/M/N/P/R/Q/T/V/W/Y, T345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y,
 S346A/C/D/F/G/K/L/M/N/P/Q/R/T/V/W/Y, or S372A/C/F/L/R/V/Y, or combinations thereof, wherein
 the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ
 ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0165] In some embodiments, the amino acid sequence of the engineered protease polypeptide
 comprises at least a substitution set at amino acid positions 135/141/160/311/315/372, 143/328/342/345,
 139/157/268/273/312/346, or 137/139/214/279, wherein the amino acid positions are relative to the
 reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the
 reference sequence corresponding to SEQ ID NO: 4 or 628.

[0166] In some embodiments, the amino acid sequence of the engineered protease polypeptide
 comprises at least a substitution set or amino acid residues 135G/141V/160L/311D/315V/372V,
 143A/328L/342G/345R, 139C/157G/268G/273T/312Q/346T, or 137N/139L/214P/279M, wherein the
 amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID
 NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0167] In some embodiments, the amino acid sequence of the engineered protease polypeptide
 comprises at least a substitution set A135G/N141V/S160L/T311D/T315V/S372V,
 H143A/V328L/S342G/T345R, N139C/S157G/S268G/V273T/S312Q/S346T, or
 H137N/C139L/S214P/Q279M, wherein the amino acid positions are relative to the reference sequence
 corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence
 corresponding to SEQ ID NO: 4 or 628.

[0168] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 185, 134, 129, 135, 184, 132, 186, 193, 263, 370, 45/134, 199, 368, 161, 141, 267, 179, 264, 160, 138, 131, 372, 151, 274, 128, 339, 313, 374, 314, 191, 324, 315, 375, 136, 220, 194, 231, 277, 369, 251, 180, 163, 343, 264/279, 279, 232, 141/300, 367, 266, 188, 130, 318, 265, 341, 190, 145, 126/192, 11/220, 192, 370/392, 99/278, 265/311, 84/159/265/279/311/370, 311/316, 342/370, 265/311/370, 192/311/316, 141/154/192, 265/311/316/342, 279/311/316, 141/265/279/311/342, 141/192/311/316/370, 141/265/311, 198/279, 392, 342/370/392, 141/198/265, 265/392, 184/267, 342, 312, 100/251, 141/220, 311/316/370, 99, 278, 405, 311/342/370, 141/198, 311/342, 141/311, 279/311/377/392, 186/198/311/342/370/392, 141/392, 311/370/392, 141/311/392, 311/370, 311/316/392, 265/311/392, 141/192, 311, 141/265/311/392, 192/311/370/392, 198/265/311/316/370, 141/186/265/311, or 141/198/265/311/370, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0169] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 185F, 134I, 129T, 135C, 184A, 129R, 132Y, 186R, 193T, 263P, 370C, 45Y/Q134W, 185M, 199K, 368T, 161E, 141T, 267L, 179S, 185V, 264L, 199C, 160M, 138Q, 131Y, 184D, 372R, 134L, 370R, 370I, 134E, 368G, 151D, 274K, 134D, 134V, 128I, 339S, 313A, 131E, 185P, 374W, 314G, 191R, 128V, 132E, 324R, 315M, 132V, 375L, 375T, 129L, 132P, 184M, 136G, 186A, 135S, 220L, 134P, 132A, 141M, 135I, 194D, 185Q, 263H, 274L, 231V, 315R, 375S, 135T, 185G, 135R, 277D, 128P, 132R, 369I, 264C, 315H, 251S, 136I, 160P, 375I, 180M, 369V, 251D, 264A, 163L, 231H, 343S, 264R/279R, 274A, 279Y, 131P, 232S, 220R, 315Q, 186T, 324V, 313S, 132D, 141R/300V, 324I, 367V, 135V, 370L, 132G, 267G, 131T, 266T, 179K, 372A, 372F, 185T, 324D, 135K, 188A, 141D, 374L, 185D, 130N, 370V, 161R, 315I, 315L, 318N, 188C, 180L, 372Y, 135P, 375E, 324A, 129K, 134M, 184G, 185A, 129H, 188D, 130F, 265C, 141W, 324W, 370E, 184R, 134A, 161L, 134T, 370G, 375A, 128G, 130V, 134N, 341G, 190S, 370P, 145R, 279H, 279S, 160Q, 370K, 126T/G192C, 374E, 128K, 160C, 186S, 11K/Q220K, 134W, 129V, 128L, 151Q, 375M, 134C, 374R, 160T, 279T, 264F, 132C, 129F, 264V, 129I, 184Q, 192M, 374S, 370F, 267A, 369W, 199L, 145M, 194A, 185S, 265R, 129S, 185R, 188W, 161G, 370G/392Y, 99V/278N, 265G/311D, 84M/159G/265G/279K/311D/370G, 311D/316K, 342N/370G, 265G/311D/370G, 192D/311D/316K, 141Q/154D/192D, 265G/311D/316K/342N, 279K/311D/316K, 141Q/265G/279K/311D/342N, 141Q/192D/311D/316K/370G, 141Q, 141Q/265G/311D, 198G/279K, 392Y, 342N/370G/392Y, 141Q/198G/265G, 265G/392Y, or 265G, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0170] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set N185F, Q134I, G129T, A135C, T184A, G129R,

T132Y, L186R, G193T, G263P, D370C, D45Y/Q134W, N185M, Q199K, N368T, S161E, N141T, Q267L, A179S, N185V, G264L, Q199C, S160M, P138Q, A131Y, T184D, S372R, Q134L, D370R, D370I, Q134E, N368G, E151D, Q274K, Q134D, Q134V, E128I, Y339S, N313A, A131E, N185P, A374W, R314G, V191R, E128V, T132E, S324R, T315M, T132V, Q375L, Q375T, G129L, T132P, T184M, V136G, L186A, A135S, Q220L, Q134P, T132A, N141M, A135I, N194D, N185Q, G263H, Q274L, D231V, T315R, Q375S, A135T, N185G, A135R, V277D, E128P, T132R, P369I, G264C, T315H, I251S, V136I, S160P, Q375I, N180M, P369V, I251D, G264A, K163L, D231H, R343S, G264R/Q279R, Q274A, Q279Y, A131P, N232S, Q220R, T315Q, L186T, S324V, N313S, T132D, N141R/A300V, S324I, A367V, A135V, D370L, T132G, Q267G, A131T, N266T, A179K, S372A, S372F, N185T, S324D, A135K, R188A, N141D, A374L, N185D, T130N, D370V, S161R, T315I, T315L, S318N, R188C, N180L, S372Y, A135P, Q375E, S324A, G129K, Q134M, T184G, N185A, G129H, R188D, T130F, Y265C, N141W, S324W, D370E, T184R, Q134A, S161L, Q134T, D370G, Q375A, E128G, T130V, Q134N, N341G, F190S, D370P, N145R, Q279H, Q279S, S160Q, D370K, A126T/G192C, A374E, E128K, S160C, L186S, N11K/Q220K, Q134W, G129V, E128L, E151Q, Q375M, Q134C, A374R, S160T, Q279T, G264F, T132C, G129F, G264V, G129I, T184Q, G192M, A374S, D370F, Q267A, P369W, Q199L, N145M, N194A, N185S, Y265R, G129S, N185R, R188W, S161G, D370G/Q392Y, I99V/A278N, Y265G/T311D, V84M/S159G/Y265G/Q279K/T311D/D370G, T311D/R316K, S342N/D370G, Y265G/T311D/D370G, G192D/T311D/R316K, N141Q/A154D/G192D, Y265G/T311D/R316K/S342N, Q279K/T311D/R316K, N141Q/Y265G/Q279K/T311D/S342N, N141Q/G192D/T311D/R316K/D370G, N141Q, N141Q/Y265G/T311D, V198G/Q279K, Q392Y, S342N/D370G/Q392Y, N141Q/V198G/Y265G, Y265G/Q392Y, or Y265G, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0171] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 135L, 194L, 128T, 134S, 313T, 184L/267L, 185E, 342G, 374Y, 141R, 186Y, 312R, 313Q, 315V, 374G, 128S, 136A, 128R, 370Q, 267V, 188M, 188F, 263S, 188S, 339W, 100V/251S, 131V, 188T, 141L, 134Y, 267M, 264N, 134G, 185L, 370S, 267W, 279M, 267R, 264T, 279L, 263R, 136C, 145E, 188G, 130A, 192N, 188L, 312I, 129E, 315E, 145A, 267H, 372V, 130G, 267T, 274W, 136M, 372C, 194T, 375V, 135G, 267I, 141L/220R, 324E, 160L, 141S, 372L, 135Y, 141V, 141A, 131R, 135E, 324Y, 311D/316K/370G, 99V, 278N, 405Q, 311D/342N/370G, 141Q/198G, 311D/342N, 141Q/311D, 279K/311D/377H/392Y, 186Y/198G/311D/342N/370G/392Y, 141Q/392Y, 311D/370G/392Y, 141Q/311D/392Y, 311D/370G, 311D/316K/392Y, 265G/311D/392Y, 141Q/192D, 311D, 141Q/265G/311D/392Y, 192D/311D/370G/392Y, 198G/265G/311D/316K/370G, 141Q/186Y/265G/311D, or 141Q/198G/265G/311D/370G, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0172] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set A135L, N194L, E128T, Q134S, N313T, T184L/Q267L, N185E, S342G, A374Y, N141R, L186Y, S312R, N313Q, T315V, A374G, E128S, V136A, E128R, D370Q, Q267V, R188M, R188F, G263S, R188S, Y339W, A100V/I251S, A131V, R188T, N141L, Q134Y, Q267M, G264N, Q134G, N185L, D370S, Q267W, Q279M, Q267R, G264T, Q279L, G263R, V136C, N145E, R188G, T130A, G192N, R188L, S312I, G129E, T315E, N145A, Q267H, S372V, T130G, Q267T, Q274W, V136M, S372C, N194T, Q375V, A135G, Q267I, N141L/Q220R, S324E, S160L, N141S, S372L, A135Y, N141V, N141A, A131R, A135E, S324Y, T311D/R316K/D370G, I99V, A278N, V405Q, T311D/S342N/D370G, N141Q/V198G, T311D/S342N, N141Q/T311D, Q279K/T311D/R377H/Q392Y, L186Y/V198G/T311D/S342N/D370G/Q392Y, N141Q/Q392Y, T311D/D370G/Q392Y, N141Q/T311D/Q392Y, T311D/D370G, T311D/R316K/Q392Y, Y265G/T311D/Q392Y, N141Q/G192D, T311D, N141Q/Y265G/T311D/Q392Y, G192D/T311D/D370G/Q392Y, V198G/Y265G/T311D/R316K/D370G, N141Q/L186Y/Y265G/T311D, or N141Q/V198G/Y265G/T311D/D370G, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0173] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 145, 145, 157, 160, 214, 221, 268, 273, 279, 311, 312, 315, 315, 342, 345, 346, 402, 409, 410, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0174] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135G, 135S, 135V, 135L, 135R, 135E, 135P, 135H, 135C, 135T, 135Y, 135W, 135M, 135N, 137S, 137A, 137N, 137D, 139R, 139E, 139F, 139L, 139K, 139D, 139H, 139I, 139S, 141T, 141S, 141V, 141L, 141R, 141M, 141G, 141Y, 141I, 141C, 141F, 141A, 141D, 141E, 141H, 143T, 143C, 143Q, 143A, 143D, 143S, 143N, 145Q, 145T, 145V, 145H, 145L, 145E, 145R, 145D, 145R, 145A, 145F, 145S, 145G, 145I, 145K, 145M, 145C, 145W, 157A, 157E, 157P, 157V, 157T, 157N, 157R, 157G, 157L, 157W, 157K, 157C, 157D, 157Q, 157M, 157H, 157I, 157F, 160R, 160V, 160C, 160Q, 160A, 160P, 160L, 160F, 160T, 160D, 160Y, 160W, 160E, 160K, 160N, 160M, 214G, 214M, 214L, 214Q, 214T, 214P, 214R, 214D, 214F, 214K, 214A, 214V, 214I, 214E, 214H, 214Y, 214C, 214W, 221L, 221T, 221I, 221R, 221D, 221A, 221C, 221V, 221F, 221G, 221P, 221K, 221Y, 221E, 221Q, 221M, 221H, 221W, 268V, 268Y, 268A, 268Q, 268P, 268G, 268T, 268H, 268I, 268F, 268N, 273S, 273C, 273A, 273L, 273F, 273T, 273M, 279R, 279E, 279F, 279G, 279T, 279M, 279L, 279S, 279A, 279K, 279V, 279W, 279Y, 279H, 311S, 311D, 311Q, 311M, 311K, 311G, 311A, 311E, 312V, 312D, 312G, 312R, 312W, 312M, 312L, 312N, 312E, 312A, 312T, 312Y, 312P, 312H,

312Q, 312K, 312C, 312I, 315E, 315S, 315L, 315R, 315G, 315A, 315M, 315Y, 315K, 315Q, 315D, 315W, 315C, 315V, 315I, 315H, 315F, 342F, 342G, 342R, 342Q, 342E, 342V, 342T, 342C, 342N, 342I, 342P, 342M, 342A, 342W, 342K, 342D, 342Y, 345G, 345R, 345L, 345V, 345A, 345M, 345W, 345I, 345S, 345E, 345Y, 345D, 345Q, 345F, 345C, 345K, 346T, 346Q, 346V, 346R, 346P, 346L, 346D, 346W, 346G, 346A, 346C, 346M, 346F, 346N, 346Y, 346K, 402*, 409*, 410*, 412*, or 413*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0175] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution A135G, A135S, A135V, A135L, A135R, A135E, A135P, A135H, A135C, A135T, A135Y, A135W, A135M, A135N, H137S, H137A, H137N, H137D, N139R, N139E, N139F, N139L, N139K, N139D, N139H, N139I, N139S, N141T, N141S, N141V, N141L, N141R, N141M, N141G, N141Y, N141I, N141C, N141F, N141A, N141D, N141E, N141H, H143T, H143C, H143Q, H143A, H143D, H143S, H143N, N145Q, N145T, N145V, N145H, N145L, N145E, N145R, N145D, N145R, N145A, N145F, N145S, N145G, N145I, N145K, N145M, N145C, N145W, S157A, S157E, S157P, S157V, S157T, S157N, S157R, S157G, S157L, S157W, S157K, S157C, S157D, S157Q, S157M, S157H, S157I, S157F, S160R, S160V, S160C, S160Q, S160A, S160P, S160L, S160F, S160T, S160D, S160Y, S160W, S160E, S160K, S160N, S160M, S214G, S214M, S214L, S214Q, S214T, S214P, S214R, S214D, S214F, S214K, S214A, S214V, S214I, S214E, S214H, S214Y, S214C, S214W, S221L, S221T, S221I, S221R, S221D, S221A, S221C, S221V, S221F, S221G, S221P, S221K, S221Y, S221E, S221Q, S221M, S221H, S221W, S268V, S268Y, S268A, S268Q, S268P, S268G, S268T, S268H, S268I, S268F, S268N, V273S, V273C, V273A, V273L, V273F, V273T, V273M, Q279R, Q279E, Q279F, Q279G, Q279T, Q279M, Q279L, Q279S, Q279A, Q279K, Q279V, Q279W, Q279Y, Q279H, T311S, T311D, T311Q, T311M, T311K, T311G, T311A, T311E, S312V, S312D, S312G, S312R, S312W, S312M, S312L, S312N, S312E, S312A, S312T, S312Y, S312P, S312H, S312Q, S312K, S312C, S312I, T315E, T315S, T315L, T315R, T315G, T315A, T315M, T315Y, T315K, T315Q, T315D, T315W, T315C, T315V, T315I, T315H, T315F, S342F, S342G, S342R, S342Q, S342E, S342V, S342T, S342C, S342N, S342I, S342P, S342M, S342A, S342W, S342K, S342D, S342Y, T345G, T345R, T345L, T345V, T345A, T345M, T345W, T345I, T345S, T345E, T345Y, T345D, T345Q, T345F, T345C, T345K, S346T, S346Q, S346V, S346R, S346P, S346L, S346D, S346W, S346G, S346A, S346C, S346M, S346F, S346N, S346Y, S346K, A402*, G409*, G410*, G412*, or G413*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0176] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 242, 157, 250, 373, 243, 336,

187, 240, 280, 271, 237, 386, 382, 328, 42, 391, 381, 275, 249, 239, 384, 139, 364, 346, 389, 254, 246, 345, 360, 303, 300, 269, 135/141/372, 311/315/372, 136/141/311, 141/188, 135/136, 135/141/315, 372, 135/141/160/267/372, 135/136/141/160/185/188/267/311/315, 160/185, 135/141/188/279/311, 135/136/141, 135/136/141/372, 135/141/160/185/267/279, 135/141/160/267, 141/188/311/372, 160/185/188/279/311, 136/141/279, 135/136/141/160/185/188, 141/372, 135/136/141/311, 185/311/315/372, 135/141/188, 136/185, 135/141, 135/136/141/279/315/372, 135/311/315, 141, 311/372, 188/311, 135/141/188/372, 141/160/279, 313/392, 342/392, 279/392, 128, 198/342, 313, 128/312, 50, 145/263, 313/342, 279/312, 312/392, 279/342, 128/342, 342, 263, 143, 262, 156, 169, 143/237, 136/160/185/267/311/372, 135/160/311/372, 135/141/311/315, 141/311/315, 136/141/160/185/188/311/315/372, 135/141/311/315/372, 135/141/160/185/311/315, 135/141/267/311/315/372, 135/136/141/160/311/315, 135/136/141/279, 135/141/267/279/311/315, 135/141/160, 135/141/160/311/315/372, 135/141/160/311/315, 135/136/141/188/311, 141/160/311, 135/141/160/279/311/315/372, 141/160/185/279/311/372, 135/136/141/160/315/372, 135/136/160/279/311/372, 128/279/312/342, 128/198/312/342, 263/342, 145/263/279/312/342/392, or 128/145/198/312/313/392, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0177] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 242E, 157V, 250N, 373F, 243R, 336F, 187A, 240A, 280K, 271A, 237G, 386W, 382G, 280S, 373Y, 328M, 157R, 157A, 42W, 243E, 382S, 391L, 381N, 243M, 275V, 157I, 373S, 157T, 280D, 249G, 239L, 384C, 139M, 240L, 243T, 250L, 250A, 382T, 364A, 346V, 373M, 389P, 373C, 382R, 373E, 254E, 246I, 250F, 280T, 373A, 139K, 345I, 360S, 275A, 249M, 364V, 303V, 300R, 239M, 269T, 135G/141Q/372L, 311D/315V/372L, 136M/141V/311D, 141V/188M, 135E/136M, 136M/141Q/311D, 135E/141V/315V, 372V, 135E/141V/160L/267I/372V, 135G/136M/141V/160L/185E/188M/267I/311D/315V, 135G/136M, 160L/185E, 135E/141V/188M/279M/311D, 135G/136M/141Q, 135G/136M/141Q/372L, 135G/141V/160L/185E/267I/279M, 135G/141V/160L/267I, 141Q/188M/311D/372V, 160L/185E/188M/279M/311D, 136M/141V/279M, 135G/136M/141V/160L/185E/188L, 141Q/372V, 135E/136M/141Q/311D, 185E/311D/315V/372V, 135G/141V/188M, 136M/185E, 135E/141Q, 135E/136M/141Q/279M/315V/372L, 135G/311D/315V, 141V, 135G/141V, 311D/372L, 188M/311D, 135E/141V/188L/372L, 141V/160L/279M, 313Q/392Y, 342G/392Y, 279L/392Y, 128T, 198G/342G, 313Q, 128T/312I, 50R, 145E/263S, 313Q/342G, 279L/312I, 312I/392Y, 279K/342G, 279K/392Y, 128T/342G, 342G, or 263S, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0178] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set T242E, S157V, D250N, V373F, Q243R, Y336F, G187A, G240A, G280K, E271A, S237G, E386W, D382G, G280S, V373Y, V328M, S157R, S157A, G42W, Q243E, D382S, T391L, R381N, Q243M, T275V, S157I, V373S, S157T, G280D, A249G, Y239L, A384C, N139M, G240L, Q243T, D250L, D250A, D382T, I364A, S346V, V373M, S389P, V373C, D382R, V373E, D254E, L246I, D250F, G280T, V373A, N139K, T345I, V360S, T275A, A249M, I364V, S303V, A300R, Y239M, M269T, A135G/N141Q/S372L, T311D/T315V/S372L, V136M/N141V/T311D, N141V/R188M, A135E/V136M, V136M/N141Q/T311D, A135E/N141V/T315V, S372V, A135E/N141V/S160L/Q267I/S372V, A135G/V136M/N141V/S160L/N185E/R188M/Q267I/T311D/T315V, A135G/V136M, S160L/N185E, A135E/N141V/R188M/Q279M/T311D, A135G/V136M/N141Q, A135G/V136M/N141Q/S372L, A135G/N141V/S160L/N185E/Q267I/Q279M, A135G/N141V/S160L/Q267I, N141Q/R188M/T311D/S372V, S160L/N185E/R188M/Q279M/T311D, V136M/N141V/Q279M, A135G/V136M/N141V/S160L/N185E/R188L, N141Q/S372V, A135E/V136M/N141Q/T311D, N185E/T311D/T315V/S372V, A135G/N141V/R188M, V136M/N185E, A135E/N141Q, A135E/V136M/N141Q/Q279M/T315V/S372L, A135G/T311D/T315V, N141V, A135G/N141V, T311D/S372L, R188M/T311D, A135E/N141V/R188L/S372L, N141V/S160L/Q279M, N313Q/Q392Y, S342G/Q392Y, Q279L/Q392Y, E128T, V198G/S342G, N313Q, E128T/S312I, K50R, N145E/G263S, N313Q/S342G, Q279L/S312I, S312I/Q392Y, Q279K/S342G, Q279K/Q392Y, E128T/S342G, S342G, or G263S, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0179] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 139C, 345R, 243L, 143A, 249S, 262S, 139R, 269Q, 328L, 157G, 156V, 242S, 139L, 262A, 169S, 346T, 143N/237A, 136M/160L/185E/267I/311D/372L, 135E/160L/311D/372L, 135G/141V/311D/315V, 141V/311D/315V, 136M/141V/160L/185E/188M/311D/315V/372V, 135G/141V/311D/315V/372L, 135G/141Q/160L/185E/311D/315V, 135G/141V/267I/311D/315V/372V, 135G/136M/141V/160L/311D/315V, 135G/136M/141V/279M, 135G/141Q/267I/279M/311D/315V, 135E/141V/311D/315V/372V, 135E/141Q/160L, 135G/141Q/311D/315V, 135G/141V/160L/311D/315V/372V, 135G/141V/160L/311D/315V, 135G/141Q/267I/311D/315V/372L, 135G/136M/141V/188M/311D, 141V/160L/311D, 135E/141V/160L/279M/311D/315V/372L, 141V/160L/185E/279M/311D/372V, 135E/141V/311D/315V, 135G/136M/141V/160L/315V/372V, 135E/141V/160L, 135E/136M/160L/279M/311D/372V, 128T/279K/312I/342G, 128T/198G/312I/342G, 263S/342G, 145E/263S/279L/312I/342G/392Y, or 128T/145E/198G/312I/313Q/392Y, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0180] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set N139C, T345R, Q243L, H143A, A249S, G262S, N139R, M269Q, V328L, S157G, T156V, T242S, N139L, G262A, T169S, S346T, H143N/S237A, V136M/S160L/N185E/Q267I/T311D/S372L, A135E/S160L/T311D/S372L, A135G/N141V/T311D/T315V, N141V/T311D/T315V, V136M/N141V/S160L/N185E/R188M/T311D/T315V/S372V, A135G/N141V/T311D/T315V/S372L, A135G/N141Q/S160L/N185E/T311D/T315V, A135G/N141V/Q267I/T311D/T315V/S372V, A135G/V136M/N141V/S160L/T311D/T315V, A135G/V136M/N141V/Q279M, A135G/N141Q/Q267I/Q279M/T311D/T315V, A135E/N141V/T311D/T315V/S372V, A135E/N141Q/S160L, A135G/N141Q/T311D/T315V, A135G/N141V/S160L/T311D/T315V/S372V, A135G/N141V/S160L/T311D/T315V, A135G/N141Q/Q267I/T311D/T315V/S372L, A135G/V136M/N141V/R188M/T311D, N141V/S160L/T311D, A135E/N141V/S160L/Q279M/T311D/T315V/S372L, N141V/S160L/N185E/Q279M/T311D/S372V, A135E/N141V/T311D/T315V, A135G/V136M/N141V/S160L/T315V/S372V, A135E/N141V/S160L, A135E/V136M/S160L/Q279M/T311D/S372V, E128T/Q279K/S312I/S342G, E128T/V198G/S312I/S342G, G263S/S342G, N145E/G263S/Q279L/S312I/S342G/Q392Y, or E128T/N145E/V198G/S312I/N313Q/Q392Y, wherein the amino acid positions are relative to the reference sequence corresponding to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0181] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set at amino acid position(s) 135/141/160/311/315/372/411, 135/141/160/311/315/372/402, 135/141/160/285/311/315/372, 135/141/160/245/311/315/372, 135/141/160/266/311/315/372, 135/141/160/311/315/355/372, 135/141/160/258/311/315/372, 135/141/160/222/311/315/372, 135/140/141/160/311/315/372, 135/141/160/268/311/315/372, 135/141/160/225/311/315/372, 135/141/160/283/311/315/372, 135/141/160/311/315/372/406, 135/141/160/311/315/372/410, 135/141/143/145/160/243/311/312/315/372, 135/139/141/143/145/157/160/311/312/315/372, 135/139/141/157/160/311/315/345/372, 135/139/141/160/269/311/315/372, 135/141/156/157/160/311/315/342/346/372, 135/139/141/143/160/311/315/372, 135/141/160/269/311/315/372, 135/139/141/160/243/311/315/328/372, 135/141/160/269/311/315/328/372, 135/141/143/145/160/169/311/315/372, 135/141/160/311/315/328/372, 135/141/143/145/160/262/311/315/372, 135/141/145/160/262/311/312/315/328/372, 135/139/141/156/157/160/311/315/372, 135/139/141/145/160/311/312/315/372, 135/141/160/311/312/315/372, 135/139/141/160/311/315/372, 135/139/141/160/311/312/315/372, 135/139/141/156/160/311/315/372, 135/139/141/143/145/160/243/311/315/372, 135/141/145/157/160/311/315/372, 135/141/145/160/311/315/346/372, 135/141/145/160/262/311/312/315/328/345/346/372, 135/141/145/160/262/311/315/372,

135/141/160/311/312/315/342/372, 135/141/143/160/243/311/315/372,
135/139/141/160/311/315/345/372, 135/141/160/311/315/342/372,
135/141/143/145/160/262/311/315/342/372, 135/139/141/143/160/169/311/315/372,
135/139/141/143/145/160/311/312/315/372, 135/141/160/169/311/315/372,
135/139/141/145/160/262/311/312/315/328/342/345/346/372, 135/139/141/160/311/315/328/372,
135/139/141/160/243/311/315/372, 135/139/141/143/160/311/315/328/372,
135/139/141/143/160/243/311/315/372, 135/139/141/145/160/311/315/372,
135/141/145/160/311/312/315/372, 135/141/145/160/169/311/315/372,
135/139/141/143/157/160/311/312/315/372, 84/135/139/143/141/160/311/315/372,
135/141/145/160/269/311/315/372, 135/141/143/145/157/160/269/311/312/315/328/372,
135/141/143/145/160/269/311/315/372, 135/141/157/160/311/315/372,
135/139/141/143/160/311/312/315/372, 135/141/160/256/311/315/372, 135/141/160/273/311/315/372,
135/141/160/311/315/372/409, 135/141/160/172/311/315/372, 135/141/160/311/315/372/401,
135/141/160/281/311/315/372, 135/141/160/253/311/315/372,
135/141/143/145/160/243/311/315/328/372, 135/141/145/160/311/315/372,
135/139/141/143/145/160/311/315/328/342/345/372, 135/141/143/160/311/315/328/342/345/372,
135/141/145/160/311/315/342/345/372, 135/141/143/160/311/315/372,
135/141/139/143/160/311/315/372, 135/139/141/145/160/311/315/328/342/345/372,
135/141/143/145/160/169/311/312/315/328/345/346/372,
135/141/143/160/243/311/315/328/342/345/346/372,
135/139/141/143/157/160/169/311/315/328/346/372, 135/143/141/145/156/160/311/312/315/328/372,
135/139/141/145/157/160/311/312/315/328/372, 135/141/143/160/311/315/328/342/345/346/372,
135/141/143/145/160/311/315/372, 135/141/143/145/160/311/312/315/342/345/372, or
135/141/143/145/160/311/315/328/372, wherein the amino acid positions are relative to the reference
sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference
sequence corresponding to SEQ ID NO: 4 or 628.

[0182] In some embodiments, the amino acid sequence of the engineered protease polypeptide
comprises at least a substitution set or amino acid residues 135G/141V/160L/311D/315V/372V/S411V,
135G/141V/160L/311D/315V/372V/411R, 135G/141V/160L/311D/315V/372V/402G,
135G/141V/160L/285S/311D/315V/372V, 135G/141V/160L/245V/311D/315V/372V,
135G/141V/160L/266H/311D/315V/372V, 135G/141V/160L/311D/315V/355A/372V,
135G/141V/160L/258W/311D/315V/372V, 135G/141V/160L/222G/311D/315V/372V,
135G/140L/141V/160L/311D/315V/372V, 135G/141V/160L/268T/311D/315V/372V,
135G/141V/160L/311D/315V/372V/411L, 135G/141V/160L/225V/311D/315V/372V,
135G/141V/160L/245L/311D/315V/372V, 135G/141V/160L/283M/311D/315V/372V,
135G/141V/160L/311D/315V/372V/406W, 135G/141V/160L/311D/315V/372V/410C,
135G/141V/160L/311D/315V/372V/406C, 135G/141V/143A/145E/160L/243L/311D/312I/315V/372V,

135G/139L/141V/143A/145E/157G/160L/311D/312I/315V/372V,
 135G/139C/141V/157G/160L/311D/315V/345R/372V, 135G/139C/141V/160L/269Q/311D/315V/372V,
 135G/141V/156V/157G/160L/311D/315V/342G/346T/372V,
 135G/139L/141V/143A/160L/311D/315V/372V, 135G/141V/160L/269Q/311D/315V/372V,
 135G/139L/141V/160L/243L/311D/315V/328L/372V, 135G/141V/160L/269Q/311D/315V/328L/372V,
 135G/141V/143A/145E/160L/169S/311D/315V/372V, 135G/141V/160L/311D/315V/328L/372V,
 135G/141V/143A/145E/160L/262S/311D/315V/372V,
 135G/141V/145E/160L/262S/311D/312I/315V/328L/372V,
 135G/139L/141V/156V/157G/160L/311D/315V/372V,
 135G/139C/141V/145E/160L/311D/312I/315V/372V, 135G/141V/160L/311D/312I/315V/372V,
 135G/139C/141V/160L/311D/315V/372V, 135G/139C/141V/160L/311D/312I/315V/372V,
 135G/139C/141V/156V/160L/311D/315V/372V,
 135G/139C/141V/143A/145E/160L/243L/311D/315V/372V,
 135G/141V/145E/157G/160L/311D/315V/372V, 135G/141V/145E/160L/311D/315V/346T/372V,
 135G/141V/145E/160L/262A/311D/312I/315V/328L/345R/346T/372V,
 135G/141V/145E/160L/262A/311D/315V/372V, 135G/141V/160L/311D/312I/315V/342G/372V,
 135G/141V/143A/160L/243L/311D/315V/372V, 135G/139C/141V/160L/311D/315V/345R/372V,
 135G/141V/160L/311D/315V/342G/372V, 135G/141V/143A/145E/160L/262S/311D/315V/342G/372V,
 135G/139L/141V/143A/160L/169S/311D/315V/372V,
 135G/139C/141V/143A/145E/160L/311D/312I/315V/372V, 135G/141V/160L/169S/311D/315V/372V,
 135G/139L/141V/145E/160L/262A/311D/312I/315V/328L/342G/345R/346T/372V,
 135G/139C/141V/160L/311D/315V/328L/372V, 135G/139L/141V/160L/243L/311D/315V/372V,
 135G/139L/141V/143A/160L/311D/315V/328L/372V, 135G/139L/141V/160L/311D/315V/372V,
 135G/139C/141V/143A/160L/243L/311D/315V/372V, 135G/139C/141V/145E/160L/311D/315V/372V,
 135G/141V/145E/160L/311D/312I/315V/372V, 135G/141V/145E/160L/169S/311D/315V/372V,
 135G/139C/141V/143A/157G/160L/311D/312I/315V/372V,
 84M/135G/139C/143A/141V/160L/311D/315V/372V, 135G/141V/145E/160L/269Q/311D/315V/372V,
 135G/141V/143A/145E/157G/160L/269Q/311D/312I/315V/328L/372V,
 135G/141V/143A/145E/160L/269Q/311D/315V/372V, 135G/141V/157G/160L/311D/315V/372V, or
 135G/139L/141V/143A/160L/311D/312I/315V/372V, wherein the amino acid positions are relative to
 the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the
 reference sequence corresponding to SEQ ID NO: 4 or 628.

[0183] In some embodiments, the amino acid sequence of the engineered protease polypeptide
 comprises at least a substitution set or amino acid residues 135G/141V/160L/256L/311D/315V/372V,
 135G/141V/160L/311D/315V/372V/411T, 135G/141V/160L/273F/311D/315V/372V,
 135G/141V/160L/311D/315V/372V/409E, 135G/141V/160L/268G/311D/315V/372V,
 135G/141V/160L/172Q/311D/315V/372V, 135G/141V/160L/311D/315V/372V/409R,

135G/141V/160L/311D/315V/372V/401L, 135G/141V/160L/281C/311D/315V/372V,
 135G/141V/160L/311D/315V/372V/410W, 135G/141V/160L/253V/311D/315V/372V,
 135G/141V/160L/311D/315V/372V/406M, 135G/141V/160L/273T/311D/315V/372V,
 135G/141V/160L/311D/315V/372V/406R, 135G/141V/160L/256M/311D/315V/372V,
 135G/141V/160L/311D/315V/372V/410I, 135G/141V/160L/273M/311D/315V/372V,
 135G/141V/160L/273L/311D/315V/372V, 135G/141V/143A/145E/160L/243L/311D/315V/328L/372V,
 135G/141V/145E/160L/311D/315V/372V,
 135G/139C/141V/143A/145E/160L/311D/315V/328L/342G/345R/372V,
 135G/139L/141V/145E/160L/311D/315V/372V,
 135G/141V/143A/160L/311D/315V/328L/342G/345R/372V,
 135G/141V/145E/160L/311D/315V/342G/345R/372V, 135G/141V/143A/160L/311D/315V/372V,
 135G/141V/139C/143A/160L/311D/315V/372V,
 135G/139C/141V/145E/160L/311D/315V/328L/342G/345R/372V,
 135G/141V/143A/145E/160L/169S/311D/312I/315V/328L/345R/346T/372V,
 135G/141V/143A/160L/243L/311D/315V/328L/342G/345R/346T/372V,
 135G/139C/141V/143A/157G/160L/169S/311D/315V/328L/346T/372V,
 135G/143A/141V/145E/156V/160L/311D/312I/315V/328L/372V,
 135G/139C/141V/145E/157G/160L/311D/312I/315V/328L/372V,
 135G/141V/143A/160L/311D/315V/328L/342G/345R/346T/372V,
 135G/139C/141V/143A/160L/311D/315V/328L/372V, 135G/141V/143A/145E/160L/311D/315V/372V,
 135G/141V/143A/145E/160L/311D/312I/315V/342G/345R/372V, or
 135G/141V/143A/145E/160L/311D/315V/328L/372V, wherein the amino acid positions are relative to
 the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the
 reference sequence corresponding to SEQ ID NO: 4 or 628.

[0184] In some embodiments, the amino acid sequence of the engineered protease polypeptide
 comprises at least a substitution set at amino acid positions

135/141/143/160/279/311/315/328/342/345/372, 135/141/143/160/250/311/315/328/342/345/372,
 135/141/143/154/160/311/315/328/342/345/372, 135/141/143/160/214/311/315/328/342/345/372,
 135/141/143/160/249/311/315/328/342/345/372, 135/141/143/160/275/311/315/328/342/345/372,
 135/137/141/143/160/311/315/328/342/345/372, 135/141/143/160/161/311/315/328/342/345/372,
 135/141/143/160/180/311/315/328/342/345/372, 135/141/143/160/174/311/315/328/342/345/372,
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 135/141/143/145/160/311/315/328/342/345/372, 135/141/143/160/278/311/315/328/342/345/372,
 135/136/141/143/160/311/315/328/342/345/372, 135/141/143/154/160/311/315/328/342/345/372/413,
 135/141/143/160/294/311/315/328/342/345/372, 135/141/143/160/237/311/315/328/342/345/372,
 135/141/143/160/311/315/328/342/345/372, 135/141/143/160/274/311/315/328/342/345/372,
 135/141/143/160/264/311/315/328/342/345/372, 135/141/143/160/185/311/315/328/342/345/372,

135/141/143/160/277/311/315/328/342/345/372, 135/141/143/160/293/311/315/328/342/345/372,
135/141/143/160/233/311/315/328/342/345/372, 135/141/143/160/173/311/315/328/342/345/372,
135/141/143/160/311/312/315/328/342/345/372, 135/141/143/160/302/311/315/328/342/345/372,
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135/141/143/160/263/311/315/328/342/345/372, 135/141/143/160/267/311/315/328/342/345/372,
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135/141/143/160/292/311/315/328/342/345/372, 135/141/143/160/246/311/315/328/342/345/372,
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135/141/143/156/160/311/315/328/342/345/372, 135/141/143/160/223/311/315/328/342/345/372,
135/141/143/160/278/311/315/328/342/345/372/413, 135/141/143/160/297/311/315/328/342/345/372,
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135/141/143/145/157/160/253/268/273/281/311/312/315/328/342/345/346/411/372,
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135/139/141/143/160/253/268/273/281/311/312/315/328/342/345/346/372/411,
135/141/143/157/160/273/311/315/328/342/345/346/372/411,
135/139/141/143/145/157/160/162/253/273/281/311/312/315/328/342/345/372,
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135/141/143/157/160/273/311/312/315/328/342/345/346/372/411,
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135/139/141/143/160/273/311/312/315/328/342/345/346/372,
135/141/143/157/160/253/268/273/311/312/315/328/342/345/346/372.
135/139/141/143/160/268/311/315/328/342/345/346/372,
135/141/143/160/268/273/311/312/315/328/342/345/346/372.
135/139/141/143/157/160/253/268/273/311/312/315/328/342/345/372,
135/139/141/143/157/160/253/311/315/328/342/345/372,
135/139/141/143/160/253/281/311/315/328/342/345/372,
135/139/141/143/157/160/253/268/273/311/315/328/342/345/372,
135/141/143/160/253/311/312/315/328/342/345/372/411, or
135/139/141/143/160/268/273/311/315/328/342/345/372, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0185] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set or amino acid residues

135G/141V/143A/160L/279Y/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/250T/311D/315V/328L/342G/345R/372V,
135G/141V/143A/154C/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/214Y/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/249S/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/275A/311D/315V/328L/342G/345R/372V,
135G/137A/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/161D/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/180H/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/174L/311D/315V/328L/342G/345R/372V,
135G/139K/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/254C/311D/315V/328L/342G/345R/372V,
135G/141V/143A/145E/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/278V/311D/315V/328L/342G/345R/372V,
135G/136M/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/154L/160L/311D/315V/328L/342G/345R/372V/413D,
135G/141V/143A/160L/294V/311D/315V/328L/342G/345R/372V,

135G/141V/143A/154R/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/237A/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/274G/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/264P/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/274T/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/278N/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/214A/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/185G/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/214V/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/279T/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/277G/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/264I/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/293A/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/233L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/278Y/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/173F/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/274L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/311D/312C/315V/328L/342G/345R/372V,
135G/141V/143A/160L/279L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/302G/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/238Q/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/294W/311D/315V/328L/342G/345R/372V,
141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/221E/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/290S/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/278S/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/263Q/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/263H/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/278L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/263E/311D/315V/328L/342G/345R/372V,
135G/141V/143A/154L/160L/311D/315V/328L/342G/345R/372V,
135G/139M/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/137S/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/233G/311D/315V/328L/342G/345R/372V,
135G/139F/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/267I/311D/315V/328L/342G/345R/372V,

135G/141V/143A/160L/221L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/173S/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/302P/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/221V/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/239M/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/290G/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/163H/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/292V/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/246V/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/214N/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/243S/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/233I/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/235Q/311D/315V/328L/342G/345R/372V,
135G/141V/143A/145D/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/274V/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/279M/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/185S/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/279K/311D/315V/328L/342G/345R/372V,
135G/141V/143A/145W/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/290E/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/214P/311D/315V/328L/342G/345R/372V,
135G/141V/143A/156V/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/156C/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/223S/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/278V/311D/315V/328L/342G/345R/372V/413D,
135G/141V/143A/160L/250C/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/267S/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/297F/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/221Q/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/194D/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/251T/311D/315V/328L/342G/345R/372V,
135G/141V/143A/145P/157R/160L/253I/268G/273T/281V/311D/312Q/315V/328L/342G/345R/346T/4
11T/372V, 135G/139C/141V/143A/160L/311D/315V/328L/342G/345R/346A/372V,
135G/141V/143A/160L/253V/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/311D/315V/328L/342G/345R/346T/372V/411T,
135G/141V/143A/160L/253V/311D/315V/328L/342G/345R/346T/372V,

135G/139C/141V/143A/160L/311D/315V/328L/342G/345R/346T/372V,
135G/141V/143A/160L/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/141V/143A/160L/273T/311D/S312Q/315V/328L/342G/345R/372V,
135G/141V/143A/160L/253I/281V/311D/315V/328L/342G/345R/372V,
135G/141V/143A/157R/160L/253V/273T/311D/312Q/315V/328L/342G/345R/346T/372V/411T,
135G/139C/141V/143A/157K/160L/253V/268G/273F/281V/311D/312Q/315V/328L/342G/345R/346A/
372V, 135G/141V/143A/160L/253V/273T/311D/315V/328L/342G/345R/372V/411T,
135G/139C/141V/143A/160L/253I/268F/273T/281V/311D/315V/328L/342G/345R/372V,
135G/139C/141V/143A/157R/160L/311D/315V/328L/342G/345R/372V/411T,
135G/141V/143A/157G/160L/311D/315V/328L/342G/345R/372V,
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135G/139C/141V/143A/160L/253V/268G/273F/281V/311D/312Q/315V/328L/342G/345R/372V/411T,
135G/141V/143A/157G/160L/253V/311D/315V/328L/342G/345R/372V/411T,
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135G/139C/141V/143A/160L/311D/315V/328L/342G/345R/372V/411T,
135G/139C/141V/143A/160L/253V/268G/273F/281C/311D/312I/315V/328L/342G/345R/346T/372V/4
11T, 135G/141V/143A/157K/160L/273F/311D/315V/328L/342G/345R/346T/372V/411T,
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72V, 135G/139C/141V/143A/160L/253V/273T/281C/311D/312Q/315V/328L/342G/345R/372V,
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135G/139C/141V/143A/160L/253I/268F/311D/315V/328L/342G/345R/372V,
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135G/139C/141V/143A/160L/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
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135G/139C/141V/143A/160L/268G/311D/315V/328L/342G/345R/346T/372V,
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135G/139C/141V/143A/157K/160L/253V/268F/273F/311D/312Q/315V/328L/342G/345R/372V,
135G/141V/143A/157K/160L/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/253V/311D/315V/328L/342G/345R/372V,

135G/139C/141V/143A/160L/311D/315V/328L/342G/345R/372V,
135G/139C/141V/143A/160L/253V/281V/311D/315V/328L/342G/345R/372V,
135G/139C/141V/143A/157G/160L/253V/268G/273T/311D/315V/328L/342G/345R/372V,
135G/141V/143A/160L/253V/311D/312Q/315V/328L/342G/345R/372V/411T,
135G/139C/141V/143A/160L/268G/273T/311D/315V/328L/342G/345R/372V, or
135G/141V/143A/160L/253I/311D/315V/328L/342G/345R/372V, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0186] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set at amino acid positions

135/139/141/143/157/160/268/273/311/312/315/31/328/342/345/346/372,
135/139/141/143/157/160/268/273/311/312/315/318/328/342/345/346/372,
135/139/141/143/157/160/268/273/296/311/312/315/328/342/345/346/372,
135/139/141/143/157/160/252/268/273/311/312/315/328/342/345/346/372,
135/139/141/143/157/160/268/273/303/311/312/315/328/342/345/346/372,
135/139/141/143/157/160/253/268/273/311/312/315/328/342/345/346/372,
135/139/141/143/157/160/268/273/311/312/315/328/342/345/346/372/413,
135/139/141/143/157/160/268/273/311/312/315/328/342/345/346/372/386,
135/139/141/143/157/160/268/273/311/312/315/328/342/345/346/372,
135/139/141/143/157/160/235/268/273/311/312/315/328/342/345/346/372,
135/139/141/143/157/160/268/273/311/312/315/328/342/345/346/372/412,
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135/139/141/143/157/160/268/273/311/312/315/328/342/345/346/371/372,
135/139/141/143/157/160/268/273/311/312/315/328/342/345/346/372/405,
135/139/141/143/157/160/268/273/311/312/315/328/342/345/346/372/389,
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139/157/160/311/315/328/342/345/346, 135/157/160/268/273/312/315/328/342/345/346/372,
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135/137/139/141/143/157/160/268/273/311/312/315/328/342/345/346/372/413,
135/137/139/141/143/157/160/221/268/273/279/311/312/315/328/342/345/346/372, or
135/137/141/143/156/157/160/214/233/268/273/311/312/315/328/342/345/346/372/413, wherein the
amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID
NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0187] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set or amino acid residues

31G/135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/318R/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/296R/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/296M/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/252P/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/303A/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/253C/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/413C,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/386P,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/413A,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312R/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/235R/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/412P,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342A/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/413S,
135G/139C/141V/143A/157G/160L/268G/273T/302P/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/371L/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/405L,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312A/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/389P,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/318P/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/391S,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/412T,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/358S/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/389C,
135G/139C/141V/143A/157G/160L/235V/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/391L,
135G/141V/143A/157G/160L/268G/312Q/315V/342G/345R/346T,
135G/139C/141V/143A/157G/160L/268G/273T/312Q/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/315V/328L/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T,
135G/141V/312Q/328L/342G/345R/346T/372V,
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135G/141V/143A/157G/268G/273T/311D/315V/328L/342G/345R/346T,

135G/139C/141V/157G/160L/268G/311D/312Q/315V/342G/345R/346T/372V,
135G/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/372V,
141V/143A/157G/273T/311D/315V/328L/345R/372V,
135G/143A/157G/160L/268G/311D/312Q/315V/328L/342G/345R/346T/372V,
139C/157G/160L/311D/315V/328L/342G/345R/346T,
135G/157G/160L/268G/273T/312Q/315V/328L/342G/345R/346T/372V,
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53A/135G/157G/160L/268G/311D/312Q/315V/328L/342G/345R/346T,
135G/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/157G/160L/268G/311D/315V/328L/342G/345R/346T/372V,
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135G/137A/141V/143A/157G/160L/221Q/233L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/
372V/413D,
135G/139C/141V/143A/157G/160L/S233L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V
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372V,
135G/137N/141V/143A/157G/160L/233L/268G/273T/279M/311D/312Q/315V/328L/342G/345R/346T/
372V,
135G/139C/141V/143A/157G/160L/221Q/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/139L/141V/143A/157G/160L/214V/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
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372V,
135G/137A/139L/141V/143A/157G/160L/221Q/233L/268G/273T/279K/311D/312Q/315V/328L/342G/
345R/346T/372V,
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372V,
135G/137N/139L/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V,
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372V,
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346T/372V,

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346T/372V,
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346T/372V,
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372V,
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372V,
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72V,
135G/137N/141V/143A/157G/160L/214V/233I/268G/273T/311D/312Q/315V/328L/342G/345R/346T/3
72V,
135G/139C/141V/143A/157G/160L/221Q/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/
413D,
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R/346T/372V,
135G/137A/139L/141V/143A/157G/160L/214P/268G/273T/311D/312Q/315V/328L/342G/345R/346T/3
72V,
135G/137N/141V/143A/157G/160L/221Q/233L/268G/273T/Q279M/311D/312Q/315V/328L/342G/345
R/346T/372V,
135G/137N/139C/141V/143A/156V/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/
372V,
135G/137N/139L/141V/143A/157G/160L/221Q/233I/268G/273T/311D/312Q/315V/328L/342G/345R/3
46T/372V,
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372V,
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372V/413D,
135G/137A/139L/141V/143A/157G/160L/221Q/233L/268G/273T/279M/311D/312Q/315V/328L/342G/
345R/346T/372V,

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135G/137N/139C/141V/143A/157G/160L/S233L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/
/372V,
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135G/137N/139C/141V/143A/157G/160L/268G/273T/311D/312Q/315V/328L/342G/345R/346T/372V/
413D,
135G/137N/139C/141V/143A/157G/160L/221Q/268G/273T/279K/311D/312Q/315V/328L/342G/345R/
346T/372V,
135G/137N/141V/143A/T156V/157G/160L/214V/233L/268G/273T/311D/312Q/315V/328L/342G/345R/
/346T/372V/413D, or
135G/137N/139L/141V/143A/156V/157G/160L/214V/268G/273T/311D/312Q/315V/328L/342G/345R/
346T/372V, wherein the amino acid positions are relative to the reference sequence corresponding to
residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ
ID NO: 4 or 628.

[0188] In some embodiments, the amino acid sequence of the engineered protease polypeptide
comprises at least a substitution set at amino acid positions

135/137/139/141/143/145/157/160/214/268/273/279/311/312/315/328/342/345/346,
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 135/137/139/141/143/157/160/214/268/273/279/311/312/315/328/342/345/346/372,
 135/137/139/141/143/157/160/212/214/268/273/279/311/312/315/328/342/345/346/372,
 135/137/139/141/143/145/157/160/214/268/273/279/311/312/315/328/342/345/346/372,
 135/137/139/141/143/157/160/179/214/268/273/279/311/312/315/328/342/345/372,
 135/137/139/141/143/157/160/214/268/273/279/311/312/315/328/342/345/346/372/375,
 135/137/139/141/143/157/160/214/264/268/273/279/311/312/315/328/342/345/346/372,
 135/137/139/141/143/157/160/179/214/268/273/279/311/312/315/328/342/345/346/372,
 135/137/139/141/143/157/160/185/214/268/273/279/311/312/315/328/342/345/346/372,
 135/137/139/141/143/157/160/214/268/273/279/311/312/315/328/342/345/346/372,
 135/137/139/141/143/157/160/214/220/268/273/279/311/312/315/328/342/345/346/372, or
 135/137/139/141/143/157/160/214/268/273/279/311/312/315/324/328/342/345/346/372, wherein the
 amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID
 NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0189] In some embodiments, the amino acid sequence of the engineered protease polypeptide
 comprises at least a substitution set or amino acid residue

135G/137N/139L/141V/143A/145E/157G/160L/214P/268G/273L/279M/311D/312Q/315V/328L/342G/
 T345R/346T,

135G/137N/139L/141V/143A/145E/157G/160L/214P/256M/268G/273L/279M/311D/312Q/315V/328L/
 342G/345R/346T/372V,

135G/137N/139L/141V/143A/157G/160L/214P/221Q/243L/268G/273L/279M/311D/312Q/315V/342G/
 345R/346T,

135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315V/328L/342G/345R/
 346T,

135G/137N/139L/141V/143A/145E/157G/160L/214P/221Q/268G/273T/279L/311D/312Q/315V/328L/3
 42G/345R/346T/372V/406R,

135G/137N/139L/141V/143A/157G/160L/214P/268G/273L/279M/311D/312Q/315V/342G/345R/346T/

372V,
135G/137N/139L/141V/143A/145E/157G/160L/169S/214P/268G/273L/279M/311D/312Q/315V/328L/342G/345R/372V/406R,
135G/137N/139L/141V/143A/157G/160L/214P/256M/268G/273L/279M/311D/312Q/315V/328L/342G/345R/346T/372V.
135G/137N/139L/141V/143A/145E/157G/160L/214P/221Q/268G/273L/279M/311D/312Q/315V/342G/345R/372V/406R,
135G/137N/139L/141V/143A/157G/160L/214P/243L/268G/273L/279M/311D/312Q/315V/328L/342G/345R/346T/372V.
135G/137N/139L/141V/143A/145E/157G/160L/214V/256M/268G/273L/279L/311D/312Q/315V/342G/345R/346T,
135G/137N/139L/141V/143A/157G/160L/T169S/214P/221Q/268G/273T/279M/311D/312Q/315V/342G/345R/346T//406R,
135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315V/328L/342G/345R/346T/406R,
135G/137N/139L/141V/143A/157G/160L/169S/214P/268G/273T/279M/311D/312Q/315V/342G/345R/346T/372V/406R,
135G/137N/139L/141V/143A/157G/160L/214P/221Q/268G/273T/279M/311D/312Q/315V/328L/342G/345R/346T,
135G/137N/139L/141V/143A/145E/157G/160L/214P/221Q/268G/273T/279L/311D/312Q/315V/342G/345R/346T,
135G/137N/139L/141V/143A/157G/160L/214V/243L/268G/273L/279M/311D/312Q/315V/342G/345R/346T/372V,
135G/137N/139L/141V/143A/145E/157G/160L/214P/221Q/268G/273T/279M/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/137N/139L/141V/143A/157G/160L/214V/256M/268G/273L/279M/311D/312Q/315V/328L/342G/345R,
135G/137N/139L/141V/143A/157G/160L/214P/221Q/268G/273T/279M/311D/312Q/315V/328L/342G/345R/346T/372V/406R,
135G/137N/139L/141V/143A/157G/160L/T169S/214P/268G/273T/279M/311D/312Q/315V/328L/342G/345R/346T,
135G/137N/139L/141V/143A/145E/157G/160L/214V/221Q/268G/273L/279M/311D/312Q/315V/328L/342G/345R/346T/372V,
135G/137N/139L/141V/143A/145E/157G/160L/214P/221Q/268G/273T/279M/311D/312Q/315V/328L/342G/345R,
135G/137N/139L/141V/143A/157G/160L/214P/243L/268G/273L/279M/311D/312Q/315V/342G/345R/346T/406R,

135G/137N/139L/141V/143A/145E/157G/160L/169S/214P/268G/273L/279M/311D/312Q/315V/342G/
345R/372V,
135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315V/342G/345R/346T/
372V,
135G/137N/139L/141V/143A/157G/160L/169S/214P/268G/273L/279M/311D/312Q/315V/328L/342G/
345R/346T,
135G/137N/139L/141V/143A/145E/157G/160L/214P/221Q/268G/273T/279M/311D/312Q/315V/342G/
345R/346T/372V.
135G/137N/139L/141V/143A/157G/160L/169S/214V/268G/273L/279M/311D/312Q/315V/328L/342G/
345R/346T/372V.
135G/137N/139L/141V/143A/157G/160L/214P/221Q/268G/273L/279M/311D/312Q/315V/342G/345R/
346T/372V,
135G/137N/139L/141V/143A/157G/160L/214P/221Q/268G/273T/279M/311D/312Q/315V/328L/342G/
345R/346T/372V,
135G/137N/139L/141V/143A/145E/157G/160L/214P/268G/273T/279M/311D/312Q/315V/342G/345R/
346T/372V,
135G/137N/139L/141V/143A/157G/160L/214N/268G/273T/279M/311D/312Q/315V/328L/342G/345R/
372V,
135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312R/315V/328L/342G/345R/
346T/372V,
135G/137N/139L/141V/143A/157G/160L/212S/214P/268G/273T/279M/311D/312Q/315V/328L/342G/
345R/346T/372V,
135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279K/311D/312Q/315V/328L/342G/345R/3
46T/372V,
135G/137N/139L/141V/143A/157G/160L/212S/214P/268G/273T/279M/311D/312R/315V/328L/342G/3
45R/346T/372V,
135G/137N/139L/141V/143A/145E/157G/160L/214P/268G/273T/279M/311D/312Q/315V/328L/342G/
345R/346T/372V,
135G/137N/139L/141V/143A/157G/160L/179S/214P/268G/273T/279M/311D/312Q/315V/328L/342G/
345R/372V,
135G/137N/139L/141V/143A/157G/160L/214N/268G/273T/279M/311D/312Q/315V/328L/342G/345R/
346T/372V,
135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315V/328L/342G/345R/
372V,
135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315R/328L/342G/345R/
346T/372Y,
135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315V/328L/342G/345R/

346T/372Y,
 135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315V/328L/342G/345R/
 346T/372V/375A,
 135G/137N/139L/141V/143A/157G/160L/214P/264F/268G/273T/279M/311D/312Q/315V/328L/342G/
 345R/346T/372V,
 135G/137N/139L/141V/143A/157G/160L/179S/214P/268G/273T/279M/311D/312Q/315V/328L/342G/
 345R/346T/372V,
 135G/137N/139L/141V/143A/157G/160L/185T/214P/268G/273T/279M/311D/312Q/315V/328L/342G/
 345R/346T/372V,
 135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315V/328L/342G/345R/
 346T/372V,
 135G/137N/139L/141V/143A/157G/160L/214P/220R/268G/273T/279M/311D/312Q/315V/328L/342G/
 345R/346T/372Y,
 135G/137N/139L/141V/143A/157G/160L/214P/268G/273T/279M/311D/312Q/315V/324D/328L/342G/
 345R/346T/372V, or
 135G/137N/139L/141V/143A/145E/157G/160L/214P/221Q/268G/273L/279M/311D/312Q/315V/342G/
 345R/346T, wherein the amino acid positions are relative to the reference sequence corresponding to
 residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ
 ID NO: 4 or 628.

[0190] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at an amino acid position set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0191] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0192] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0193] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set of an engineered protease polypeptide variant set forth

in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0194] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to an amino acid sequence comprising at least a substitution or substitution set of an engineered protease polypeptide set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0195] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0196] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242.

[0197] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or to the reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0198] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413

of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0199] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 11, 31, 42, 45, 50, 53, 84, 99, 100, 126, 128, 129, 130, 131, 132, 134, 135, 136, 137, 138, 139, 140, 141, 143, 145, 151, 154, 156, 157, 159, 160, 161, 162, 163, 169, 172, 173, 174, 179, 180, 184, 185, 186, 187, 188, 190, 191, 192, 193, 194, 198, 199, 212, 214, 220, 221, 222, 223, 225, 231, 232, 233, 235, 237, 238, 239, 240, 242, 243, 245, 246, 249, 250, 251, 252, 253, 254, 256, 258, 262, 263, 264, 265, 266, 267, 268, 269, 271, 273, 274, 275, 277, 278, 279, 280, 281, 283, 285, 290, 292, 293, 294, 296, 297, 300, 302, 303, 311, 312, 313, 314, 315, 316, 318, 324, 328, 336, 339, 341, 342, 343, 345, 346, 355, 358, 360, 364, 367, 368, 369, 370, 371, 372, 373, 374, 375, 377, 381, 382, 384, 386, 389, 391, 392, 401, 402, 405, 406, 409, 410, 411, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0200] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 11K, 31G, 42W, 45Y, 50R, 53A, 84M, 99V, 100V, 126T, 128G/I/K/L/P/R/S/T/V, 129E/F/H/I/K/L/R/S/T/V, 130A/F/G/N/V, 131E/P/R/T/V/Y, 132A/C/D/E/G/P/R/V/Y, 134A/C/D/E/G/I/L/M/N/P/S/T/V/W/Y, 135A/C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 136C/G/I/M, 137A/D/N/S, 138Q, 139C/D/E/F/H/I/K/L/M/N/R/S, 140L, 141A/C/D/E/F/G/H/I/L/M/N/Q/R/S/T/V/W/Y, 143A/C/D/H/N/Q/S/T, 145A/C/D/E/F/G/H/I/K/L/P/Q/R/S/T/V/W, 151D/Q, 154C/D/L/R, 156C/V, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/S/T/V/W, 159G, 160A/C/D/E/F/K/L/M/N/P/R/Q/S/T/V/W/Y, 161D/E/G/L/R, 162I, 163H/L, 169S, 172Q, 173F/S, 174L, 179K/S, 180H/L/M, 184A/D/G/L/M/Q/R, 185A/D/E/F/G/L/M/P/Q/R/S/T/V, 186A/R/S/T/Y, 187A, 188A/C/D/F/G/L/M/S/T/W, 190S, 191R, 192C/D/M/N, 193T, 194A/D/L/T, 198G, 199C/K/L, 212S, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 220K/L/R, 221A/C/D/E/F/G/H/I/K/L/M/P/Q/R/T/V/W/Y, 222G, 223S, 225V, 231H/V, 232S, 233G/I/L, 235Q/R/V, 237A/G, 238Q, 239L/M, 240A/L, 242E/S, 243E/L/M/R/S/T, 245L/V, 246I/V, 249G/M/S, 250A/C/F/L/N/T, 251D/S/T, 252P, 253C/I/V, 254C/E, 256L/M, 258W, 262A/S, 263E/H/P/Q/R/S, 264A/C/F/I/L/N/P/R/T/V, 265C/G/R, 266H/T/Y, 267A/G/H/I/L/M/R/S/T/V/W, 268A/F/G/H/I/N/P/Q/S/T/V/Y, 269Q/T, 271A, 273A/C/F/L/M/S/T/V, 274A/G/K/L/T/V/W, 275A/V, 277D/G, 278L/N/S/V/Y, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 280D/K/S/T, 281C/V, 283M, 285S, 290E/G/S, 292V, 293A, 294V/W, 296M/R, 297F, 300R/V, 302G/P, 303A/V, 311A/E/D/G/K/M/Q/S/T, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/S/T/V/W/Y, 313A/Q/S/T, 314G, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/T/V/W/Y, 316K, 318N/P/R, 324A/D/E/I/R/V/W/Y, 328L/M/V, 336F, 339S/W, 341G, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/S/T/V/W/Y, 343S,

345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/S/T/V/W/Y, 355A, 358S, 360S, 364A/V, 367V, 368G/T, 369I/V/W, 370C/E/F/G/I/K/L/P/Q/R/S/V, 371L, 372A/C/F/L/R/S/V/Y, 373A/C/E/F/M/S/Y, 374E/G/L/R/S/W/Y, 375A/E/I/L/M/S/T/V, 377H, 381N, 382G/R/S/T, 384C, 386P/W, 389C/P, 391L/S, 392Y, 401L, 402G/*, 405L/Q, 406C/M/R/W, 409E/R/*, 410C/I/W/*, 411L/R/T/V, 412P/T/*, or 413A/C/D/S/*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0201] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 157, 160, 214, 268, 273, 279, 311, 312, 315, 328, 342, 345, 346, or 372, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0202] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135A/C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 137A/D/N/S, 139C/D/E/F/H/I/K/L/M/N/R/S, 141A/C/D/E/F/G/H/I/L/M/N/Q/R/S/T/V/W/Y, 143A/C/D/H/N/Q/S/T, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/S/T/V/W, 160A/C/D/E/F/K/L/M/N/P/R/Q/S/T/V/W/Y, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 268A/F/G/H/I/N/P/Q/S/T/V/Y, 273A/C/F/L/M/S/T/V, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 311A/E/D/G/K/M/Q/S/T, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/S/T/V/W/Y, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/T/V/W/Y, 328L/M/V, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/S/T/V/W/Y, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/S/T/V/W/Y, or 372A/C/F/L/R/S/V/Y, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0203] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135G, 137N, 139L/C, 141V, 143A, 157G, 160L, 214P, 268G, 273T, 279M, 311D, 312Q, 315V, 328L, 342G, 345R, 346T, or 372V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0204] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or to the reference sequence corresponding to

SEQ ID NO: 948, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0205] In some embodiments, the engineered protease polypeptide of comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 950-1154, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 950-1154, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0206] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 411, 402, 285, 245, 266, 355, 258, 222, 140, 268, 225, 283, 406, 410, 143/145/243/312, 139/143/145/157/312, 139/157/345, 139/269, 156/157/342/346, 139/143, 269, 139/243/328, 269/328, 143/145/169, 328, 143/145/262, 145/262/312/328, 139/156/157, 139/145/312, 312, 139, 139/312, 139/156, 139/143/145/243, 145/157, 145/346, 145/262/312/328/345/346, 145/262, 312/342, 143/243, 139/345, 342, 143/145/262/342, 139/143/169, 139/143/145/312, 169, 139/145/262/312/328/342/345/346, 139/328, 139/243, 139/143/328, 139/143/243, 139/145, 145/312, 145/169, 139/143/157/312, 84/139/143, 145/269, 143/145/157/269/312/328, 143/145/269, 157, 139/143/312, 256, 273, 409, 172, 401, 281, 253, 143/145/243/328, 145, 139/143/145/328/342/345, 143/328/342/345, 145/342/345, 143, 139/145/328/342/345, 143/145/169/312/328/345/346, 143/243/328/342/345/346, 139/143/157/169/328/346, 143/145/156/312/328, 139/145/157/312/328, 143/328/342/345/346, 143/145, 143/145/312/342/345, or 143/145/328, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0207] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 411V, 411R, 402G, 285S, 245V, 266H, 355A, 258W, 222G, 140L, 268T, 411L, 225V, 245L, 283M, 406W, 410C, 406C, 143A/145E/243L/312I, 139L/143A/145E/157G/312I, 139C/157G/345R, 139C/269Q, 156V/157G/342G/346T, 139L/143A, 269Q, 139L/243L/328L, 269Q/328L, 143A/145E/169S, 328L, 143A/145E/262S, 145E/262S/312I/328L, 139L/156V/157G, 139C/145E/312I, 312I, 139C, 139C/312I, 139C/156V, 139C/143A/145E/243L, 145E/157G, 145E/346T, 145E/262A/312I/328L/345R/346T, 145E/262A, 312I/342G, 143A/243L, 139C/345R, 342G, 143A/145E/262S/342G, 139L/143A/169S, 139C/143A/145E/312I, 169S, 139L/145E/262A/312I/328L/342G/345R/346T, 139C/328L, 139L/243L, 139L/143A/328L, 139L, 139C/143A/243L, 139C/145E, 145E/312I, 145E/169S, 139C/143A/157G/312I,

84M/139C/143A, 145E/269Q, 143A/145E/157G/269Q/312I/328L, 143A/145E/269Q, 157G, or 139L/143A/312I, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0208] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set S411V, S411R, A402G, A285S, I245V, N266H, P355A, M258W, A222G, Q140L, S268T, S411L, I225V, I245L, V283M, A406W, G410C, A406C, H143A/N145E/Q243L/S312I, N139L/H143A/N145E/S157G/S312I, N139C/S157G/T345R, N139C/M269Q, T156V/S157G/S342G/S346T, N139L/H143A, M269Q, N139L/Q243L/V328L, M269Q/V328L, H143A/N145E/T169S, V328L, H143A/N145E/G262S, N145E/G262S/S312I/V328L, N139L/T156V/S157G, N139C/N145E/S312I, S312I, N139C, N139C/S312I, N139C/T156V, N139C/H143A/N145E/Q243L, N145E/S157G, N145E/S346T, N145E/G262A/S312I/V328L/T345R/S346T, N145E/G262A, S312I/S342G, H143A/Q243L, N139C/T345R, S342G, H143A/N145E/G262S/S342G, N139L/H143A/T169S, N139C/H143A/N145E/S312I, T169S, N139L/N145E/G262A/S312I/V328L/S342G/T345R/S346T, N139C/V328L, N139L/Q243L, N139L/H143A/V328L, N139L, N139C/H143A/Q243L, N139C/N145E, N145E/S312I, N145E/T169S, N139C/H143A/S157G/S312I, V84M/N139C/H143A, N145E/M269Q, H143A/N145E/S157G/M269Q/S312I/V328L, H143A/N145E/M269Q, S157G, or N139L/H143A/S312I, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0209] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 256L, 411T, 273F, 409E, 268G, 172Q, 409R, 401L, 281C, 410W, 253V, 406M, 273T, 406R, 256M, 410I, 273M, 273L, 143A/145E/243L/328L, 145E, 139C/143A/145E/328L/342G/345R, 139L/145E, 143A/328L/342G/345R, 145E/342G/345R, 143A, 139C/143A, 139C/145E/328L/342G/345R, 143A/145E/169S/312I/328L/345R/346T, 143A/243L/328L/342G/345R/346T, 139C/143A/157G/169S/328L/346T, 143A/145E/156V/312I/328L, 139C/145E/157G/312I/328L, 143A/328L/342G/345R/346T, 139C/143A/328L, 143A/145E, 143A/145E/312I/342G/345R, or 143A/145E/328L, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0210] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set I256L, S411T, V273F, G409E, S268G, D172Q, G409R, H401L, T281C, G410W, A253V, A406M, V273T, A406R, I256M, G410I, V273M, V273L, H143A/N145E/Q243L/V328L, N145E, N139C/H143A/N145E/V328L/S342G/T345R, N139L/N145E, H143A/V328L/S342G/T345R, N145E/S342G/T345R, H143A, N139C/H143A,

N139C/N145E/V328L/S342G/T345R, H143A/N145E/T169S/S312I/V328L/T345R/S346T, H143A/Q243L/V328L/S342G/T345R/S346T, N139C/H143A/S157G/T169S/V328L/S346T, H143A/N145E/T156V/S312I/V328L, N139C/N145E/S157G/S312I/V328L, H143A/V328L/S342G/T345R/S346T, N139C/H143A/V328L, H143A/N145E, H143A/N145E/S312I/S342G/T345R, or H143A/N145E/V328L, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0211] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or to the reference sequence corresponding to SEQ ID NO: 1126, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0212] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1156-1422, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1156-1422, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0213] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s), or amino acid residue(s) 279, 250, 154, 214, 249, 275, 137, 161, 180, 174, 139, 254, 145, 278, 136, 154/413, 294, 237, 274, 264, 185, 277, 293, 233, 173, 312, 302, 238, 135, 221, 290, 263, 267, 239, 163, 292, 246, 243, 235, 156, 223, 278/413, 297, 194, 251, 253/411, 145/157/253/268/273/281/312/346/411, 139/346, 253, 346/411, 253/346, 312/346, 273/312, 253/281, 157/253/273/312/346/411, 139/157/253/268/273/281/312/346, 253/273/411, 139/253/268/273/281, 139/157/411, 157, 273, 139/253/268/273/281/312/411, 157/253/411, 139/145/253/346, 139/157/253/273/312, 139/157/268/273/312/346, 157/273/312/346, 139/411, 139/253/268/273/281/312/346/411, 157/273/346/411, 139/145/157/162/253/273/281/312, 139/253/273/281/312, 157/253/268/273/281/312, 139/253/268, 139/157/312, 253/273/281/346, 157/253/312/346/411, 157/273/312/346/411, 139/145/157/253/268/281/312, 139/273/312/346, 157/253/268/273/312/346, 139/268/346, 268/273/312/346, 139/157/253/268/273/312, 139/157/253, 139/253/281, 139/157/253/268/273, 253/312/411, or 139/268/273, wherein the amino acid positions are

relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0214] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 279Y, 250T, 154C, 214Y, 249S, 275A, 137A, 161D, 180H, 174L, 139K, 254C, 145E, 278V, 136M, 154L/413D, 294V, 154R, 237A, 274G, 137N, 264P, 274T, 278N, 214A, 185G, 214V, 279T, 277G, 264I, 293A, 233L, 278Y, 173F, 274L, 312C, 279L, 302G, 238Q, 294W, 135A, 221E, 290S, 278S, 263Q, 263H, 278L, 263E, 154L, 139M, 137S, 233G, 139F, 267I, 221L, 173S, 302P, 221V, 239M, 290G, 163H, 292V, 246V, 214N, 243S, 233I, 235Q, 145D, 274V, 279M, 185S, 279K, 145W, 290E, 214P, 156V, 156C, 223S, 278V/413D, 250C, 267S, 297F, 221Q, 194D, 251T, 253V/411T, 145P/157R/253I/268G/273T/281V/312Q/346T/411T, 139C/346A, 253V, 346T/411T, 253V/346T, 139C/346T, 312Q/346T, 273T/312Q, 253I/281V, 157R/253V/273T/312Q/346T/411T, 139C/157K/253V/268G/273F/281V/312Q/346A, 253V/273T/411T, 139C/253I/268F/273T/281V, 139C/157R/411T, 157G, 273T, 139C/253V/268G/273F/281V/312Q/411T, 157G/253V/411T, 139C/145E/253V/346T, 139C/157K/253V/273T/312Q, 139C/157G/268G/273T/312Q/346T, 157G/273T/312Q/346T, 139C/411T, 139C/253V/268G/273F/281C/312I/346T/411T, 157K/273F/346T/411T, 139C/145E/157G/162I/253V/273F/281V/312Q, 139C/253V/273T/281C/312Q, 157G/253V/268F/273F/281V/312Q, 139C/253I/268F, 139C/157R/312Q, 253V/273T/281C/346T, 157K/253V/312I/346T/411T, 157R/273T/312Q/346T/411T, 139C/145E/157K/253V/268G/281C/312Q, 139C/273T/312Q/346T, 157K/253V/268F/273T/312I/346T, 139C/268G/346T, 157K, 268G/273F/312Q/346T, 139C/157K/253V/268F/273F/312Q, 157K/273T/312Q/346T, 139C/157G/253V, 139C, 139C/253V/281V, 139C/157G/253V/268G/273T, 253V/312Q/411T, 139C/268G/273T, or 253I, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0215] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set Q279Y, D250T, A154C, S214Y, A249S, T275A, H137A, S161D, N180H, N174L, N139K, D254C, N145E, A278V, V136M, A154L/G413D, S294V, A154R, S237A, Q274G, H137N, G264P, Q274T, A278N, S214A, N185G, S214V, Q279T, V277G, G264I, S293A, S233L, A278Y, H173F, Q274L, S312C, Q279L, S302G, L238Q, S294W, G135A, S221E, D290S, A278S, G263Q, G263H, A278L, G263E, A154L, N139M, H137S, S233G, N139F, Q267I, S221L, H173S, S302P, S221V, Y239M, D290G, K163H, A292V, L246V, S214N, Q243S, S233I, S235Q, N145D, Q274V, Q279M, N185S, Q279K, N145W, D290E, S214P, T156V, T156C, T223S, A278V/G413D, D250C, Q267S, Y297F, S221Q, N194D, I251T, A253V/S411T, N145P/S157R/A253I/S268G/V273T/T281V/S312Q/S346T/S411T, N139C/S346A, A253V, S346T/S411T, A253V/S346T, N139C/S346T, S312Q/S346T, V273T/S312Q, A253I/T281V, S157R/A253V/V273T/S312Q/S346T/S411T,

N139C/S157K/A253V/S268G/V273F/T281V/S312Q/S346A, A253V/V273T/S411T, N139C/A253I/S268F/V273T/T281V, N139C/S157R/S411T, S157G, V273T, N139C/A253V/S268G/V273F/T281V/S312Q/S411T, S157G/A253V/S411T, N139C/N145E/A253V/S346T, N139C/S157K/A253V/V273T/S312Q, N139C/S157G/S268G/V273T/S312Q/S346T, S157G/V273T/S312Q/S346T, N139C/S411T, N139C/A253V/S268G/V273F/T281C/S312I/S346T/S411T, S157K/V273F/S346T/S411T, N139C/N145E/S157G/V162I/A253V/V273F/T281V/S312Q, N139C/A253V/V273T/T281C/S312Q, S157G/A253V/S268F/V273F/T281V/S312Q, N139C/A253I/S268F, N139C/S157R/S312Q, A253V/V273T/T281C/S346T, S157K/A253V/S312I/S346T/S411T, S157R/V273T/S312Q/S346T/S411T, N139C/N145E/S157K/A253V/S268G/T281C/S312Q, N139C/V273T/S312Q/S346T, S157K/A253V/S268F/V273T/S312I/S346T, N139C/S268G/S346T, S157K, S268G/V273F/S312Q/S346T, N139C/S157K/A253V/S268F/V273F/S312Q, S157K/V273T/S312Q/S346T, N139C/S157G/A253V, N139C, N139C/A253V/T281V, N139C/S157G/A253V/S268G/V273T, A253V/S312Q/S411T, N139C/S268G/V273T, or A253I, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0216] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or to the reference sequence corresponding to SEQ ID NO: 1368, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0217] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1424-1608, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1424-1608, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0218] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 31, 318, 296, 252, 303, 253, 413, 386, 312, 235, 412, 342, 302, 371, 405, 389, 391, 358, 139/273/311/328/372, 311, 372, 139/143/157/160/268/273/311/315, 143, 139/143, 139/160/312/372, 143/273/328, 346, 135/139/160/268/312/342/346, 139/141/273, 135/141/143/268/273/312/372, 139/141/143/311,

139/157/268/328/346/372, 53/139/141/143/273/372, 139, 139/141/143/273/312, 137/139/221/233/413, 233, 221/279, 137/139/233/279, 221, 139/214, 137/139/156, 139/214/221, 214/233, 137/139/221/233/279, 137/139/221, 137/139, 137/139/279, 137, 137/139/214/279, 266, 139/221, 137/221, 137/156/214/312, 137/221/233, 137/139/156/221, 137/139/214, 279, 137/139/233, 137/139/214/233, 221/413, 137/214/233, 137/156, 137/139/221/233, 214/221, 137/221/413, 214, 137/233, 137/413, 137/221/279, 137/139/156/214/233/413, or 137/139/156/214, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0219] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 31G, 318R, 296R, 296M, 252P, 303A, 253C, 413C, 386P, 413A, 312R, 235R, 412P, 342A, 413S, 302P, 371L, 405L, 312A, 389P, 318P, 391S, 412T, 358S, 389C, 235V, 391L, 139N/273V/311T/328V/372S, 311T, 312S, 372S, 139N/143H/157S/160S/268S/273V/311T/315T, 143H, 139N/143H, 139N/160S/312S/372S, 143H/273V/328V, 346S, 135A/139N/160S/268S/312S/342S/346S, 139N/141N/273V, 135A/141N/143H/268S/273V/312S/372S, 139N/141N/143H/311T, 139N/157S/268S/328V/346S/372S, 53A/139N/141N/143H/273V/372S, 139N, 139N/141N/143H/273V/312S, 139L, 137A/139N/221Q/233L/413D, 233L, 221Q/279K, 137N/139N/233L/279M, 221Q, 139L/214V, 137N/139N/156V, 139L/214V/221Q, 214V/233L, 137A/139L/221Q/233L/279K, 137A/139L/221Q, 137N/139L, 137N/139L/279K, 137A/139N, 137N, 137N/139L/221Q, 137N/139L/214P/279M, 266Y, 139L/221Q, 137N/221Q, 137N/139N, 137N/156V/214V/312C, 137N/221Q/233L, 137N/139N/156V/221Q, 137A/139L/214V, 413D, 279K, 279M, 137A/139L/233L, 137N/139N/214V/233L, 221Q/413D, 137N/214V/233L, 137A/139L/214P, 137N/139N/221Q/233L/279M, 137N/156V, 137N/139L/221Q/233L, 214V/221Q, 137N/221Q/413D, 137A/139L/221Q/233L/279M, 214V, 137A/139L, 137N/233L, 137A, 137N/413D, 137N/221Q/279K, 137N/139N/156V/214V/233L/413D, or 137N/139L/156V/214V, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0220] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set D31G, S318R, S296R, S296M, D252P, S303A, A253C, G413C, E386P, G413A, Q312R, S235R, G412P, G342A, G413S, S302P, I371L, V405L, Q312A, S389P, S318P, T391S, G412T, A358S, S389C, S235V, T391L, C139N/T273V/D311T/L328V/V372S, D311T, Q312S, V372S, C139N/A143H/G157S/L160S/G268S/T273V/D311T/V315T, A143H, C139N/A143H, C139N/L160S/Q312S/V372S, A143H/T273V/L328V, T346S, G135A/C139N/L160S/G268S/Q312S/G342S/T346S, C139N/V141N/T273V, G135A/V141N/A143H/G268S/T273V/Q312S/V372S, C139N/V141N/A143H/D311T,

C139N/G157S/G268S/L328V/T346S/V372S, T53A/C139N/V141N/A143H/T273V/V372S, C139N, C139N/V141N/A143H/T273V/Q312S, C139L, H137A/C139N/S221Q/S233L/G413D, S233L, S221Q/Q279K, H137N/C139N/S233L/Q279M, S221Q, C139L/S214V, H137N/C139N/T156V, C139L/S214V/S221Q, S214V/S233L, H137A/C139L/S221Q/S233L/Q279K, H137A/C139L/S221Q, H137N/C139L, H137N/C139L/Q279K, H137A/C139N, H137N, H137N/C139L/S221Q, H137N/C139L/S214P/Q279M, N266Y, C139L/S221Q, H137N/S221Q, H137N/C139N, H137N/T156V/S214V/Q312C, H137N/S221Q/S233L, H137N/C139N/T156V/S221Q, H137A/C139L/S214V, G413D, Q279K, Q279M, H137A/C139L/S233L, H137N/C139N/S214V/S233L, S221Q/G413D, H137N/S214V/S233L, H137A/C139L/S214P, H137N/C139N/S221Q/S233L/Q279M, H137N/T156V, H137N/C139L/S221Q/S233L, S214V/S221Q, H137N/S221Q/G413D, H137A/C139L/S221Q/S233L/Q279M, S214V, H137A/C139L, H137N/S233L, H137A, H137N/G413D, H137N/S221Q/Q279K, H137N/C139N/T156V/S214V/S233L/G413D, or H137N/C139L/T156V/S214V, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0221] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or to the reference sequence corresponding to SEQ ID NO: 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0222] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1610-1710, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1610-1710, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0223] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 145/273/372, 145/256/273, 221/243/273/328/372, 372, 145/221/279/372/406, 273/328, 145/169/273/346/406, 256/273, 145/221/273/328/346/406, 243/273, 145/214/256/273/279/328/372, 169/221/328/372/406, 372/406, 169/328/372/406, 221/372, 145/221/273/328/372, 214/243/273/328, 145/221, 214/256/273/346/372, 221/406, 169/372, 145/214/221/273, 145/221/346/372, 243/273/328/372/406, 145/169/273/328/346, 328, 169/273/372, 145/221/328, 169/214/273, 221/273/328, 221, 145/328, 214/346, 312, 212, 279, 212/312,

145, 179/346, 214, 346, 315/372, 375, 264, 179, 185, 220/372, or 324, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0224] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set, or amino acid residue(s) 145E/273L/372S, 145E/256M/273L, 221Q/243L/273L/328V/372S, 372S, 145E/221Q/279L/372S/406R, 273L/328V, 145E/169S/273L/346S/406R, 256M/273L, 145E/221Q/273L/328V/346S/406R, 243L/273L, 145E/214V/256M/273L/279L/328V/372S, 169S/221Q/328V/372S/406R, 372S/406R, 169S/328V/372S/406R, 221Q/372S, 145E/221Q/273L/328V/372S, 214V/243L/273L/328V, 145E/221Q, 214V/256M/273L/346S/372S, 221Q/406R, 169S/372S, 145E/214V/221Q/273L, 145E/221Q/346S/372S, 243L/273L/328V/372S/406R, 145E/169S/273L/328V/346S, 328V, 169S/273L/372S, 145E/221Q/328V, 169S/214V/273L, 221Q/273L/328V, 221Q, 145E/328V, 214N/346S, 312R, 212S, 279K, 212S/312R, 145E, 179S/346S, 214N, 346S, 315R/372Y, 372Y, 375A, 264F, 179S, 185T, 220R/372Y, 324D, 145E/221Q/273L/328V/372S, or 145E/221Q/273L/328V/372S, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0225] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set N145E/T273L/V372S, N145E/I256M/T273L, S221Q/Q243L/T273L/L328V/V372S, V372S, N145E/S221Q/M279L/V372S/A406R, T273L/L328V, N145E/T169S/T273L/T346S/A406R, I256M/T273L, N145E/S221Q/T273L/L328V/T346S/A406R, Q243L/T273L, N145E/P214V/I256M/T273L/M279L/L328V/V372S, T169S/S221Q/L328V/V372S/A406R, V372S/A406R, T169S/L328V/V372S/A406R, S221Q/V372S, N145E/S221Q/T273L/L328V/V372S, P214V/Q243L/T273L/L328V, N145E/S221Q, P214V/I256M/T273L/T346S/V372S, S221Q/A406R, T169S/V372S, N145E/P214V/S221Q/T273L, N145E/S221Q/T346S/V372S, Q243L/T273L/L328V/V372S/A406R, N145E/T169S/T273L/L328V/T346S, L328V, T169S/T273L/V372S, N145E/S221Q/L328V, T169S/P214V/T273L, S221Q/T273L/L328V, S221Q, N145E/L328V, P214N/T346S, Q312R, Y212S, M279K, Y212S/Q312R, N145E, A179S/T346S, P214N, T346S, V315R/V372Y, V372Y, Q375A, G264F, A179S, N185T, Q220R/V372Y, S324D, N145E/S221Q/T273L/L328V/V372S, or N145E/S221Q/T273L/L328V/V372S, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0226] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at an amino acid position set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence

corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0227] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0228] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0229] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set of a reference engineered protease polypeptide set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0230] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence comprising at least a substitution or substitution set of an engineered protease polypeptide variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0231] In some embodiments, the amino acid sequence of the engineered protease polypeptide comprises amino acid residues 135-413, or comprises amino acid residues 128-413, wherein the engineered protease polypeptide is proteolytically active or is an active protease.

[0232] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to an amino acid sequence comprising residues 135-413 of an amino acid sequence of a protease variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, or to an amino acid sequence comprising an amino acid sequence of a variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**.

[0233] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence comprising residues 135-413, or residues 128-413 of SEQ ID NO: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, 976, 978, 980, 982, 984, 986, 988, 990, 992, 994, 996, 998, 1000, 1002, 1004, 1006, 1008, 1010, 1012, 1014, 1016, 1018, 1020, 1022, 1024, 1026, 1028, 1030, 1032, 1034, 1036, 1038, 1040, 1042, 1044, 1046, 1048, 1050, 1052, 1054, 1056, 1058, 1060, 1062, 1064, 1066, 1068, 1070, 1072, 1074, 1076, 1078, 1080, 1082, 1084, 1086, 1088, 1090, 1092, 1094, 1096, 1098, 1100, 1102, 1104, 1106, 1108, 1110, 1112, 1114, 1116, 1118, 1120, 1122, 1124, 1126, 1128, 1130, 1132, 1134, 1136, 1138, 1140, 1142, 1144, 1146, 1148, 1150, 1152, 1154, 1156, 1158, 1160, 1162, 1164, 1166, 1168, 1170, 1172, 1174, 1176, 1178, 1180, 1182, 1184, 1186, 1188, 1190, 1192, 1194, 1196, 1198, 1200, 1202, 1204, 1206, 1208, 1210, 1212, 1214, 1216, 1218, 1220, 1222, 1224, 1226, 1228, 1230, 1232, 1234, 1236, 1238, 1240, 1242, 1244, 1246, 1248, 1250, 1252, 1254, 1256, 1258, 1260, 1262, 1264, 1266, 1268, 1270, 1272, 1274, 1276, 1278, 1280, 1282, 1284, 1286, 1288, 1290, 1292, 1294, 1296, 1298, 1300, 1302, 1304, 1306, 1308, 1310, 1312, 1314, 1316, 1318, 1320, 1322, 1324, 1326, 1328, 1330, 1332, 1334, 1336, 1338, 1340, 1342, 1344, 1346, 1348, 1350, 1352, 1354, 1356, 1358, 1360, 1362, 1364, 1366, 1368, 1370, 1372, 1374, 1376, 1378, 1380, 1382, 1384, 1386, 1388, 1390, 1392, 1394, 1396, 1398, 1400, 1402, 1404, 1406, 1408, 1410, 1412, 1414, 1416,

1418, 1420, 1422, 1424, 1426, 1428, 1430, 1432, 1434, 1436, 1438, 1440, 1442, 1444, 1446, 1448, 1450, 1452, 1454, 1456, 1458, 1460, 1462, 1464, 1468, 1470, 1472, 1474, 1476, 1478, 1480, 1482, 1484, 1486, 1488, 1490, 1492, 1494, 1496, 1498, 1500, 1502, 1504, 1506, 1508, 1510, 1512, 1514, 1516, 1518, 1520, 1522, 1524, 1526, 1528, 1530, 1532, 1534, 1536, 1538, 1540, 1542, 1544, 1546, 1548, 1550, 1552, 1554, 1556, 1558, 1560, 1562, 1564, 1566, 1568, 1570, 1572, 1574, 1576, 1578, 1580, 1582, 1584, 1586, 1588, 1590, 1592, 1594, 1596, 1598, 1600, 1602, 1604, 1606, 1608, 1610, 1612, 1614, 1616, 1618, 1620, 1622, 1624, 1626, 1628, 1630, 1632, 1634, 1636, 1638, 1640, 1642, 1644, 1646, 1648, 1650, 1652, 1654, 1656, 1658, 1660, 1662, 1664, 1666, 1668, 1670, 1672, 1674, 1676, 1678, 1680, 1682, 1684, 1686, 1688, 1690, 1692, 1694, 1696, 1698, 1700, 1702, 1704, 1706, 1708, 1710, 1712, 1714, 1716, 1718, 1720, 1722, 1724, 1726, 1728, 1730, 1732, 1734, 1736, 1738, 1740, 1742, 1744, 1746, 1748, 1750, 1752, 1754, 1756, 1758, 1760, 1762, 1764, 1766, 1768, 1770, 1772, 1774, 1776, 1778, 1780, 1782, 1784, 1786, 1788, 1790, 1792, 1794, 1796, 1798, 1800, 1802, 1804, 1806, 1808, 1810, 1812, 1814, 1816, 1818, 1820, 1822, 1824, 1826, 1828, 1830, 1832, 1834, 1836, 1838, 1840, 1842, 1844, 1846, 1848, 1850, 1852, 1854, 1856, 1858, 1860, 1862, 1864, 1866, 1868, 1870, 1872, 1874, 1876, 1878, 1880, 1882, 1884, 1886, 1888, 1890, 1892, 1894, 1896, 1898, 1900, 1902, 1904, 1906, 1908, 1910, 1912, 1914, 1916, 1918, 1920, 1922, 1924, 1926, 1928, 1930, 1932, 1934, 1936, 1938, 1940, 1942, 1944, 1946, 1948, 1950, 1952, 1954, 1956, 1958, 1960, 1962, 1964, 1966, 1968, 1970, 1972, 1974, 1976, 1978, 1980, 1982, 1984, 1986, 1988, 1990, 1992, 1994, 1996, 1998, 2000, 2002, 2004, 2006, 2008, 2010, 2012, 2014, 2016, 2018, 2020, 2022, 2024, 2026, 2028, 2030, 2032, 2034, 2036, 2038, 2040, 2042, 2044, 2046, 2048, 2050, 2052, 2054, 2056, 2058, 2060, 2062, 2064, 2066, 2068, 2070, 2072, 2074, 2076, 2078, 2080, 2082, 2084, 2086, 2088, 2090, 2092, 2094, 2096, 2098, 2100, 2102, 2104, 2106, 2108, 2110, 2112, 2114, 2116, 2118, 2120, 2122, 2124, 2126, 2128, 2130, 2132, 2134, 2136, 2138, 2140, 2142, 2144, 2146, 2148, 2150, 2152, 2154, 2156, 2158, 2160, 2162, 2164, 2166, 2168, 2170, 2172, 2174, 2176, 2178, 2180, 2182, 2184, 2186, 2188, 2190, 2192, 2194, 2196, 2198, 2200, 2202, 2204, 2206, 2208, 2210, 2212, 2214, 2216, 2218, 2220, 2222, 2224, 2226, 2228, 2230, 2232, 2234, 2236, 2238, 2240, or 2242. In some embodiments, the amino acid sequence of the engineered protease polypeptide optionally has 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 insertions, substitutions, and/or deletions. In some embodiments, the amino acid sequence optionally has 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 substitutions. In some of embodiments, the amino acid sequence of the engineered protease polypeptide optionally has 1, 2, 3, 4, up to 5 insertions, substitutions, and/or deletions. In some embodiments, the amino acid sequence optionally has 1, 2, 3, 4, up to 5 substitutions. In some embodiments, the substitutions comprise non-conservative and/or conservative substitutions. In some embodiments, the substitutions comprise conservative substitutions.

[0234] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence comprising SEQ ID NO: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186,

188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, 976, 978, 980, 982, 984, 986, 988, 990, 992, 994, 996, 998, 1000, 1002, 1004, 1006, 1008, 1010, 1012, 1014, 1016, 1018, 1020, 1022, 1024, 1026, 1028, 1030, 1032, 1034, 1036, 1038, 1040, 1042, 1044, 1046, 1048, 1050, 1052, 1054, 1056, 1058, 1060, 1062, 1064, 1066, 1068, 1070, 1072, 1074, 1076, 1078, 1080, 1082, 1084, 1086, 1088, 1090, 1092, 1094, 1096, 1098, 1100, 1102, 1104, 1106, 1108, 1110, 1112, 1114, 1116, 1118, 1120, 1122, 1124, 1126, 1128, 1130, 1132, 1134, 1136, 1138, 1140, 1142, 1144, 1146, 1148, 1150, 1152, 1154, 1156, 1158, 1160, 1162, 1164, 1166, 1168, 1170, 1172, 1174, 1176, 1178, 1180, 1182, 1184, 1186, 1188, 1190, 1192, 1194, 1196, 1198, 1200, 1202, 1204, 1206, 1208, 1210, 1212, 1214, 1216, 1218, 1220, 1222, 1224, 1226, 1228, 1230, 1232, 1234, 1236, 1238, 1240, 1242, 1244, 1246, 1248, 1250, 1252, 1254, 1256, 1258, 1260, 1262, 1264, 1266, 1268, 1270, 1272, 1274, 1276, 1278, 1280, 1282, 1284, 1286, 1288, 1290, 1292, 1294, 1296, 1298, 1300, 1302, 1304, 1306, 1308, 1310, 1312, 1314, 1316, 1318, 1320, 1322, 1324, 1326, 1328, 1330, 1332, 1334, 1336, 1338, 1340, 1342, 1344, 1346, 1348, 1350, 1352, 1354, 1356, 1358, 1360, 1362, 1364, 1366, 1368, 1370, 1372, 1374, 1376, 1378, 1380, 1382, 1384, 1386, 1388, 1390, 1392, 1394, 1396, 1398, 1400, 1402, 1404, 1406, 1408, 1410, 1412, 1414, 1416, 1418, 1420, 1422, 1424, 1426, 1428, 1430, 1432, 1434, 1436, 1438, 1440, 1442, 1444, 1446, 1448, 1450, 1452, 1454, 1456, 1458, 1460, 1462, 1464, 1468, 1470, 1472, 1474, 1476, 1478, 1480, 1482, 1484, 1486, 1488, 1490, 1492, 1494, 1496, 1498, 1500, 1502, 1504, 1506, 1508, 1510, 1512, 1514, 1516, 1518, 1520, 1522, 1524, 1526, 1528, 1530, 1532, 1534, 1536, 1538, 1540, 1542, 1544, 1546, 1548, 1550, 1552, 1554, 1556, 1558, 1560, 1562, 1564, 1566, 1568, 1570, 1572, 1574, 1576, 1578, 1580, 1582, 1584, 1586, 1588, 1590, 1592, 1594, 1596, 1598, 1600, 1602,

1604, 1606, 1608, 1610, 1612, 1614, 1616, 1618, 1620, 1622, 1624, 1626, 1628, 1630, 1632, 1634, 1636, 1638, 1640, 1642, 1644, 1646, 1648, 1650, 1652, 1654, 1656, 1658, 1660, 1662, 1664, 1666, 1668, 1670, 1672, 1674, 1676, 1678, 1680, 1682, 1684, 1686, 1688, 1690, 1692, 1694, 1696, 1698, 1700, 1702, 1704, 1706, 1708, 1710, 1712, 1714, 1716, 1718, 1720, 1722, 1724, 1726, 1728, 1730, 1732, 1734, 1736, 1738, 1740, 1742, 1744, 1746, 1748, 1750, 1752, 1754, 1756, 1758, 1760, 1762, 1764, 1766, 1768, 1770, 1772, 1774, 1776, 1778, 1780, 1782, 1784, 1786, 1788, 1790, 1792, 1794, 1796, 1798, 1800, 1802, 1804, 1806, 1808, 1810, 1812, 1814, 1816, 1818, 1820, 1822, 1824, 1826, 1828, 1830, 1832, 1834, 1836, 1838, 1840, 1842, 1844, 1846, 1848, 1850, 1852, 1854, 1856, 1858, 1860, 1862, 1864, 1866, 1868, 1870, 1872, 1874, 1876, 1878, 1880, 1882, 1884, 1886, 1888, 1890, 1892, 1894, 1896, 1898, 1900, 1902, 1904, 1906, 1908, 1910, 1912, 1914, 1916, 1918, 1920, 1922, 1924, 1926, 1928, 1930, 1932, 1934, 1936, 1938, 1940, 1942, 1944, 1946, 1948, 1950, 1952, 1954, 1956, 1958, 1960, 1962, 1964, 1966, 1968, 1970, 1972, 1974, 1976, 1978, 1980, 1982, 1984, 1986, 1988, 1990, 1992, 1994, 1996, 1998, 2000, 2002, 2004, 2006, 2008, 2010, 2012, 2014, 2016, 2018, 2020, 2022, 2024, 2026, 2028, 2030, 2032, 2034, 2036, 2038, 2040, 2042, 2044, 2046, 2048, 2050, 2052, 2054, 2056, 2058, 2060, 2062, 2064, 2066, 2068, 2070, 2072, 2074, 2076, 2078, 2080, 2082, 2084, 2086, 2088, 2090, 2092, 2094, 2096, 2098, 2100, 2102, 2104, 2106, 2108, 2110, 2112, 2114, 2116, 2118, 2120, 2122, 2124, 2126, 2128, 2130, 2132, 2134, 2136, 2138, 2140, 2142, 2144, 2146, 2148, 2150, 2152, 2154, 2156, 2158, 2160, 2162, 2164, 2166, 2168, 2170, 2172, 2174, 2176, 2178, 2180, 2182, 2184, 2186, 2188, 2190, 2192, 2194, 2196, 2198, 2200, 2202, 2204, 2206, 2208, 2210, 2212, 2214, 2216, 2218, 2220, 2222, 2224, 2226, 2228, 2230, 2232, 2234, 2236, 2238, 2240, or 2242. In some embodiments, the amino acid sequence of the engineered protease polypeptide optionally has 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 insertions, substitutions, and/or deletions. In some embodiments, the amino acid sequence optionally has 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 substitutions. In some of embodiments, the amino acid sequence of the engineered protease polypeptide optionally has 1, 2, 3, 4, up to 5 insertions, substitutions, and/or deletions. In some embodiments, the amino acid sequence optionally has 1, 2, 3, 4, up to 5 substitutions. In some embodiments, the substitutions comprise non-conservative and/or conservative substitutions. In some embodiments, the substitutions comprise conservative substitutions.

[0235] In some embodiments, the engineered protease polypeptide comprises an amino acid sequence comprising residues 135-413 of SEQ ID NO: 628, 948, 1126, 1368, 1547, 1640, or 1710, or an amino acid sequence comprising SEQ ID NO: 628, 948, 1126, 1368, 1547, 1640, or 1710. In some embodiments, the amino acid sequence of the engineered protease polypeptide optionally has 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 insertions, substitutions, and/or deletions. In some embodiments, the amino acid sequence optionally has 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 substitutions. In some of embodiments, the amino acid sequence of the engineered protease polypeptide optionally has 1, 2, 3, 4, up to 5 insertions, substitutions, and/or deletions. In some embodiments, the amino acid sequence optionally has 1, 2, 3, 4, up to 5 substitutions. In some embodiments, the substitutions comprise non-conservative and/or conservative substitutions. In some embodiments, the substitutions comprise conservative substitutions.

[0236] In some embodiments, the engineered protease polypeptide described herein, particularly the pro-polypeptide form, is capable of converting or forming a proteolytically active polypeptide or an active protease. In some embodiments, the formation of a proteolytically active polypeptide or an active protease is by auto-proteolysis. In some embodiments, the formation of a proteolytically active polypeptide or an active protease is by proteolysis by another protease, including any of the proteolytically active polypeptide or active protease of the engineered protease polypeptide described herein.

[0237] In some embodiments, the engineered protease polypeptide comprises a proteolytically active polypeptide or is an active protease. In some embodiments, the proteolytically active polypeptide or active protease comprises amino acid residues 135-413, or comprises amino acid residues 128-413 of any of the engineered protease polypeptide described herein.

[0238] In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has an improved property as compared to a reference protease polypeptide.

[0239] In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has increased protease activity as compared to a reference protease. In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has at least 1.1, 1.2, 1.3, 1.4, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 or greater fold increase in protease activity as compared to the reference protease. Exemplary increases in protease activity compared to the reference protease are provided in the Examples.

[0240] In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has increased resistance to a gastric protease as compared to a reference protease. In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has increased resistance to pepsin as compared to the reference protease. In some embodiments, the increased resistance to the gastric protease is at acidic pH conditions.

[0241] In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has increased stability and/or activity at acidic pH or neutral pH as compared to a reference protease. In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has at least 1.1, 1.2, 1.3, 1.4, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 or greater fold increase in protease activity at acidic pH or neutral as compared to the reference protease. In some embodiments, the acidic pH is from about 2.8 to 4.5.

[0242] In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has increased thermostability as compared to a reference protease. In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide has increased thermostability at temperature of about 64 °C or 71 °C as compared to the reference protease.

[0243] In some embodiments, the proteolytically active polypeptide or active protease of the engineered protease polypeptide is characterized by an improved property selected from: i) increased protease activity, ii) increased resistance to pepsin, iii) increased stability and/or activity at acidic pH, iv) increased stability and/or activity at neutral pH, or v) increased thermostability, or any combination of i), ii), iii), iv), and v) as compared to a reference protease.

[0244] In some embodiments, the reference protease has an amino acid sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548; residues 128-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548; or a mature active protease of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548. In some embodiments, the reference protease has an amino acid sequence corresponding to residues 135-413 or residues 128-413 of SEQ ID NO: 4 or 628, or a mature active protease of SEQ ID NO: 4 or 628. In some embodiments, the reference protease is a proteolytically active polypeptide of SEQ ID NO: 2.

[0245] In some embodiments, the pro-polypeptide or proteolytically active polypeptide of the engineered protease polypeptide described herein includes or further comprises at the carboxy terminal region a Big-1 domain. In some embodiments, the Big-1 domain is that of SEQ ID NO: 2. In some embodiments, the Big-1 domain comprises amino acid residues 426-522 of SEQ ID NO: 2.

[0246] In some embodiments, the engineered protease polypeptide comprises a deletion of the protease of SEQ ID NO: 2, where the deletion is of at least the carboxy terminal region of SEQ ID NO: 2, and wherein the deletion maintains the protease activity of the mature form of SEQ ID NO: 2. In some embodiments, the carboxy terminal region deleted comprises deletion of the Big-1 domain. In some embodiments, the carboxy terminal deletion is up to and including amino acid residue 426, or up to and including amino acid residue 414 of SEQ ID NO: 2. In some embodiments, the engineered protease polypeptide further comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 amino acid deletions of the carboxy terminus at amino acid residue 413 of SEQ ID NO: 2, wherein the further amino acid deletion(s) maintain proteolytic activity of the mature form of SEQ ID NO: 2 having the further amino acid deletions. In some embodiments, the mature form of the protease polypeptide has an amino terminus at amino acid residue 128 or 135 of SEQ ID NO: 2.

[0247] In some embodiments, the engineered protease polypeptide further comprises a fusion polypeptide or a fusion protein. In some embodiments, the engineered protease polypeptide further comprises a heterologous fusion polypeptide or a fusion protein. In some embodiments, the engineered protease polypeptide described herein can be fused to a variety of polypeptide or protein sequences, such as, by way of example and not limitation, polypeptide tags that can be used for detection and/or purification. In some embodiments, the fusion polypeptide of the recombinant protease polypeptide comprises a glycine-histidine or histidine-tag (His-tag). In some embodiments, the fusion polypeptide of the engineered protease polypeptide comprises an epitope tag, such as c-myc, FLAG, V5, or hemagglutinin (HA). In some embodiments, the fusion polypeptide of the engineered protease

polypeptide comprises a GST, SUMO, Strep, MBP, or GFP tag. In some embodiments, the fusion is to the amino (N-) terminus of the engineered protease polypeptide. In some embodiments, the fusion is to the carboxy (C-) terminus of the engineered protease polypeptide. In some embodiments, the fusion polypeptide is inserted following a signal sequence and before the engineered protease polypeptide to allow expression and secretion of the protease polypeptide comprising the fusion polypeptide (e.g., polypeptide tag) and engineered protease polypeptide.

[0248] In some embodiments, the engineered protease polypeptide further comprises a signal sequence or signal peptide. In some embodiments, the signal sequence or signal peptide is functional in the host cell used or to be used for expression of the engineered protease polypeptide. In some embodiments the signal sequence or signal peptide is fused to a pro-polypeptide form of the engineered protease polypeptide, e.g., for forming a pre-pro-polypeptide. In some embodiments, the signal sequence or signal peptide is fused to the polypeptide that includes the proteolytically active polypeptide or active protease of the engineered protease polypeptide. In some embodiments, the signal sequence or signal peptide can be a bacterial, fungal, or mammalian signal sequence or signal polypeptide. In some embodiments, the signal sequence or signal peptide can be a naturally occurring signal sequence or a synthetic signal sequence, including a hybrid signal sequence.

[0249] In some embodiments, the signal sequence or signal peptide is a bacterial signal sequence or signal peptide, or a signal sequence or signal peptide functional in bacterial cells. In some embodiments, the bacterial signal sequence or signal peptide is recognized by the general secretion (Sec) or twin-arginine translocation (Tat) pathway (see, e.g., Freudl, R., *Microbial Cell Factories*, 2018, 17(52):1-10). In some embodiments, the signal sequence or signal peptide can be a Sec signal sequence or signal peptide from genes encoding, among others, LamB, MalE, OmpA, OmpF, OmpN, OmpC, OmpX, PhoA, PhoE, GBP, TolC, TolB, or CirA. In some embodiments, the signal sequence or signal peptide can be a Tat signal sequence or signal peptide from genes encoding, among others, TorA, TorZ, AmiA, AmiC, FtsP, EfeB, YcbK, NrfC, WcaM, YahJ, MdoD, or FhuD.

[0250] In some embodiments, signal sequence or signal peptide is a fungal (e.g., yeast) signal sequence or signal peptide or a signal sequence, or a signal peptide functional in fungal cells. Exemplary fungal signal sequence or signal peptide includes, among others, those found in *Pichia pastoris* Ost1, *Pichia pastoris* Pst1, *S. cerevisiae* α -mating factor pre-pro sequence, *S. cerevisiae* invertase, *Komagataella pastoris* yeast α -factor, *S. cerevisiae* CYP, *Pichia pastoris* PH08, *S. cerevisiae* PEP4, *S. cerevisiae* SUC2, *Pichia pastoris* KAR2, *Pichia pastoris* DSE4, *Pichia pastoris* EXG1, or *Pichia pastoris* SCW10.

[0251] In some embodiments, the signal sequence or signal peptide is a mammalian or insect cell signal sequence or signal peptide, or a signal sequence or signal peptide functional in mammalian cells or insect cells. Exemplary mammalian or insect signal sequence or signal peptide includes those from, among others, human OSM, VSV-G, mouse Ig Kappa, mouse Ig heavy, BM40, Scrccon, human IgKVIII, CD33, tPA, human chymotrypsinogen, human trypsinogen-2, human IL-2, human serum albumin (HSA),

influenza haemagglutinin, human insulin, silkworm Fibroin LC, and honeybee melittin signal peptide of gp64 or gp67.

[0252] In some embodiments, for any of the engineered protease polypeptide disclosed herein, the engineered protease polypeptide is purified or is a purified preparation or composition. In some embodiments, the purified preparation comprises the pro-polypeptide of the engineered protease polypeptide. In some embodiments, the purified preparation comprises the proteolytically active polypeptide or the active protease form of the engineered protease polypeptide.

[0253] In some embodiments, the present disclosure further provides a functional or biologically active fragment of an engineered protease polypeptide described herein. In some embodiments, functional or biologically active fragments have at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the activity of the proteolytically active polypeptide or the active protease of the engineered protease polypeptide from which it was derived (i.e., the parent engineered protease polypeptide). In some embodiments, a functional or biologically active fragment comprises at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the parent sequence of the engineered protease polypeptide. In some embodiments the functional or biologically active fragment is truncated by less than 5, less than 10, less than 15, less than 20, less than 25, less than 30, less than 35, less than 40, less than 45, and less than 50 amino acids of the parent engineered protease polypeptide.

[0254] In some embodiments, the functional or biologically active fragment of the engineered protease polypeptide described herein includes at least a mutation or mutation set in the amino acid sequence of the parent engineered protease polypeptide variant described herein. Accordingly, in some embodiments, the functional or biologically active fragments of the engineered protease polypeptide displays the enhanced or improved property associated with the mutation or mutation set in the parent engineered protease polypeptide variant.

Polynucleotides Encoding Engineered Protease Polypeptides, Expression Vectors, and Host Cells

[0255] In a further aspect, the present disclosure provides a recombinant polynucleotide encoding an engineered protease polypeptide described herein, expression vectors comprising the recombinant polynucleotide operably linked to one or more control sequences, and appropriate host cells comprising the expression vector for expression of the engineered protease polypeptide.

[0256] As will be apparent to the skilled artisan, availability of a protein sequence and the knowledge of the codons corresponding to the various amino acids provide a description of all the polynucleotides capable of encoding the subject polypeptides. The degeneracy of the genetic code, where the same amino acids are encoded by alternative or synonymous codons, allows an extremely large number of nucleic acids to be made, all of which encode the engineered protease polypeptide. Thus, having knowledge of a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids

by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the encoded protein. In this regard, the present disclosure specifically contemplates each and every possible variation of polynucleotides that could be made encoding the engineered protease polypeptide described herein by selecting combinations based on the possible codon choices, and all such polynucleotide variations are to be considered specifically disclosed for any polypeptide described herein, including the engineered protease polypeptide set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, and the accompanying Sequence Listing.

[0257] In some embodiments, the codons are preferably optimized for utilization by the chosen host cell for protein production. In some embodiments, the polynucleotide encoding the engineered protease polypeptide preferably uses preferred codons used in bacterial cells for expression in bacterial cells. In some embodiments, the polynucleotide encoding the engineered protease polypeptide preferably uses preferred codons used in fungal cells for expression in fungal cells. In some embodiments, the polynucleotide encoding the engineered protease polypeptide preferably uses preferred codons used in insect cells for expression in insect cells. In some embodiments, the polynucleotide encoding the engineered protease polypeptide preferably uses preferred codons used in mammalian cells for expression in mammalian cells. In some embodiments, codon optimized polynucleotides encoding an engineered protease polypeptide described herein contain preferred codons at about 40%, 50%, 60%, 70%, 80%, 90%, or greater than 90% of the codon positions in the coding region.

[0258] As discussed above, it is to be understood that the present disclosure provides recombinant polynucleotides encoding each and every engineered protease polypeptide described herein, including a pre-pro-polypeptide, pro-polypeptide, or proteolytically active polypeptide thereof, or a biologically or functionally active fragment thereof.

[0259] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0260] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or to the reference sequence corresponding to SEQ ID NO: 4 or 628,

wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0261] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0262] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, or to the reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0263] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution at amino acid position 11, 31, 42, 45, 50, 53, 84, 99, 100, 126, 128, 129, 130, 131, 132, 134, 135, 136, 137, 138, 139, 140, 141, 143, 145, 151, 154, 156, 157, 159, 160, 161, 162, 163, 169, 172, 173, 174, 179, 180, 184, 185, 186, 187, 188, 190, 191, 192, 193, 194, 198, 199, 212, 214, 220, 221, 222, 223, 225, 231, 232, 233, 235, 237, 238, 239, 240, 242, 243, 245, 246, 249, 250, 251, 252, 253, 254, 256, 258, 262, 263, 264, 265, 266, 267, 268, 269, 271, 273, 274, 275, 277, 278, 279, 280, 281, 283, 285, 290, 292, 293, 294, 296, 297, 300, 302, 303, 311, 312, 313, 314, 315, 316, 318, 324, 328, 336, 339, 341, 342, 343, 345, 346, 355, 358, 360, 364, 367, 368, 369, 370, 371, 372, 373, 374, 375, 377, 381, 382, 384, 386, 389, 391, 392, 401, 402, 405, 406, 409, 410, 411, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0264] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution at amino acid position 135, 137, 139, 141, 143, 157, 160, 214, 268, 273, 279, 311, 312, 315, 328, 342, 345, 346, or 372, or combinations thereof, wherein the amino acid positions are relative to the

reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0265] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution set at amino acid positions 135/141/160/311/315/372, 143/328/342/345, 139/157/268/273/312/346, or 137/139/214/279, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0266] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set at amino acid position(s) 185, 134, 129, 135, 184, 132, 186, 193, 263, 370, 45/134, 199, 368, 161, 141, 267, 179, 264, 160, 138, 131, 372, 151, 274, 128, 339, 313, 374, 314, 191, 324, 315, 375, 136, 220, 194, 231, 277, 369, 251, 180, 163, 343, 264/279, 279, 232, 141/300, 367, 266, 188, 130, 318, 265, 341, 190, 145, 126/192, 11/220, 192, 370/392, 99/278, 265/311, 84/159/265/279/311/370, 311/316, 342/370, 265/311/370, 192/311/316, 141/154/192, 265/311/316/342, 279/311/316, 141/265/279/311/342, 141/192/311/316/370, 141/265/311, 198/279, 392, 342/370/392, 141/198/265, 265/392, 184/267, 342, 312, 100/251, 141/220, 311/316/370, 99, 278, 405, 311/342/370, 141/198, 311/342, 141/311, 279/311/377/392, 186/198/311/342/370/392, 141/392, 311/370/392, 141/311/392, 311/370, 311/316/392, 265/311/392, 141/192, 311, 141/265/311/392, 192/311/370/392, 198/265/311/316/370, 141/186/265/311, or 141/198/265/311/370, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0267] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set at amino acid position(s) 242, 157, 250, 373, 243, 336, 187, 240, 280, 271, 237, 386, 382, 328, 42, 391, 381, 275, 249, 239, 384, 139, 364, 346, 389, 254, 246, 345, 360, 303, 300, 269, 135/141/372, 311/315/372, 136/141/311, 141/188, 135/136, 135/141/315, 372, 135/141/160/267/372, 135/136/141/160/185/188/267/311/315, 160/185, 135/141/188/279/311, 135/136/141, 135/136/141/372, 135/141/160/185/267/279, 135/141/160/267, 141/188/311/372, 160/185/188/279/311, 136/141/279, 135/136/141/160/185/188, 141/372, 135/136/141/311, 185/311/315/372, 135/141/188, 136/185, 135/141, 135/136/141/279/315/372, 135/311/315, 141, 311/372, 188/311, 135/141/188/372, 141/160/279, 313/392, 342/392, 279/392, 128, 198/342, 313, 128/312, 50, 145/263, 313/342, 279/312, 312/392, 279/342, 128/342, 342, 263, 143, 262, 156, 169, 143/237, 136/160/185/267/311/372, 135/160/311/372, 135/141/311/315, 141/311/315, 136/141/160/185/188/311/315/372, 135/141/311/315/372, 135/141/160/185/311/315, 135/141/267/311/315/372, 135/136/141/160/311/315, 135/136/141/279, 135/141/267/279/311/315,

135/141/160, 135/141/160/311/315/372, 135/141/160/311/315, 135/136/141/188/311, 141/160/311, 135/141/160/279/311/315/372, 141/160/185/279/311/372, 135/136/141/160/315/372, 135/136/160/279/311/372, 128/279/312/342, 128/198/312/342, 263/342, 145/263/279/312/342/392, or 128/145/198/312/313/392, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0268] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution at amino acid position 135, 137, 139, 141, 143, 145, 145, 157, 160, 214, 221, 268, 273, 279, 311, 312, 315, 315, 342, 345, 346, 402, 409, 410, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0269] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution at an amino acid position set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0270] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution as set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0271] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set at amino acid position(s) set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0272] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set of an engineered protease polypeptide set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0273] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence having a substitution or substitution set as set forth in Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

[0274] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0275] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or to the reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242.

[0276] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0277] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or to the reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0278] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising at least a substitution at amino acid position 11, 31, 42, 45, 50, 53, 84, 99, 100, 126, 128, 129, 130, 131, 132, 134, 135, 136, 137, 138, 139, 140, 141, 143, 145, 151, 154, 156, 157, 159, 160, 161, 162, 163, 169, 172, 173, 174, 179, 180, 184, 185, 186, 187, 188, 190, 191, 192, 193, 194, 198, 199, 212, 214, 220, 221, 222, 223, 225, 231, 232, 233, 235, 237, 238, 239, 240, 242, 243, 245, 246, 249, 250, 251, 252, 253, 254, 256, 258, 262, 263, 264, 265, 266, 267, 268, 269, 271, 273, 274, 275, 277, 278, 279, 280, 281, 283, 285, 290, 292, 293, 294, 296, 297, 300, 302, 303, 311, 312, 313, 314, 315, 316, 318, 324, 328, 336, 339, 341, 342, 343, 345, 346, 355, 358, 360, 364, 367, 368, 369, 370, 371, 372, 373, 374, 375, 377, 381, 382, 384, 386, 389, 391, 392, 401, 402, 405, 406, 409, 410, 411, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0279] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising at least a substitution at amino acid position 135, 137, 139, 141, 143, 157, 160, 214, 268, 273, 279, 311, 312, 315, 328, 342, 345, 346, or 372, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

[0280] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or to the reference sequence corresponding to SEQ ID NO: 948, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0281] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 950-1154, or to the reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 950-1154, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0282] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set at amino acid position(s) 411, 402, 285, 245, 266, 355, 258, 222, 140, 268, 225, 283, 406, 410, 143/145/243/312, 139/143/145/157/312, 139/157/345, 139/269, 156/157/342/346, 139/143, 269, 139/243/328, 269/328, 143/145/169, 328, 143/145/262, 145/262/312/328, 139/156/157, 139/145/312, 312, 139, 139/312, 139/156, 139/143/145/243, 145/157, 145/346, 145/262/312/328/345/346, 145/262, 312/342, 143/243, 139/345, 342, 143/145/262/342, 139/143/169, 139/143/145/312, 169, 139/145/262/312/328/342/345/346, 139/328, 139/243, 139/143/328, 139/143/243, 139/145, 145/312, 145/169, 139/143/157/312, 84/139/143, 145/269, 143/145/157/269/312/328, 143/145/269, 157, 139/143/312, 256, 273, 409, 172, 401, 281, 253, 143/145/243/328, 145, 139/143/145/328/342/345, 143/328/342/345, 145/342/345, 143, 139/145/328/342/345, 143/145/169/312/328/345/346, 143/243/328/342/345/346, 139/143/157/169/328/346, 143/145/156/312/328, 139/145/157/312/328, 143/328/342/345/346, 143/145, 143/145/312/342/345, or 143/145/328, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

[0283] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or to the reference sequence corresponding to SEQ ID NO: 1126, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0284] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1156-1422, or to the reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1156-1422, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0285] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set at amino acid position(s) 279, 250, 154, 214, 249, 275, 137, 161, 180, 174,

139, 254, 145, 278, 136, 154/413, 294, 237, 274, 264, 185, 277, 293, 233, 173, 312, 302, 238, 135, 221, 290, 263, 267, 239, 163, 292, 246, 243, 235, 156, 223, 278/413, 297, 194, 251, 253/411, 145/157/253/268/273/281/312/346/411, 139/346, 253, 346/411, 253/346, 312/346, 273/312, 253/281, 157/253/273/312/346/411, 139/157/253/268/273/281/312/346, 253/273/411, 139/253/268/273/281, 139/157/411, 157, 273, 139/253/268/273/281/312/411, 157/253/411, 139/145/253/346, 139/157/253/273/312, 139/157/268/273/312/346, 157/273/312/346, 139/411, 139/253/268/273/281/312/346/411, 157/273/346/411, 139/145/157/162/253/273/281/312, 139/253/273/281/312, 157/253/268/273/281/312, 139/253/268, 139/157/312, 253/273/281/346, 157/253/312/346/411, 157/273/312/346/411, 139/145/157/253/268/281/312, 139/273/312/346, 157/253/268/273/312/346, 139/268/346, 268/273/312/346, 139/157/253/268/273/312, 139/157/253, 139/253/281, 139/157/253/268/273, 253/312/411, or 139/268/273, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

[0286] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or to the reference sequence corresponding to SEQ ID NO: 1368, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0287] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1424-1608, or to the reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1424-1608, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0288] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set at amino acid position(s) 31, 318, 296, 252, 303, 253, 413, 386, 312, 235, 412, 342, 302, 371, 405, 389, 391, 358, 139/273/311/328/372, 311, 372, 139/143/157/160/268/273/311/315, 143, 139/143, 139/160/312/372, 143/273/328, 346, 135/139/160/268/312/342/346, 139/141/273, 135/141/143/268/273/312/372, 139/141/143/311,

139/157/268/328/346/372, 53/139/141/143/273/372, 139, 139/141/143/273/312, 137/139/221/233/413, 233, 221/279, 137/139/233/279, 221, 139/214, 137/139/156, 139/214/221, 214/233, 137/139/221/233/279, 137/139/221, 137/139, 137/139/279, 137, 137/139/214/279, 266, 139/221, 137/221, 137/156/214/312, 137/221/233, 137/139/156/221, 137/139/214, 279, 137/139/233, 137/139/214/233, 221/413, 137/214/233, 137/156, 137/139/221/233, 214/221, 137/221/413, 214, 137/233, 137/413, 137/221/279, 137/139/156/214/233/413, or 137/139/156/214, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

[0289] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or to the reference sequence corresponding to SEQ ID NO: 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0290] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1610-1710, or to the reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1610-1710, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0291] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set at amino acid position(s) 145/273/372, 145/256/273, 221/243/273/328/372, 372, 145/221/279/372/406, 273/328, 145/169/273/346/406, 256/273, 145/221/273/328/346/406, 243/273, 145/214/256/273/279/328/372, 169/221/328/372/406, 372/406, 169/328/372/406, 221/372, 145/221/273/328/372, 214/243/273/328, 145/221, 214/256/273/346/372, 221/406, 169/372, 145/214/221/273, 145/221/346/372, 243/273/328/372/406, 145/169/273/328/346, 328, 169/273/372, 145/221/328, 169/214/273, 221/273/328, 221, 145/328, 214/346, 312, 212, 279, 212/312, 145, 179/346, 214, 346, 315/372, 375, 264, 179, 185, 220/372, or 324, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

[0292] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution at an amino acid position set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0293] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution as set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0294] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising at least a substitution or substitution set at amino acid position(s) set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0295] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising at least a substitution or substitution set of an engineered protease polypeptide variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0296] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence having a substitution or substitution set as set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

[0297] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising an amino acid sequence comprising residues

135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242, or an amino acid sequence comprising an even-numbered SEQ ID NO. of SEQ ID NOs: 6-2242.

[0298] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 403 to 1239 of SEQ ID NO: 3, 627, 947, 1125, 1367, or 1547, or to a reference polynucleotide sequence corresponding to SEQ ID NO: 3, 627, 947, 1125, 1367, or 1547, wherein the recombinant polynucleotide encodes an engineered protease polypeptide.

[0299] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 403-1239 of an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-2241, or to a reference polynucleotide corresponding to an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-2241, wherein the recombinant polynucleotide encodes an engineered protease polypeptide.

[0300] As discussed herein, the polynucleotide sequence of the recombinant polynucleotide encoding an engineered protease polypeptide is codon optimized. In some embodiments, the polynucleotide sequence is codon optimized for expression in a selected host cell. In some embodiments, the polynucleotide sequence is codon optimized for expression in a bacterial cell, fungal cell, insect cell, or mammalian cell.

[0301] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence comprising residues 403-1239, or residues 382-1239 of SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587, 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713,

715, 717, 719, 721, 723, 725, 727, 7299, 731, 733, 735, 737, 739, 741, 743, 745, 7437, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, 975, 977, 979, 981, 983, 985, 987, 989, 991, 993, 995, 997, 999, 1001, 1003, 1005, 1007, 1009, 1011, 1013, 1015, 1017, 1019, 1021, 1023, 1025, 1027, 1029, 1031, 1033, 1035, 1037, 1039, 1041, 1043, 1045, 1047, 1049, 1051, 1053, 1055, 1057, 1059, 1061, 1063, 1065, 1067, 1069, 1071, 1073, 1075, 1077, 1079, 1081, 1083, 1085, 1087, 1089, 1091, 1093, 1095, 1097, 1099, 1101, 1103, 1105, 1107, 1109, 1111, 1113, 1115, 1117, 1119, 1121, 1123, 1125, 1127, 1129, 1131, 1133, 1135, 1137, 1139, 1141, 1143, 1145, 1147, 1149, 1151, 1153, 1155, 1157, 1159, 1161, 1163, 1165, 1167, 1169, 1171, 1173, 1175, 1177, 1179, 1181, 1183, 1185, 1187, 1189, 1191, 1193, 1195, 1197, 1199, 1201, 1203, 1205, 1207, 1209, 1211, 1213, 1215, 1217, 1219, 1221, 1223, 1225, 1227, 1229, 1231, 1233, 1235, 1237, 1239, 1241, 1243, 1245, 1247, 1249, 1251, 1253, 1255, 1257, 1259, 1261, 1263, 1265, 1267, 1269, 1271, 1273, 1275, 1277, 1279, 1281, 1283, 1285, 1287, 1289, 1291, 1293, 1295, 1297, 1299, 1301, 1303, 1305, 1307, 1309, 1311, 1313, 1315, 1317, 1319, 1321, 1323, 1325, 1327, 1329, 1331, 1333, 1335, 1337, 1339, 1341, 1343, 1345, 1347, 1349, 1351, 1353, 1355, 1357, 1359, 1361, 1363, 1365, 1367, 1369, 1371, 1373, 1375, 1377, 1379, 1381, 1383, 1385, 1387, 1389, 1391, 1393, 1395, 1397, 1399, 1401, 1403, 1405, 1407, 1409, 1411, 1413, 1415, 1417, 1419, 1421, 1423, 1425, 1427, 1429, 1431, 1433, 1435, 1437, 1439, 1441, 1443, 1445, 1447, 1449, 1451, 1453, 1455, 1457, 1459, 1461, 1463, 1465, 1467, 1469, 1471, 1473, 1475, 1477, 1479, 1481, 1483, 1485, 1487, 1489, 1491, 1493, 1495, 1497, 1499, 1501, 1503, 1505, 1507, 1509, 1511, 1513, 1515, 1517, 1519, 1521, 1523, 1525, 1527, 1529, 1531, 1533, 1535, 1537, 1539, 1541, 1543, 1545, 1547, 1549, 1551, 1553, 1555, 1557, 1559, 1561, 1563, 1565, 1567, 1569, 1571, 1573, 1575, 1577, 1579, 1581, 1583, 1585, 1587, 1589, 1591, 1593, 1595, 1597, 1599, 1601, 1603, 1605, 1607, 1609, 1611, 1613, 1615, 1617, 1619, 1621, 1623, 1625, 1627, 1629, 1631, 1633, 1635, 1637, 1639, 1641, 1643, 1645, 1647, 1649, 1651, 1653, 1655, 1657, 1659, 1661, 1663, 1665, 1667, 1669, 1671, 1673, 1675, 1677, 1679, 1681, 1683, 1685, 1687, 1689, 1691, 1693, 1695, 1697, 1699, 1701, 1703, 1705, 1707, 1709, 1711, 1713, 1715, 1717, 1719, 1721, 1723, 1725, 1727, 1729, 1731, 1733, 1735, 1737, 1739, 1741, 1743, 1745, 1747, 1749, 1751, 1753, 1755, 1757, 1759, 1761, 1763, 1765, 1767, 1769, 1771, 1773, 1775, 1777, 1779, 1781, 1783, 1785, 1787, 1789, 1791, 1793, 1795, 1797, 1799, 1801, 1803, 1805, 1807, 1809, 1811, 1813, 1815, 1817, 1819, 1821, 1823, 1825, 1827, 1829, 1831, 1833, 1835, 1837, 1839, 1841, 1843, 1845, 1847, 1849, 1851, 1853, 1855, 1857, 1859, 1861, 1863, 1865, 1867, 1869, 1871, 1873, 1875, 1877, 1879, 1881, 1883, 1885, 1887, 1889, 1891, 1893, 1895, 1897, 1899, 1901, 1903, 1905, 1907, 1909, 1911, 1913, 1915, 1917, 1919, 1921, 1923, 1925, 1927, 1929, 1931, 1933, 1935, 1937, 1939, 1941, 1943, 1945, 1947, 1949, 1951, 1953, 1955, 1957, 1959, 1961, 1963, 1965, 1967, 1969, 1971, 1973, 1975, 1977, 1979, 1981, 1983, 1985, 1987, 1989, 1991, 1993, 1995, 1997, 1999, 2001, 2003, 2005, 2007, 2009, 2011, 2013, 2015, 2017, 2019, 2021, 2023,

2025, 2027, 2029, 2031, 2033, 2035, 2037, 2039, 2041, 2043, 2045, 2047, 2049, 2051, 2053, 2055, 2057, 2059, 2061, 2063, 2065, 2067, 2069, 2071, 2073, 2075, 2077, 2079, 2081, 2083, 2085, 2087, 2089, 2091, 2093, 2095, 2097, 2099, 2101, 2103, 2105, 2107, 2109, 2111, 2113, 2115, 2117, 2119, 2121, 2123, 2125, 2127, 2129, 2131, 2133, 2135, 2137, 2139, 2141, 2143, 2145, 2147, 2149, 2151, 2153, 2155, 2157, 2159, 2161, 2163, 2165, 2167, 2169, 2171, 2173, 2175, 2177, 2179, 2181, 2183, 2185, 2187, 2189, 2191, 2193, 2195, 2197, 2199, 2201, 2203, 2205, 2207, 2209, 2211, 2213, 2215, 2217, 2219, 2221, 2223, 2225, 2227, 2229, 2231, 2233, 2235, 2237, 2239, or 2241.

[0302] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence comprising SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587, 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 717, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, 975, 977, 979, 981, 983, 985, 987, 989, 991, 993, 995, 997, 999, 1001, 1003, 1005, 1007, 1009, 1011, 1013, 1015, 1017, 1019, 1021, 1023, 1025, 1027, 1029, 1031, 1033, 1035, 1037, 1039, 1041, 1043, 1045, 1047, 1049, 1051, 1053, 1055, 1057, 1059, 1061, 1063, 1065, 1067, 1069, 1071, 1073, 1075, 1077, 1079, 1081, 1083, 1085, 1087, 1089, 1091, 1093, 1095, 1097, 1099, 1101, 1103, 1105, 1107, 1109, 1111, 1113, 1115, 1117, 1119, 1121, 1123, 1125, 1127, 1129, 1131, 1133, 1135, 1137, 1139, 1141, 1143, 1145, 1147, 1149, 1151, 1153, 1155, 1157, 1159, 1161, 1163, 1165, 1167, 1169, 1171, 1173, 1175, 1177, 1179, 1181, 1183, 1185, 1187, 1189,

1191, 1193, 1195, 1197, 1199, 1201, 1203, 1205, 1207, 1209, 1211, 1213, 1215, 1217, 1219, 1221, 1223, 1225, 1227, 1229, 1231, 1233, 1235, 1237, 1239, 1241, 1243, 1245, 1247, 1249, 1251, 1253, 1255, 1257, 1259, 1261, 1263, 1265, 1267, 1269, 1271, 1273, 1275, 1277, 1279, 1281, 1283, 1285, 1287, 1289, 1291, 1293, 1295, 1297, 1299, 1301, 1303, 1305, 1307, 1309, 1311, 1313, 1315, 1317, 1319, 1321, 1323, 1325, 1327, 1329, 1331, 1333, 1335, 1337, 1339, 1341, 1343, 1345, 1347, 1349, 1351, 1353, 1355, 1357, 1359, 1361, 1363, 1365, 1367, 1369, 1371, 1373, 1375, 1377, 1379, 1381, 1383, 1385, 1387, 1389, 1391, 1393, 1395, 1397, 1399, 1401, 1403, 1405, 1407, 1409, 1411, 1413, 1415, 1417, 1419, 1421, 1423, 1425, 1427, 1429, 1431, 1433, 1435, 1437, 1439, 1441, 1443, 1445, 1447, 1449, 1451, 1453, 1455, 1457, 1459, 1461, 1463, 1465, 1467, 1469, 1471, 1473, 1475, 1477, 1479, 1481, 1483, 1485, 1487, 1489, 1491, 1493, 1495, 1497, 1499, 1501, 1503, 1505, 1507, 1509, 1511, 1513, 1515, 1517, 1519, 1521, 1523, 1525, 1527, 1529, 1531, 1533, 1535, 1537, 1539, 1541, 1543, 1545, 1547, 1549, 1551, 1553, 1555, 1557, 1559, 1561, 1563, 1565, 1567, 1569, 1571, 1573, 1575, 1577, 1579, 1581, 1583, 1585, 1587, 1589, 1591, 1593, 1595, 1597, 1599, 1601, 1603, 1605, 1607, 1609, 1611, 1613, 1615, 1617, 1619, 1621, 1623, 1625, 1627, 1629, 1631, 1633, 1635, 1637, 1639, 1641, 1643, 1645, 1647, 1649, 1651, 1653, 1655, 1657, 1659, 1661, 1663, 1665, 1667, 1669, 1671, 1673, 1675, 1677, 1679, 1681, 1683, 1685, 1687, 1689, 1691, 1693, 1695, 1697, 1699, 1701, 1703, 1705, 1707, 1709, 1711, 1713, 1715, 1717, 1719, 1721, 1723, 1725, 1727, 1729, 1731, 1733, 1735, 1737, 1739, 1741, 1743, 1745, 1747, 1749, 1751, 1753, 1755, 1757, 1759, 1761, 1763, 1765, 1767, 1769, 1771, 1773, 1775, 1777, 1779, 1781, 1783, 1785, 1787, 1789, 1791, 1793, 1795, 1797, 1799, 1801, 1803, 1805, 1807, 1809, 1811, 1813, 1815, 1817, 1819, 1821, 1823, 1825, 1827, 1829, 1831, 1833, 1835, 1837, 1839, 1841, 1843, 1845, 1847, 1849, 1851, 1853, 1855, 1857, 1859, 1861, 1863, 1865, 1867, 1869, 1871, 1873, 1875, 1877, 1879, 1881, 1883, 1885, 1887, 1889, 1891, 1893, 1895, 1897, 1899, 1901, 1903, 1905, 1907, 1909, 1911, 1913, 1915, 1917, 1919, 1921, 1923, 1925, 1927, 1929, 1931, 1933, 1935, 1937, 1939, 1941, 1943, 1945, 1947, 1949, 1951, 1953, 1955, 1957, 1959, 1961, 1963, 1965, 1967, 1969, 1971, 1973, 1975, 1977, 1979, 1981, 1983, 1985, 1987, 1989, 1991, 1993, 1995, 1997, 1999, 2001, 2003, 2005, 2007, 2009, 2011, 2013, 2015, 2017, 2019, 2021, 2023, 2025, 2027, 2029, 2031, 2033, 2035, 2037, 2039, 2041, 2043, 2045, 2047, 2049, 2051, 2053, 2055, 2057, 2059, 2061, 2063, 2065, 2067, 2069, 2071, 2073, 2075, 2077, 2079, 2081, 2083, 2085, 2087, 2089, 2091, 2093, 2095, 2097, 2099, 2101, 2103, 2105, 2107, 2109, 2111, 2113, 2115, 2117, 2119, 2121, 2123, 2125, 2127, 2129, 2131, 2133, 2135, 2137, 2139, 2141, 2143, 2145, 2147, 2149, 2151, 2153, 2155, 2157, 2159, 2161, 2163, 2165, 2167, 2169, 2171, 2173, 2175, 2177, 2179, 2181, 2183, 2185, 2187, 2189, 2191, 2193, 2195, 2197, 2199, 2201, 2203, 2205, 2207, 2209, 2211, 2213, 2215, 2217, 2219, 2221, 2223, 2225, 2227, 2229, 2231, 2233, 2235, 2237, 2239, or 2241.

[0303] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence comprising residues 403-1239, or residues 382-1239 of SEQ ID NO: 627, 947, 1125, 1367, 1547, 1639, or 1709, or a polynucleotide sequence comprising SEQ ID NO: 627, 947, 1125, 1367, 1547, 1639, or 1709.

[0304] In some embodiments, the present disclosure provides a recombinant polynucleotide capable of hybridizing under highly stringent conditions to a reference polynucleotide encoding an engineered protease polypeptide described herein, e.g., a recombinant polynucleotide provided in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, or a reverse complement thereof. In some embodiments, the present disclosure provides a recombinant polynucleotide capable of hybridizing under highly stringent conditions to a reverse complement of a reference polynucleotide encoding an engineered protease polypeptide described herein, wherein the recombinant polynucleotide hybridizing under stringent conditions encodes an protease polypeptide comprising an amino acid sequence having one or more residue differences as compared to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, at residue positions selected from any positions as set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**. In some embodiments, the recombinant polynucleotide that hybridizes under highly stringent conditions comprises a polynucleotide sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 403-1239, or residues 382-1239 of SEQ ID NO: 3, 627, 947, 1125, 1367, or 1547, or to a reference polynucleotide sequence corresponding to SEQ ID NO: 3, 627, 947, 1125, 1367, or 1547. In some additional embodiments, the polynucleotide hybridizing under highly stringent conditions comprises a polynucleotide sequence having at least 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to at least one polynucleotide reference sequence corresponding to nucleotide residues 403-1239, or residues 382-1239 of a polynucleotide sequence provided in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, or a polynucleotide sequence provided in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the recombinant polynucleotide hybridizing under stringent conditions encodes an engineered protease polypeptide.

[0305] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising a signal sequence or a signal peptide, as described herein. In some embodiments, the encoded signal sequence or signal peptide is functional in the host cell used or to be used for expression of the engineered protease polypeptide. In some embodiments the encoded signal sequence or signal peptide is fused to a pro-polypeptide form of the engineered protease to form a pre-pro-polypeptide. In some embodiments, the encoded signal sequence or signal peptide is fused to the polypeptide that includes the mature, active form of the engineered protease. In some embodiments, the encoded signal sequence can be a naturally occurring signal sequence or a synthetic signal sequence, including a hybrid signal sequence.

[0306] In some embodiments, the recombinant polynucleotide comprises a polynucleotide sequence encoding an engineered protease polypeptide comprising a fusion polypeptide. In some embodiments, the encoded engineered protease polypeptide can be fused to a variety of polypeptide sequences as described above. In some embodiments, the encoded fusion polypeptide of the engineered protease

polypeptides comprises a glycine-histidine or histidine-tag (His-tag), such as provided at the carboxy terminal region of SEQ ID NO: 2. In some embodiments, the encoded fusion protein of the engineered protease polypeptides comprise an epitope tag, such as c-myc, FLAG, V5, or hemagglutinin (HA). In some embodiments, the fusion protein of the engineered protease polypeptides comprises a GST, SUMO, Strep, MBP, or GFP tag.

[0307] In another aspect, the present disclosure further provides an expression vector comprising a recombinant polynucleotide encoding an engineered protease polypeptide described herein, e.g., for expression of the encoded engineered protease polypeptide. In some embodiments, the expression vector comprises one or more control sequences operably linked to the recombinant polynucleotide to regulate the expression of the recombinant polynucleotide and/or encoded polypeptide. In some embodiments, the control sequences include, among others, promoters, leader sequences, polyadenylation sequences, pro-peptide sequences, signal peptide sequences, and transcription terminators. In some embodiments, the control sequences, such as promoters, leader sequences, polyadenylation sequences, pro-peptide sequences, signal peptide sequences, and transcription terminators, are selected depending on the type chosen host cell into which the expression vector is to be introduced.

[0308] In some embodiments, suitable promoters are selected based on the host cells. In some embodiments, the promoter is a heterologous promoter. In some embodiments, for bacterial host cells, suitable promoters include, among others, promoters obtained from the *E. coli* lac operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus subtilis* *xylA* and *xylB* genes, and prokaryotic beta-lactamase gene (see, e.g., Villa-Kamaroff et al., Proc. Natl Acad. Sci. USA, 1978, 75:3727-3731), as well as the *tac* promoter (see, e.g., DeBoer et al., Proc. Natl Acad. Sci. USA, 1983, 80:21-25). In some embodiments, for fungal host cells, suitable promoters include, among others, promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, and *Fusarium oxysporum* trypsin-like protease (see, e.g., WO 96/00787), as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase), and mutant, truncated, and hybrid promoters thereof. Exemplary yeast cell promoters can be from the genes for *Saccharomyces cerevisiae* enolase (*ENO-1*), *Saccharomyces cerevisiae* galactokinase (*GAL1*), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (*ADH2/GAP*), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. In some embodiments, promoters effective in *Pichia* cells are used. In some embodiments, for insect host cells, suitable

promoters include, among others, baculovirus promoters (e.g., P10 and polyhedron promoters), OpIE2 promoter, and *Nephotettix cincticeps* actin promoters. In some embodiments, for mammalian host cells, suitable promoters include, among others, promoters of cytomegalovirus (CMV), chicken β -actin promoter fused with the CMV enhancer, simian virus 40 (SV40), human phosphoglycerate kinase, beta actin, elongation factor-1a or glyceraldehyde-3-phosphate dehydrogenase, or *Gallus* β -actin.

[0309] In some embodiments, the control sequence is a suitable transcription terminator sequence (i.e., a sequence recognized by a host cell to terminate transcription). In some embodiments, the terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the engineered protease polypeptide. Any suitable terminator which is functional in the host cell of choice finds use for the purposes in the present disclosure. In some embodiments, for bacterial expression, suitable transcription terminators include, among others, Rho-dependent terminators that rely on a Rho transcription factor, or Rho-independent, or intrinsic terminators, which do not require a transcription factor. Exemplary bacterial transcription terminators are described in Peters et al., *J Mol Biol.*, 2011, 412(5):793-813. In some embodiments, for fungal host cells, suitable transcription terminators include, among others, terminators from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease. Exemplary terminators for yeast host cells can be obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are known in the art (see, e.g., Romanos et al., *supra*). In some embodiments, for mammalian host cells, suitable terminators include, among others, transcription terminators of cytomegalovirus (CMV), Simian virus 40 (SV40), human growth hormone hGH, bovine growth hormone BGH, and human or rabbit beta globulin.

[0310] In some embodiments, the control sequence is also a suitable leader sequence (i.e., a non-translated region of an mRNA that is important for translation by the host cell). In some embodiments, the leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the engineered protease polypeptide. Any suitable leader sequence that is functional in the host cell of choice find use in expression of the engineered protease polypeptide. Exemplary leader sequences for mammalian and insect cells include, among others, leader sequences of expressed genes (e.g., heat shock protein, myosin, BIP immunoglobulin binding protein, GRP glucose regulated protein, etc.), viral leader sequences (e.g., EMC virus) and synthetic leader sequences, e.g., hTEE-658 and those described in, for example Cao et al., *Nature Commun.*, 2021, 12:4138, incorporated herein by reference. Exemplary leader sequences for fungal expression include, among others, those from *Aspergillus oryzae* TAKA amylase, and *Aspergillus nidulans* triose phosphate isomerase. Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-

phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

[0311] In some embodiments, the control sequence is also a polyadenylation sequence (i.e., a sequence operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA). Any suitable polyadenylation sequence which is functional in the host cell of choice finds use in the present invention. Exemplary polyadenylation sequences for mammalian and insect cells include, among others, those of genes for human and mouse alpha-globin, mouse kappa light chain, chicken ovalbumin, SV40, as well as a synthetic polyA sequences (see, e.g., Clerici et al., *eLife*, 2017, 6:e33111). Exemplary polyadenylation sequences for fungal host cells include, but not limited to, the genes for *Aspergillus oryzae* TKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase. Useful polyadenylation sequences for yeast host cells are known in the art (see, e.g., Guo and Sherman, *Mol. Cell. Bio.*, 1995, 15:5983-5990).

[0312] In some embodiments, the control sequence comprises one or more regulatory sequences that facilitate regulation of the expression of the polynucleotide and/or corresponding encoded polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. In prokaryotic host cells, suitable regulatory sequences include, among others, the lac, tac, and trp operator systems. In yeast host cells, suitable regulatory systems include, among others the ADH2 system or GAL1 system. In filamentous fungi, suitable regulatory sequences include, among others, the TKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter. In mammalian cells, suitable regulatory systems include, among others, zinc-inducible sheep metallothionein (MT) promoter, dexamethasone (Dex)-inducible promoter, mouse mammary tumor virus (MMTV) promoter; ecdysone insect promoter, tetracycline-inducible promoter system, RU486-inducible promoter system, and the rapamycin-inducible promoter system.

[0313] In some embodiments, the recombinant expression vector may be any suitable vector (e.g., a plasmid or virus), that can be conveniently subjected to recombinant DNA procedures and bring about the expression of the protease-encoding polynucleotide. The choice of the vector typically depends on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

[0314] In some embodiments, the expression vector is an autonomously replicating vector (i.e., a vector that exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid, an extra-chromosomal element, a minichromosome, or an artificial chromosome). The vector may contain any means for assuring self-replication. In some alternative

embodiments, the vector is one in which, when introduced into the host cell, it is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, in some embodiments, a single vector or plasmid, or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, and/or a transposon is utilized. In some embodiment, recombinant polynucleotides may be provided on a non-replicating expression vector or plasmid. In some embodiments, the non-replicating expression vector or plasmid can be based on viral vectors defective in replication (see, e.g., Travieso et al., *Vaccines*, 2022, Vol. 7, Article 75).

[0315] In some embodiments, the expression vector contains one or more selectable markers, which permit selection of transformed cells. A “selectable marker” is a gene, the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers include, among others, the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers, which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, or tetracycline resistance. Suitable markers for yeast host cells include, among others, *ADE2*, *HIS3*, *LEU2*, *LYS2*, *MET3*, *TRP1*, and *URA3*. Selectable markers for use in filamentous fungal host cells include, among others, *amdS* (acetamidase; e.g., from *A. nidulans* or *A. oryzae*), *argB* (ornithine carbamoyltransferases), *bar* (phosphinothricin acetyltransferase; e.g., from *S. hygrosopicus*), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase; e.g., from *A. nidulans* or *A. oryzae*), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Selectable marker for mammalian cells include, among others, chloramphenicol acetyl transferase (CAT), nourseothricin N-acetyl transferase, blasticidin-S deaminase, blasticidin S acetyltransferase, *Sh ble* (Zeocin® resistance), aminoglycoside 3'-phosphotransferase (neomycin resistance), *hph* (hygromycin resistance), thymidine kinase, and puromycin N-acetyl-transferase.

[0316] In another aspect, the present disclosure provides a host cell comprising a recombinant polynucleotide encoding an engineered protease polypeptide described herein, the polynucleotide(s) being operably linked to one or more control sequences for expression of the encoded engineered protease polypeptide(s) in the host cell. In some embodiments, the host cell is a bacterial cell, fungal cell, insect cell, or mammalian cell.

[0317] In some embodiments, the host cell is a bacterial cell, including, among others, *E. coli*, *B. subtilis*, *Vibrio fluvialis*, *Streptomyces* and *Salmonella typhimurium* cell. Exemplary bacterial host cells also include various *Escherichia coli* strains (e.g., W3110 (Δ fhuA) and BL21). In some embodiments, the host cell is a fungal cell, such as filamentous fungal cell or yeast cell. In some embodiments, suitable fungal host cells include, among others, *Pichia*, *Saccharomyces*, *Yarrowia*, *Kluyveromyces*, *Aspergillus*, *Trichoderma*, *Neurospora*, *Mucor*, *Penicillium* T. *Trichoderma*, or *Myceliophthora* fungal cell. Exemplary fungal host cell includes, among others, *Pichia pastoris*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus fumigatus*

Trichoderma reesei, *Neurospora crassa*, *Mucor circinelloides*, *Penicillium chrysogenum* T. *reesei*, *Trichoderma harzianum*, *Saccharomyces cerevisiae*, or *Myceliophthora thermophila*. In some embodiments, the host cell is an insect cell. In some embodiments, a suitable insect host cell is a lepidopteran or dipteran insect cell. Exemplary insect host cell includes, among others, Sf9 cell, Sf21 cell, Schneider 2 cell, and BTI-TN-5B1-4 (High Five) cell. In some embodiments, the host cell is a mammalian cell. In some embodiments, the host cell is a human cell or rodent cell. Exemplary mammalian cells include, among others, Expi293, HeLa, U2OS, A549, HT1080, CAD, P19, NIH 3T3, L929, Hek 293, 293F, 293E, 293T, COS, Vero, NS0, Sp2/0 cell, DUKX-X11, MCF-7, Y79, SO-Rb50, Hep G2, J558L, and CHO cell.

[0318] In some embodiments, the host cell expresses an engineered protease polypeptide described herein. In some embodiments, the engineered protease polypeptide expressed in the host cell is a pre-pro-polypeptide or pre-pro-enzyme form of the engineered protease polypeptide. In some embodiments, the engineered protease polypeptide expressed in the host cell is a pro-polypeptide or pro-enzyme form of an engineered protease polypeptide. In some embodiments, the engineered protease polypeptide expressed in the host cell is a proteolytically active polypeptide or an active protease of the engineered protease polypeptide. In some embodiments, the engineered protease polypeptide expressed in the host cell is mature, active protease of the engineered protease polypeptide.

[0319] In some embodiments, any suitable method for introducing polynucleotides for expression of the engineered protease polypeptides into cells will find use for the purposes herein. Suitable techniques include, among others electroporation, biolistic particle bombardment, liposome mediated transfection, calcium chloride transfection, and protoplast fusion.

[0320] In some embodiments, recombinant polynucleotides encoding the engineered protease polypeptide can be produced using any suitable methods known the art. For example, a wide variety of different mutagenesis techniques are available to the skilled artisan. Methods are available to make specific substitutions at defined amino acids (site-directed), specific or random mutations in a localized region of the gene (region-specific), or random mutagenesis over the entire gene (e.g., saturation mutagenesis). Numerous methods known to those in the art to generate polypeptide variants, include, by way of example and not limitation, site-directed mutagenesis of single-stranded DNA or double-stranded DNA using PCR, cassette mutagenesis, gene synthesis, error-prone PCR, shuffling, and chemical saturation mutagenesis. Non-limiting examples of methods used for DNA and protein engineering are provided in the following references: US Pat. No. 6,117,679; US Pat. No. 6,420,175; US Pat. No. 6,376,246; US Pat. No. 6,586,182; US Pat. No. 7,747,391; US Pat. No. 7,747,393; US Pat. No. 7,783,428; and US Pat. No. 8,383,346. After the variants are produced, they can be screened for any desired property (e.g., increased activity, increased thermal activity, increased stability, increased thermostability, increased resistance to gastric proteases, increased pH stability, etc.).

[0321] In some embodiments, the engineered protease polypeptides with the properties disclosed herein can be obtained by subjecting the polynucleotide encoding the naturally occurring or engineered protease polypeptide to a suitable mutagenesis and/or directed evolution methods known in the art, such as provided in the Examples. An exemplary directed evolution technique is mutagenesis and/or DNA shuffling (see, e.g., Stemmer, *Proc. Natl. Acad. Sci. USA*, 1994, 91:10747-10751; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767 and U.S. Pat. 6,537,746). Other directed evolution procedures that can be used include, among others, staggered extension process (StEP), in vitro recombination (see, e.g., Zhao et al., *Nat. Biotechnol.*, 1998, 16:258-261), mutagenic PCR (see, e.g., Caldwell et al., *PCR Methods Appl.*, 1994, 3:S136-S140), and cassette mutagenesis (see, e.g., Black et al., *Proc. Natl. Acad. Sci. USA*, 1996, 93:3525-3529).

[0322] Guidance for other suitable mutagenesis and directed evolution methods are described in, among others, US Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, 5,837,458, 5,928,905, 6,096,548, 6,117,679, 6,132,970, 6,165,793, 6,180,406, 6,251,674, 6,265,201, 6,277,638, 6,287,861, 6,287,862, 6,291,242, 6,297,053, 6,303,344, 6,309,883, 6,319,713, 6,319,714, 6,323,030, 6,326,204, 6,335,160, 6,335,198, 6,344,356, 6,352,859, 6,355,484, 6,358,740, 6,358,742, 6,365,377, 6,365,408, 6,368,861, 6,372,497, 6,337,186, 6,376,246, 6,379,964, 6,387,702, 6,391,552, 6,391,640, 6,395,547, 6,406,855, 6,406,910, 6,413,745, 6,413,774, 6,420,175, 6,423,542, 6,426,224, 6,436,675, 6,444,468, 6,455,253, 6,479,652, 6,482,647, 6,483,011, 6,484,105, 6,489,146, 6,500,617, 6,500,639, 6,506,602, 6,506,603, 6,518,065, 6,519,065, 6,521,453, 6,528,311, 6,537,746, 6,573,098, 6,576,467, 6,579,678, 6,586,182, 6,602,986, 6,605,430, 6,613,514, 6,653,072, 6,686,515, 6,703,240, 6,716,631, 6,825,001, 6,902,922, 6,917,882, 6,946,296, 6,961,664, 6,995,017, 7,024,312, 7,058,515, 7,105,297, 7,148,054, 7,220,566, 7,288,375, 7,384,387, 7,421,347, 7,430,477, 7,462,469, 7,534,564, 7,620,500, 7,620,502, 7,629,170, 7,702,464, 7,747,391, 7,747,393, 7,751,986, 7,776,598, 7,783,428, 7,795,030, 7,853,410, 7,868,138, 7,783,428, 7,873,477, 7,873,499, 7,904,249, 7,957,912, 7,981,614, 8,014,961, 8,029,988, 8,048,674, 8,058,001, 8,076,138, 8,108,150, 8,170,806, 8,224,580, 8,377,681, 8,383,346, 8,457,903, 8,504,498, 8,589,085, 8,762,066, 8,768,871, 9,593,326, 9,665,694, 9,684,771, and all related PCT and non-US counterparts; Ling et al., *Anal. Biochem.*, 1997, 254(2):157-78; Dale et al., *Meth. Mol. Biol.*, 1996, 57:369-74; Smith, *Ann. Rev. Genet.*, 1985, 19:423-462; Botstein et al., *Science*, 1985, 229:1193-1201; Carter, *Biochem. J.*, 1986, 237:1-7; Kramer et al., *Cell*, 1984, 38:879-887; Wells et al., *Gene*, 1985, 34:315-323; Minshull et al., *Curr. Op. Chem. Biol.*, 1999, 3:284-290; Christians et al., *Nat. Biotechnol.*, 1999, 17:259-264; Cramer et al., *Nature*, 1998, 391:288-291; Cramer et al., *Nat. Biotechnol.*, 1997, 15:436-438; Zhang et al., *Proc. Nat. Acad. Sci. U.S.A.*, 1997, 94:4504-4509; Cramer et al., *Nat. Biotechnol.*, 1996, 14:315-319; Stemmer, *Nature*, 1994, 366:389-391; Stemmer, *Proc. Nat. Acad. Sci. USA*, 1994, 91:10747-10751; EP 3 049 973; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767; WO 2009/152336; and WO 2015/048573, all of which are incorporated herein by reference.

[0323] In some embodiments, the clones obtained following mutagenesis treatment are screened by subjecting polypeptide preparations to a defined treatment conditions or assay conditions (e.g., temperature, pH condition, gastric protease, etc.) and measuring polypeptide activity after the treatments or other suitable assay conditions. Clones containing a polynucleotide encoding the polypeptide of interest are then isolated, the polynucleotide sequenced to identify the nucleotide sequence changes (if any), and used to express the polypeptide in a host cell. Measuring polypeptide activity from the expression libraries can be performed using any suitable method known in the art and as described in the Examples.

[0324] For engineered polypeptides of known sequence, the polynucleotides encoding the subject polypeptide can be prepared by standard solid-phase methods, according to known synthetic methods. In some embodiments, fragments of up to about 100 bases can be individually synthesized, then joined (e.g., by enzymatic or chemical ligation methods, or polymerase mediated methods) to form any desired continuous sequence. For example, polynucleotides and oligonucleotides disclosed herein can be prepared by chemical synthesis using the classical phosphoramidite method (see, e.g., Beaucage et al., Tet. Lett., 1981, 22:1859-69; and Matthes et al., EMBO J., 1984, 3:801-05), as it is typically practiced in automated synthetic methods. According to the phosphoramidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated, and cloned in appropriate vectors.

[0325] In some embodiments, a method for preparing the engineered protease polypeptide can comprise: (a) synthesizing a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from the amino acid sequence of any engineered protease polypeptide as described herein, and (b) expressing the engineered protease polypeptide encoded by the polynucleotide. In some embodiments of the method, the amino acid sequence encoded by the polynucleotide can optionally have one or several (e.g., up to 3, 4, 5, or up to 10) amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-15, 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-30, 1-35, 1-40, 1-45, or 1-50 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, or 50 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 22, 23, 24, or 25 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the substitutions are conservative or non-conservative substitutions.

Methods of Preparing Engineered Protease Polypeptide and Proteolytically Active Polypeptide

[0326] In another aspect, the present disclosure provides a method of producing an engineered protease polypeptide, where the method comprises culturing a host cell comprising an expression vector capable of expressing a polynucleotide encoding the engineered protease polypeptide under suitable conditions

such that the engineered protease polypeptide is expressed or produced. Appropriate culture media and growth conditions for various host cells are known in the art.

[0327] In some embodiments, the method further comprises a step of isolating the expressed protease polypeptide, such as from the culture medium and/or cells. In some embodiments, the method further comprises purifying the expressed engineered protease polypeptide. In some embodiments, isolating and/or purifying the engineered protease polypeptide can be done by using any one or more of the known techniques for protein purification, including, among others, detergent lysis, sonication, filtration, salting-out, selective precipitation, ultra-centrifugation, and chromatography.

[0328] Chromatographic techniques for isolation and/or purification of polypeptides and proteins include, among others, reverse phase chromatography, high-performance liquid chromatography, ion-exchange chromatography, hydrophobic-interaction chromatography, size-exclusion chromatography, gel electrophoresis, and affinity chromatography. Conditions for purifying a particular polypeptide may depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, molecular weight, molecular shape, etc., and will be apparent to those having skill in the art. In some embodiments, affinity techniques may be used to isolate the engineered protease polypeptides. For affinity chromatography purification, any antibody that specifically binds the engineered protease polypeptide of interest can be used. Where the engineered protease includes a fusion polypeptide that includes an affinity tag, such as a His-tag, standard affinity methods for the particular fusion polypeptide can be used.

[0329] In some embodiments, the present disclosure provides a method of preparing a proteolytically active polypeptide or an active protease of an engineered protease polypeptide. In some embodiments, a method of preparing a proteolytically active protease polypeptide comprises incubating or reacting an engineered protease polypeptide described herein under suitable conditions such that the proteolytically active protease or active protease is produced. In some embodiments, the method is used to prepare a mature proteolytically active polypeptide or active protease of an engineered protease polypeptide.

[0330] In some embodiments, the proteolytically active polypeptide or active protease is prepared from an engineered protease polypeptide that contains a pro-domain of the protease. In some embodiments, the proteolytically active polypeptide or active protease is prepared from a pro-polypeptide form of the engineered protease polypeptide. In some embodiments, the proteolytically active polypeptide or active protease prepared by the method has an amino terminus within amino acid residues 128-135, particularly where the amino terminus is at amino acid residue 128 or 135, wherein the amino acid positions are numbered with respect to SEQ ID NO: 2, or an equivalent position for any engineered protease polypeptide variant described herein.

[0331] In some embodiments, the suitable conditions for preparing the proteolytically active polypeptide or active protease is sufficient for activation of autoproteolysis of the appropriate engineered protease polypeptide. Without being bound by any theory of operation, an engineered protease polypeptide

containing a pro-domain can undergo auto-proteolysis to generate a proteolytically active polypeptide or an active protease in which autoproteolysis occurs at least at amino acid position 128 and/or 135, where the amino acid positions are numbered with respect to SEQ ID NO: 2, or an equivalent position for any of the engineered protease polypeptide variants described herein.

[0332] In some embodiments, the proteolytically active polypeptide or active protease can be prepared by subjecting the pro-polypeptide form of the engineered protease polypeptide to another protease that can cleave the pro-polypeptide and separate the pro-domain from the protease domain. In some embodiments, the other protease comprises a proteolytically active polypeptide or active protease of an engineered protease polypeptide described herein. Exemplary conditions for auto-proteolysis or proteolysis with an engineered protease are provided in the Examples.

Compositions and Pharmaceutical Compositions

[0333] In a further aspect, the present disclosure provides a composition comprising an engineered protease polypeptide. In some embodiments, the composition comprises an engineered protease polypeptide, wherein the engineered protease polypeptide is in the form of a pre-pro-polypeptide or pre-pro-enzyme; a pro-polypeptide or pro-enzyme; or a proteolytically active polypeptide or active protease, including mature, proteolytically active polypeptide, as described herein. In some embodiments, the pro-polypeptide or pro-enzyme form of the engineered protease polypeptide if a form with reduced or low protease activity is desired, for example for storage and/or prior to activation of the protease.

[0334] In some embodiments, the composition comprises an engineered protease polypeptide as a dietary/nutritional supplement, or in combination with food or drink. In some embodiments, the engineered protease polypeptide may be used in any suitable edible enzyme delivery matrix. In some embodiments, engineered protease polypeptide are present in an edible enzyme delivery matrix designed for rapid dispersal of the protease within the digestive tract of an animal or subject upon ingestion of the polypeptide. In some embodiments, an engineered protease polypeptide is mixed or admixed with protein-containing food or a drink. Non-limiting examples of such foods include a protein-containing powder, a spread, a spray, a sauce, a dip, a cream, dressing, cheese, butter, margarines, spreads, butter, dairy products, nut butters, seed butters, kernel butters, peanut butter, vegetables, meats, poultry, and fish. In some embodiments, the engineered protease polypeptide is mixed or admixed with infant formula or with breast milk.

[0335] In some embodiments, the engineered protease is formulated as a pharmaceutical composition. Depending on the mode of administration, the compositions comprise a therapeutically effective amount of an engineered protease polypeptide and can be in the form of a solid, semi-solid, or liquid. In some embodiments, the pharmaceutical composition comprises an engineered protease polypeptide, and a pharmaceutically acceptable carrier, excipient, or diluent. The carrier can be a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be

sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers.

[0336] In some embodiments, the excipient includes, among others, starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain appropriate amounts of wetting or emulsifying agents, and/or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Examples of suitable pharmaceutical carriers are described in Remington: The Science and Practice of Pharmacy, 23rd Ed, A. Adejare ed., Academic Press, 2020, incorporated in its entirety by reference herein. Such compositions will contain a therapeutically effective amount of the engineered protease polypeptide, preferably in purified form, together with a suitable amount of carrier and/or excipient so as to provide the form for proper administration to the subject.

[0337] In some embodiments, the engineered protease polypeptide is formulated for use as oral pharmaceutical compositions (e.g., for oral administration). Any suitable format for use in delivering the protease polypeptide may be used, including but not limited to pills, tablets, gel tabs, capsules, lozenges, dragees, powders, soft gels, sol-gels, gels, emulsions, sprays, ointments, liniments, creams, pastes, jellies, demulcents, sticks, suspensions (including but not limited to oil-based suspensions, oil-in water emulsions, etc.), slurries, syrups, controlled release formulations. For oral administration, the pharmaceutical composition can be used alone or in combination with appropriate additives to make the tablets, powders, granules, capsules, syrups, liquids, suspensions, etc. For example, solid oral forms of the composition can be prepared with conventional additives, disintegrators, lubricants, diluents, buffering agents, moistening agents, preservatives and flavoring agents. Non-limiting examples of excipients include sugars (e.g., lactose, sucrose, mannitol, and/or sorbitol), starches (e.g., corn, wheat, rice, potato, or other plant starch), cellulose (e.g., methyl cellulose, hydroxypropylmethyl cellulose, sodium carboxy-methylcellulose), gums (e.g., arabic, tragacanth, guar, etc.), and/or proteins (e.g., gelatin, collagen, etc.). Additional components in oral formulations may include coloring and or sweetening agents (e.g., glucose, sucrose, and mannitol) and lubricating agents (e.g., magnesium stearate), as well as enteric coatings (e.g., methacrylate polymers, hydroxyl propyl methyl cellulose phthalate, and/or any other suitable enteric coating known in the art). In some embodiments, the formulation releases the enzyme(s) in the stomach of the subject so that target proteins can be degraded by the engineered protease.

[0338] In some embodiments, the engineered protease polypeptide is provided as a unit dose formulation. For example, and without limitation, the unit dose may be present in a tablet, a capsule, and the like. The unit dose may be in solid, liquid, powder, or any other form. A unit dose formulation of the

pharmaceutical composition will allow for appropriate dosing while avoiding potential negative side effects of administering an excessive amount of the composition.

[0339] In some embodiments, the engineered protease polypeptide or composition thereof, including as a pharmaceutical composition, can be lyophilized from an aqueous solution, optionally in the presence of appropriate buffers (e.g., phosphate, citrate, histidine, imidazole buffers) and excipients (e.g., cryoprotectants such as sucrose, lactose, trehalose, etc.). Lyophilizates can optionally be blended with excipients and made into different forms.

[0340] In some embodiments, the composition, including a pharmaceutical composition, further comprises a lipase. In some embodiments, the lipase can be any lipase suitable for treating exocrine pancreatic insufficiency. In some embodiments, the composition, including a pharmaceutical composition further comprises an amylase. In some embodiments, the amylase can be any amylase suitable for treating exocrine pancreatic insufficiency. In some embodiments, the composition, including a pharmaceutical composition, further comprises a lipase and an amylase. In some embodiments, the protease and amylase are suitable for treating pancreatic insufficiency.

Uses and Methods

[0341] In another aspect, the present disclosure provides use of the engineered protease polypeptides for degrading a target protein or polypeptide. In some embodiments, a method of degrading a target protein or polypeptide comprises contacting a target protein or polypeptide with an engineered protease polypeptide described under suitable conditions for proteolytically active polypeptide or an active protease of the engineered protease polypeptide to degrade the target protein or polypeptide. In some embodiments, the target protein or polypeptide comprises a mixture or proteins and/polypeptides. In some embodiments, the mixture of protein and/or polypeptides is protein(s) in food or drink.

[0342] In some embodiments, the engineered protease polypeptide is a pro-polypeptide form, and the suitable conditions promote formation of the proteolytically active polypeptide or active protease of the engineered protease polypeptide. In some embodiments, the engineered protease polypeptide is the proteolytically active protease or active protease, e.g., protease polypeptide comprising amino acid residues 135-413 or 128-413, that does not need any further activation.

[0343] In some embodiments, the engineered protease polypeptides are applied in the treatment of exocrine pancreatic insufficiency or a deficiency in pancreatic enzymes required for efficient digestion of proteins in food. In some embodiments, a method of treating a deficiency in pancreatic enzymes required for efficient digestion of proteins in food, comprises administering to a subject in need thereof an effective amount of an engineered protease polypeptide or a pharmaceutical composition thereof described herein. In some embodiments, the subject is a human patient with exocrine pancreatic insufficiency.

[0344] In some embodiments, the engineered protease polypeptide administered is the pro-polypeptide form, e.g., protease polypeptide of residues 1-413 of an engineered protease polypeptide, where the engineered protease polypeptide is activated to the proteolytically active polypeptide or active protease prior to administration, or administered under conditions that result in activation to the proteolytically active polypeptide or active protease. In some embodiments, the engineered protease polypeptide administered is the proteolytically active polypeptide or the active protease, e.g., protease polypeptide comprising residues 128-413 or 135-413 of an engineered protease polypeptide.

[0345] In some embodiments, the engineered protease polypeptide or pharmaceutical composition thereof is administered immediately prior to, concurrently with, or subsequent to consumption of a protein-containing food or drink. In some embodiments, the engineered protease polypeptide is preferably administered concurrently with the ingestion of the food or drink.

[0346] In some embodiments, the subject for treatment is a human infant. In some embodiments, the engineered protease polypeptide is administered with infant formula or during breast feeding.

[0347] In some embodiments, the subject for treatment is a child, e.g., 12 months or older and up to 4 years old. In some embodiments, the subject for treatment is a child older than 4 years old, or a young adult, up to 18 years of age. In some embodiments, the subject for treatment is a human adult.

[0348] In some embodiments, the present disclosure provides use of an engineered protease polypeptide described herein for treating exocrine pancreatic insufficiency.

[0349] In some embodiments, the present disclosure provides use of an engineered protease polypeptide described herein in the preparation of a medicament for treating exocrine pancreatic insufficiency.

EXAMPLES

[0350] The following Examples, including experiments and results achieved, are provided for illustrative purposes only and are not to be construed as limiting the present invention. In the embodiments herein, the abbreviations and technical terms are those commonly used and known in the art.

Example 1

Protease Gene Acquisition and Construction of Expression Vectors

[0351] A wild-type protease (WP_077617485) of *Bacillus sinesaloumensis* Marseille P3516 from the serine peptidase S8 family with a C-terminal 6X-histidine tag (SEQ ID NO: 2) was codon optimized for expression in *E. coli* and cloned into an *E. coli* expression vector system (see, e.g., US Pat. Application WO2021/061915A2). In addition, in some embodiments, expression vectors lacking antimicrobial resistance markers are used. The plasmid construct was transformed into an *E. coli* strain derived from W3110. Directed evolution techniques were used to generate libraries of gene variants from this plasmid construct (see e.g., US Pat. No. 8,383,346, and WO2010/144103) as well as its derivatives.

Example 2

High-Throughput (HTP) Growth of Protease Variants and Screening Conditions

2.1: HTP Growth of *Bacillus sinesaloumensis* Marseille P3516 Protease and Variants

[0352] Transformed *E. coli* cells were selected by plating onto Luria Broth (LB) agar plates containing 1% glucose with selection. After overnight incubation at 37 °C, colonies were placed into the wells of 96-well shallow flat bottom plates (NUNC™, Thermo-Scientific) filled with 180 µl/well LB supplemented with 1% glucose and selection (e.g., chloramphenicol). The cultures were allowed to grow overnight for 18-20 hours in a shaker (200 rpm, 30 °C, and 85% relative humidity; Kuhner).

[0353] Overnight growth samples (20 µL) were transferred into Costar 96-well deep plates filled with 380 µL of Terrific Broth (TB) supplemented with a selection. The plates were incubated for 130 minutes in a shaker (250 rpm, 30 °C, and 85% relative humidity; Kuhner). The cells were then induced with 40 µL of 10 mM isopropylthiogalactoside (IPTG) in sterile water and incubated overnight for 20-24 hours in a shaker (250 rpm, 30 °C, and 85% relative humidity; Kuhner). The cells were pelleted (4000 rpm x 20 min), the supernatants were discarded, and the cells were frozen at -80 °C prior to analysis.

2.2: Lysis of HTP Cell Pellets

[0354] For cell lysis, 200, 300, or 400 µL of lysis buffer (1X PBS, 1 mg/ml lysozyme, 0.5 mg/ml polymyxin B sulfate, 0.4U/mL DNase I from New England Biolabs) was added to the cell pellets. The mixture was agitated for 1.5-2 hours at room temperature, and centrifuged (4000 rpm x 15 min) prior to further analysis. At this stage, the sample is referred to as “clarified lysate.” Sometimes additional dilutions of clarified lysate were performed in PBS buffer prior to subsequent challenges and activity assays.

2.4: Activation of Clarified Lysates

[0355] To activate the protease, a small quantity of previously purified and activated protease was added to the clarified lysate to degrade the pro-peptide and facilitate the activation of the protease present in the lysate. Clarified lysate was mixed 1:1 with PBS containing 0.02 g/L of purified protease in a BioRad hardshell plate (final concentration 50% clarified lysate and 0.01 g/L purified protease). Samples were mixed and incubated at 37 °C in a thermocycler for 16+ hours. At this stage, the sample is referred to as “activated lysate.” Sometimes additional dilutions of activated lysate were performed in PBS buffer prior to subsequent challenges and activity assays.

2.5: Analysis of Activated Lysates for Protease Activity with Casein Activity Assay

[0356] The activity of protease variants was determined by measuring the degradation of Casein using an activity assay adapted for high throughput from the United States Pharmacopeia protease assay. For this assay, 80 µL of reaction buffer (50 mM Potassium Phosphate buffer, pH 7.5, or 100 mM sodium phosphate buffer, pH 6.5) was added to a Costar deepwell plate. Next, 80 µL of 15 g/L casein sodium salt

dissolved in water was added to the reaction plate. Finally, 40 μ L of the sample to be analyzed was added to the reaction plate, starting the reaction. Reaction plates were incubated at 40 °C in a multitrion shaker with shaking at 400 rpm for 1 hour. After 1 hour, 200 μ L of 50 g/L of trichloroacetic acid (TCA) was added to the reaction plate, simultaneously quenching the reaction and precipitating out any whole proteins. Quenched reactions were thoroughly shaken and centrifuged to pellet out any precipitated protein. After centrifugation, 200 μ L of supernatant was transferred to a Greiner UV-star flat bottom plate and the absorbance was read at 280nm using a Molecular Devices SpectraMax M2 plate reader. To ensure that samples did not saturate the assay, samples were often diluted prior to analysis based on a pre-determined dilution factor (up to 128x). The term “unchallenged activity” is defined as protease activity without any prior challenge described in Examples 2.7-2.8.

2.6: Analysis of Clarified Lysates for Protease Activity with BODIPY-Casein Assay

[0357] In some cases, the activity of protease variants was determined by measuring the degradation of Casein using the EnzChek Protease Assay Kit (Thermo Fisher Scientific). For this assay, 90 μ L of 10 μ g/mL BODIPY-casein substrate in aqueous buffer (100 mM sodium phosphate buffer, pH 7) was added to a 96-well, opaque, black microtiter plate (Costar). To start the assay, 10 μ L of sample (challenged, diluted cell lysate) was added to the reaction mix and incubated at 37 °C in a multitrion shaker with shaking at 400rpm. After 1 hour, plates were read on a Molecular Devices SpectraMax M2 plate reader for fluorescence (Excitation: 485, Emission: 530). To ensure that samples did not saturate the assay, samples were diluted in assay buffer prior to analysis based on a pre-determined dilution factor (up to 250x). The term “unchallenged activity” is defined as protease activity without any prior challenge described in Examples 2.8.

2.7: HTP Analysis of Activated Lysates Pre-Incubated with Heat Challenge

[0358] Thermostability of protease variants were assessed as described herein. Clarified lysate or activated lysate was transferred to a PCR plate (BioRad) and incubated for 1 hour at 63 °C, 64 °C or 71 °C in a thermocycler. After incubation, samples were centrifuged and the supernatant was analyzed for residual protease activity as described in Example 2.5.

2.8: HTP Analysis of Clarified Lysates or Activated Lysates Pre-Incubated at Reduced pH in the Presence of Pepsin

[0359] The activities of protease variants were determined after pre-incubation at low pH in the presence of pepsin to simulate the environment of the stomach. Clarified lysate, activated lysate, or activated lysate further diluted in PBS was mixed 1:1 with McIlvaine buffer, pH 2.8 - 4.5, + 4000 U/mL (1.6 mg/mL) pepsin from porcine gastric mucosa (Sigma) in a PCR plate (BioRad), for a final challenge pH 2.8–4.5 and a final pepsin concentration of 2000 U/mL (0.8 mg/mL). Samples were mixed then incubated for 1 hour at 37 °C in a thermocycler. To stop the challenges, samples were mixed 1:1 with 400 mM sodium phosphate buffer, pH 7.0, neutralizing the pH and inactivating the pepsin. Neutralized challenge

samples were then further diluted and analyzed for residual protease activity as described in Examples 2.5 – 2.6.

Example 3

Screening Results of Protease Variants derived from SEQ ID NO: 4

3.1: HTP Growth of *Bacillus sinosaloumensis* Marseille P3516 Protease and Variants

[0360] The polynucleotide sequence of SEQ ID NO: 1 encoding the polypeptide of SEQ ID NO: 2 was subcloned into a different *E. coli* expression vector system (see e.g., US Pat. Application WO2021061915A2) and was used as the backbone for the construction of protease variants in this Example. Within the Sequence Listing, for purposes of consistency in description, the protease variants studied in this Example are represented as a fragment (residues 1-413, SEQ ID NO: 4) of their full-length sequence of actual variants examined. In this Example, the actual variants studied include residues 414-533 of SEQ ID NO: 2 and the His-tag sequence (see **FIG. 1**). New variants were generated by known protein evolution techniques and the variants screened using the BODIPY-casein activity assay after a one hour pre-incubation at pH 4.5 in the presence of pepsin, as described in Example 2. Some variants were subjected to a modified pre-incubation with more lysate present in the challenge prior to screening with the BODIPY-casein activity assay. Analysis of the data relative to SEQ ID NO: 4 is listed in **Table 3.1**. Some variants from these libraries were assayed in triplicate in the BODIPY-casein activity assay after no prior challenge, and after a 1 hour pre-incubation at pH 4.5 in the presence of pepsin, as described in Example 2. Analysis of the average data relative to SEQ ID NO: 4 is listed in **Table 3.2**.

Table 3.1: Protease activity relative to SEQ ID NO: 4			
SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 4)	High lysate pH 4.5 and Pepsin Challenge Improvement Relative to SEQ ID NO: 4	pH 4.5 and Pepsin Challenge Improvement Relative to SEQ ID NO: 4 ¹
3/4		+	+
5/6	N185F	++	
7/8	Q134I	++	
9/10	G129T	+++	
11/12	A135C	++	
13/14	T184A	++	
15/16	G129R	+++	
17/18	T132Y	++	
19/20	L186R	++	
21/22	G193T	+	
23/24	G263P	++	
25/26	D370C	+	
27/28	D45Y/Q134W	++	
29/30	N185M	+	

Table 3.1: Protease activity relative to SEQ ID NO: 4			
31/32	Q199K	++	
33/34	N368T	+++	
35/36	S161E	+++	
37/38	N141T	++	
39/40	Q267L	++	
41/42	A179S	++	
43/44	N185V	+	
45/46	G264L	+++	
47/48	Q199C	+	
49/50	S160M	++	
51/52	P138Q	+	
53/54	A131Y	+	
55/56	T184D	+	
57/58	S372R	+	
59/60	Q134L	++	
61/62	D370R	++	
63/64	D370I	++	
65/66	Q134E	+++	
67/68	N368G	++	
69/70	E151D	+	
71/72	Q274K	+	
73/74	Q134D	+++	
75/76	Q134V	++	
77/78	E128I	++	
79/80	Y339S	+++	
81/82	N313A	++	
83/84	A131E	+	
85/86	N185P	++	
87/88	A374W	+	
89/90	R314G	++	
91/92	V191R	+	
93/94	E128V	++	
95/96	T132E	++	
97/98	S324R	+	
99/100	T315M	+	
101/102	T132V	+	
103/104	Q375L	+	
105/106	Q375T	++	
107/108	G129L	+	
109/110	T132P	++	
111/112	T184M	+	
113/114	V136G	+	
115/116	L186A	+	
117/118	A135S	+	

Table 3.1: Protease activity relative to SEQ ID NO: 4			
119/120	Q220L	+	
121/122	Q134P	++	
123/124	T132A	+	
125/126	N141M	++	
127/128	A135I	+	
129/130	N194D	++	
131/132	N185Q	++	
133/134	G263H	++	
135/136	Q274L	++	
137/138	D231V	++	
139/140	T315R	++	
141/142	Q375S	++	
143/144	A135T	++	
145/146	N185G	++	
147/148	A135R	+	
149/150	V277D	+	
151/152	E128P	++	
153/154	T132R	+	
155/156	P369I	+	
157/158	G264C	++	
159/160	T315H	+	
161/162	I251S	+	
163/164	V136I	+	
165/166	S160P	++	
167/168	Q375I	++	
169/170	N180M	+	
171/172	P369V	++	
173/174	I251D	++	
175/176	G264A	+	
177/178	K163L	++	
179/180	D231H	++	
181/182	R343S	++	
183/184	G264R/Q279R	++	
185/186	Q274A	++	
187/188	Q279Y	++	
189/190	A131P	++	
191/192	N232S	++	
193/194	Q220R	++	
195/196	T315Q	+	
197/198	L186T	+	
199/200	S324V	++	
201/202	N313S	+	
203/204	T132D	++	
205/206	N141R/A300V	++	

Table 3.1: Protease activity relative to SEQ ID NO: 4			
207/208	S324I	++	
209/210	A367V	++	
211/212	A135V	++	
213/214	D370L	+	
215/216	T132G	++	
217/218	Q267G	++	
219/220	A131T	+	
221/222	N266T	+	
223/224	A179K	+	
225/226	S372A	+	
227/228	S372F	++	
229/230	N185T	++	
231/232	S324D	++	
233/234	A135K	+	
235/236	R188A	++	
237/238	N141D	++	
239/240	A374L	+	
241/242	N185D	++	
243/244	T130N	+	
245/246	D370V	+	
247/248	S161R	++	
249/250	T315I	+	
251/252	T315L	+++	
253/254	S318N	++	
255/256	R188C	++	
257/258	N180L	++	
259/260	S372Y	++	
261/262	A135P	++	
263/264	Q375E	++	
265/266	S324A	++	
267/268	G129K	++	
269/270	Q134M	+	
271/272	T184G	+++	
273/274	N185A	++	
275/276	G129H	++	
277/278	R188D	+	
279/280	T130F	+	
281/282	Y265C	++	
283/284	N141W		++
285/286	S324W		+
287/288	D370E		++
289/290	T184R		+
291/292	Q134A		+
293/294	S161L		++

Table 3.1: Protease activity relative to SEQ ID NO: 4			
295/296	Q134T		+
297/298	D370G		++
299/300	Q375A		++
301/302	E128G		++
303/304	T130V		++
305/306	Q134N		++
307/308	N341G		++
309/310	F190S		++
311/312	D370P		+
313/314	N145R		+
315/316	Q279H		+
317/318	Q279S		+
319/320	S160Q		+
321/322	D370K		+
323/324	A126T/G192C		+
325/326	A374E		++
327/328	E128K		++
329/330	S160C		+
331/332	L186S		++
333/334	N11K/Q220K		+
335/336	Q134W		++
337/338	G129V		++
339/340	E128L		++
341/342	E151Q		++
343/344	Q375M		++
345/346	Q134C		+
347/348	A374R		++
349/350	S160T		+
351/352	Q279T		++
353/354	G264F		++
355/356	T132C		++
357/358	G129F		++
359/360	G264V		++
361/362	G129I		++
363/364	T184Q		+
365/366	G192M		+
367/368	A374S		++
369/370	D370F		++
371/372	Q267A		++
373/374	P369W		+
375/376	Q199L		+
377/378	N145M		+
379/380	N194A		++
381/382	N185S		++

Table 3.1: Protease activity relative to SEQ ID NO: 4			
383/384	Y265R		+
385/386	G129S		++
387/388	N185R		+
389/390	R188W		++
391/392	S161G	++	
393/394	D370G/Q392Y	++	
395/396	I99V/A278N	++	
397/398	Y265G/T311D	++	
399/400	V84M/S159G/Y265G/Q279K/T311D/ D370G	++	
401/402	T311D/R316K	++	
403/404	S342N/D370G	++	
405/406	Y265G/T311D/D370G	++	
407/408	G192D/T311D/R316K	++	
409/410	N141Q/A154D/G192D	++	
411/412	Y265G/T311D/R316K/S342N	++	
413/414	Q279K/T311D/R316K	++	
415/416	N141Q/Y265G/Q279K/T311D/S342N	++	
417/418	N141Q/G192D/T311D/R316K/D370G	++	
419/420	N141Q	++	
421/422	N141Q/Y265G/T311D	++	
423/424	V198G/Q279K	++	
425/426	Q392Y	+	
427/428	S342N/D370G/Q392Y	++	
429/430	N141Q/V198G/Y265G	++	
431/432	Y265G/Q392Y	++	
433/434	Y265G	++	
All activities were determined relative to the reference polypeptide of SEQ ID NO: 4. Levels of increased activity are defined as follows: "+" 0.9 to 1.2, "++" > 1.2, "+++" > 1.8. The reference sequence and all variants within this table are represented in the Sequence Listing as a fragment (residues 1-413) of their full length sequence, as described in Example 3			

Table 3.2: Protease activity relative to SEQ ID NO: 4			
SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 4)	pH 4.5 pepsin challenge improvement relative to SEQ ID NO: 4	Unchallenged activity improvement relative to SEQ ID NO: 4
435/436	A135L	+	
437/438	N194L	+	+
439/440	E128T	+	+
441/442	Q134S	+	+

Table 3.2: Protease activity relative to SEQ ID NO: 4			
443/444	N313T	+	+
445/446	T184L/Q267L	+	+
447/448	N185E	+	+
449/450	S342G	++	+
451/452	A374Y	+	+
453/454	N141R	+	
455/456	L186Y	+	+
457/458	S312R	+	
459/460	N313Q	+	+
461/462	T315V	+	+
463/464	A374G	+	+
465/466	E128S	+	+
467/468	V136A	+	+
469/470	E128R	+	
471/472	D370Q	+	
473/474	Q267V	+	+
475/476	R188M	+	+
477/478	R188F	+	++
479/480	G263S	+	++
481/482	R188S	+	+
483/484	Y339W	+	+
485/486	A100V/I251S	+	+++
487/488	A131V	+	+
489/490	R188T	+	+
491/492	N141L	++	
493/494	Q134Y	+	
495/496	Q267M	+	+
497/498	G264N	+	+
499/500	Q134G	+	+
501/502	N185L	+	+
503/504	D370S	+	
505/506	Q267W	+	+
507/508	Q279M	+	++
509/510	Q267R	+	+
511/512	G264T	+	+
513/514	Q279L	+	++
515/516	G263R	+	
517/518	V136C	+	
519/520	N145E	++	+
521/522	R188G	+	+
523/524	T130A	+	+
525/526	G192N	+	+
527/528	R188L	+	+
529/530	S312I	++	

Table 3.2: Protease activity relative to SEQ ID NO: 4			
531/532	G129E	+	
533/534	T315E	+	++
535/536	N145A	+	+
537/538	Q267H	+	+
539/540	S372V	+	+
541/542	T130G	+	+
543/544	Q267T		++
545/546	Q274W	+	+
547/548	V136M	+	+
549/550	S372C	+	+
551/552	N194T	+	+
553/554	Q375V	+	+
555/556	A135G	+	+
557/558	Q267I	+	+
559/560	N141L/Q220R	+	+
561/562	S324E	+	+
563/564	S160L	++	+
565/566	N141S	++	
567/568	S372L	+	++
569/570	A135Y	+	
571/572	N141V	+	+
573/574	N141A	+	+
575/576	A131R	+	+
577/578	A135E	+	+
579/580	S324Y	+	
581/582	T311D/R316K/D370G	+	++
583/584	I99V	+	+
585/586	A278N	+	+
587/588	V405Q		+
589/590	T311D/S342N/D370G	++	++
591/592	N141Q/V198G	++	++
593/594	T311D/S342N	++	++
595/596	N141Q/T311D	++	++
597/598	Q279K/T311D/R377H/Q392Y	+	+
599/600	L186Y/V198G/T311D/S342N/D370G/Q392Y	++	+++
601/602	N141Q/Q392Y	++	+
603/604	T311D/D370G/Q392Y	++	++
605/606	N141Q/T311D/Q392Y	+	++
607/608	T311D/D370G	++	++
609/610	T311D/R316K/Q392Y	++	++
611/612	Y265G/T311D/Q392Y	+	+++
613/614	N141Q/G192D	++	++
615/616	T311D	++	++
617/618	N141Q/Y265G/T311D/Q392Y	++	++

Table 3.2: Protease activity relative to SEQ ID NO: 4

619/620	G192D/T311D/D370G/Q392Y	++	++
621/622	V198G/Y265G/T311D/R316K/D370G	+	+++
623/624	N141Q/L186Y/Y265G/T311D	++	+++
625/626	N141Q/V198G/Y265G/T311D/D370G	++	+++
627/628		+	+

All activities were determined relative to the reference polypeptide of SEQ ID NO: 4. Levels of increased activity are defined as follows: "+" 0.9 to 1.2, "++" > 1.2, "+++" > 2. The reference sequence and all variants within this table are represented in the Sequence Listing as a fragment (i.e., residues 1-413) of their full length sequence, as described in Example 3.

Example 4

Screening Results of Protease Variants derived from SEQ ID NO: 628

[0361] The engineered protease variant used in this Example and represented by SEQ ID NO: 628 has amino acid residues 426-522 of SEQ ID NO: 2 deleted while retaining the His-tag sequence (see **FIG. 1**). This construct was used as the backbone for the construction of protease variants in this Example. Within the Sequence Listing, for purposes of consistency in description, SEQ ID NO: 628 and variants derived therefrom are represented as a fragment (residues 1-413) of their full-length sequence of the actual variants studied. The actual variants examined include residues 414-425 of SEQ ID NO: 2 and the His-tag sequence (see **FIG. 1**). Mutations identified in **Tables 3.1** and **3.2** were recombined on this backbone and additional mutagenesis was performed. Variants were screened using the casein activity assay at pH 7.5 after a 1 hour pre-incubation at pH 4.5 in the presence of pepsin, as described in Example 2. Analysis of the data relative to SEQ ID NO: 628 is listed in **Table 4.1**. Some variants from these libraries were assayed in triplicate in the casein activity assay at pH 7.5 after no prior challenge, after a 1 hour pre-incubation at pH 4.5 in the presence of Pepsin, after a one hour pre-incubation at pH 4.45 in the presence of Pepsin, and after a one hour heat challenge at 64 °C, as described in Example 2. Analysis of the average data relative to SEQ ID NO: 628 are listed in **Table 4.2**.

Table 4.1: Protease activity relative to SEQ ID NO: 628

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 628)	pH 4.5 Pepsin Challenge Improvement Relative to SEQ ID NO: 628
629/630	T242E	+
631/632	S157V	++
633/634	D250N	+
635/636	V373F	+
637/638	Q243R	+
639/640	Y336F	+

Table 4.1: Protease activity relative to SEQ ID NO: 628		
641/642	G187A	+
643/644	G240A	+
645/646	G280K	+
647/648	E271A	+
649/650	S237G	+
651/652	E386W	++
653/654	D382G	++
655/656	G280S	+
657/658	V373Y	+
659/660	V328M	+
661/662	S157R	+
663/664	S157A	+
665/666	G42W	+
667/668	Q243E	+
669/670	D382S	+
671/672	T391L	++
673/674	R381N	+
675/676	Q243M	+
677/678	T275V	+
679/680	S157I	++
681/682	V373S	+
683/684	S157T	+
685/686	G280D	+
687/688	A249G	+
689/690	Y239L	+
691/692	A384C	+
693/694	N139M	++
695/696	G240L	+
697/698	Q243T	++
699/700	D250L	+
701/702	D250A	+
703/704	D382T	++
705/706	I364A	+
707/708	S346V	+
709/710	V373M	+
711/712	S389P	++
713/714	V373C	+
715/716	D382R	+
717/718	V373E	+
719/720	D254E	+
721/722	L246I	+
723/724	D250F	+
725/726	G280T	+
727/728	V373A	++

Table 4.1: Protease activity relative to SEQ ID NO: 628		
729/730	N139K	+
731/732	T345I	+
733/734	V360S	+
735/736	T275A	+
737/738	A249M	+
739/740	I364V	+
741/742	S303V	+
743/744	A300R	+
745/746	Y239M	+
747/748	M269T	+
749/750	A135G/N141Q/S372L	++
751/752	T311D/T315V/S372L	++
753/754	V136M/N141V/T311D	++
755/756	N141V/R188M	++
757/758	A135E/V136M	+
759/760	V136M/N141Q/T311D	++
761/762	A135E/N141V/T315V	++
763/764	S372V	+
765/766	A135E/N141V/S160L/Q267I/S372V	++
767/768	A135G/V136M/N141V/S160L/N185E/R188M/Q267I/T311D/T315V	++
769/770	A135G/V136M	++
771/772	S160L/N185E	+
773/774	A135E/N141V/R188M/Q279M/T311D	++
775/776	A135G/V136M/N141Q	++
777/778	A135G/V136M/N141Q/S372L	++
779/780	A135G/N141V/S160L/N185E/Q267I/Q279M	++
781/782	A135G/N141V/S160L/Q267I	++
783/784	N141Q/R188M/T311D/S372V	++
785/786	S160L/N185E/R188M/Q279M/T311D	++
787/788	V136M/N141V/Q279M	++
789/790	A135G/V136M/N141V/S160L/N185E/R188L	++
791/792	N141Q/S372V	++
793/794	A135E/V136M/N141Q/T311D	++
795/796	N185E/T311D/T315V/S372V	++
797/798	A135G/N141V/R188M	++
799/800	V136M/N185E	++
801/802	A135E/N141Q	++
803/804	A135E/V136M/N141Q/Q279M/T315V/S372L	++
805/806	A135G/T311D/T315V	++
807/808	N141V	++
809/810	A135G/N141V	++
811/812	T311D/S372L	++
813/814	R188M/T311D	++

Table 4.1: Protease activity relative to SEQ ID NO: 628		
815/816	A135E/N141V/R188L/S372L	++
817/818	N141V/S160L/Q279M	++
819/820	N313Q/Q392Y	++
821/822	S342G/Q392Y	++
823/824	Q279L/Q392Y	+
825/826	E128T	+
827/828	V198G/S342G	++
829/830	N313Q	+
831/832	E128T/S312I	++
833/834	K50R	+
835/836	N145E/G263S	+
837/838	N313Q/S342G	+
839/840	Q279L/S312I	+
841/842	S312I/Q392Y	++
843/844	Q279K/S342G	+
845/846	Q279K/Q392Y	+
847/848	E128T/S342G	+
849/850	S342G	+
851/852	G263S	+
All activities were determined relative to the reference polypeptide of SEQ ID NO: 628. Levels of increased activity are defined as follows: "+" 1 to 1.2, "++" > 1.2. The reference sequence and all variants within this table are represented in the Sequence Listing as a fragment (residue 1-413) of their full length sequence, as described in Example 4		

Table 4.2: Protease activity relative to SEQ ID NO: 628					
SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 628)	pH 4.5 and Pepsin Challenge Improvement Relative to SEQ ID NO: 628	pH 4.45 and Pepsin Challenge Improvement Relative to SEQ ID NO: 628	Unchallenged Activity Improvement Relative to SEQ ID NO: 628	Heat Challenge Improvement Relative to SEQ ID NO: 628
853/854	N139C	++	+	+	++
855/856	T345R	++	++	+	+
857/858	Q243L	+	+	+	++
859/860	H143A	+	++		
861/862	A249S	+	+	+	++
863/864	G262S	++	++	+	++
865/866	N139R	+	+	+	+
867/868	M269Q	++	++	+	+++
869/870	V328L	++	++	+	+++
871/872	S157G	+	+	+	+
873/874	T156V	+	+	+	+

Table 4.2: Protease activity relative to SEQ ID NO: 628					
875/876	T242S	+	+	+	
877/878	N139L	++	++	+	++
879/880	G262A	++	++	+	++
881/882	T169S	++	++	+	+
883/884	S346T	++	++	+	++
885/886	H143N/S237A	++	++	+	++
887/888	V136M/S160L/N185E/Q267I/T311D/S372L	++	++	+	+++
889/890	A135E/S160L/T311D/S372L	++	+++	+	++++
891/892	A135G/N141V/T311D/T315V	++	++	+	++++
893/894	N141V/T311D/T315V	++	++	++	++++
895/896	V136M/N141V/S160L/N185E/R188M/T311D/T315V/S372V	++	++	+	++++
897/898	A135G/N141V/T311D/T315V/S372L	++	+++	+	++++
899/900	A135G/N141Q/S160L/N185E/T311D/T315V	++	++	+	++++
901/902	A135G/N141V/Q267I/T311D/T315V/S372V	++	++	+	++++
903/904	A135G/V136M/N141V/S160L/T311D/T315V	++	++	+	++++
905/906	A135G/V136M/N141V/Q279M	++	++	+	+++
907/908	A135G/N141Q/Q267I/Q279M/T311D/T315V	++	++	+	++++
909/910	A135E/N141V/T311D/T315V/S372V	++	+++	+	++++
911/912	A135E/N141Q/S160L	++	++	+	+++
913/914	A135G/N141Q/T311D/T315V	++	++	+	++++
915/916	A135G/N141V/S160L/T311D/T315V/S372V	++	+++	+	++++
917/918	A135G/N141V/S160L/T311D/T315V	++	+++	+	++++
919/920	A135G/N141Q/Q267I/T311D/T315V/S372L	++	++	+	++++
921/922	A135G/V136M/N141V/R188M/T311D	++	+++	+	++++
923/924	N141V/S160L/T311D	++	+++	++	++++

Table 4.2: Protease activity relative to SEQ ID NO: 628					
925/926	A135E/N141V/S160L/Q279M/T311D/T315V/S372L	++	++	+	++++
927/928	N141V/S160L/N185E/Q279M/T311D/S372V	++	++	+	++++
929/930	A135E/N141V/T311D/T315V	++	+++	+	++++
931/932	A135G/V136M/N141V/S160L/T315V/S372V	++	++	+	++
933/934	A135E/N141V/S160L	++	++	+	+++
935/936	A135E/V136M/S160L/Q279M/T311D/S372V	++	++	++	++++
937/938	E128T/Q279K/S312I/S342G	++	++	+	+++
939/940	E128T/V198G/S312I/S342G	++	++	+	+++
941/942	G263S/S342G	+	++	+	
943/944	N145E/G263S/Q279L/S312I/S342G/Q392Y	++	++	+	++
945/946	E128T/N145E/V198G/S312I/N313Q/Q392Y	++	++	+	+
All activities were determined relative to the reference polypeptide of SEQ ID NO: 628. Levels of increased activity are defined as follows: "+" 0.9 to 1.2, "++" > 1.2, "+++" > 2, "++++" > 3. The reference sequence and all variants within this table are represented in the Sequence Listing as a fragment (residues 1-413) of their full length sequence, as described in Example 4					

Example 5

Screening Results of Protease Variants derived from SEQ ID NO: 948

[0362] The engineered protease variant SEQ ID NO: 916 was truncated at the C-termini by 24 residues, including the removal of the 6XHis tag, resulting in SEQ ID NO: 948. As such, SEQ ID NO: 948 has a carboxy terminus at amino acid residue 413. SEQ ID NO: 948 was used as the backbone for the construction of additional protease variants. Previously identified mutations were recombined on this backbone and additional mutagenesis was performed. Variants were screened using the casein activity assay at pH 7.5 after a 1 hour pre-incubation at pH 3.9 in the presence of pepsin as described in Example 2. Analysis of the data relative to SEQ ID NO: 948 is listed in **Table 5.1**. Some variants generated from these libraries were assayed in triplicate in the casein activity assay at pH 7.5 after no prior challenge, after a 1 hour pre-incubation at pH 3.8, 3.9, or 4.0 in the presence of pepsin, and after a 1 hour heat

challenge at 71 °C, as described in Example 2. Additionally, additional variants were assayed in triplicate in the casein activity assay at pH 6.5 unchallenged, as described in Example 2. Analysis of the average data relative to SEQ ID NO: 948 are listed in **Table 5.2**.

Table 5.1: Protease activity relative to SEQ ID NO: 948		
SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 948)	pH 3.9 pepsin challenge improvement relative to SEQ ID NO: 948
949/950	S411V	++
951/952	S411R	++
953/954	A402G	++
955/956	A285S	+
957/958	I245V	++
959/960	N266H	++
961/962	P355A	++
963/964	M258W	++
965/966	A222G	+
967/968	Q140L	++
969/970	S268T	+
971/972	S411L	++
973/974	I225V	++
975/976	I245L	++
977/978	V283M	+
979/980	A406W	+
981/982	G410C	+
983/984	A406C	++
985/986	H143A/N145E/Q243L/S312I	+++
987/988	N139L/H143A/N145E/S157G/S312I	+++
989/990	N139C/S157G/T345R	+++
991/992	N139C/M269Q	++
993/994	T156V/S157G/S342G/S346T	+++
995/996	N139L/H143A	+++
997/998	M269Q	++
999/1000	N139L/Q243L/V328L	+++
1001/1002	M269Q/V328L	++
1003/1004	H143A/N145E/T169S	+++
1005/1006	V328L	+++
1007/1008	H143A/N145E/G262S	+++
1009/1010	N145E/G262S/S312I/V328L	+++
1011/1012	N139L/T156V/S157G	+++
1013/1014	N139C/N145E/S312I	+++
1015/1016	S312I	++
1017/1018	N139C	+++
1019/1020	N139C/S312I	+++

Table 5.1: Protease activity relative to SEQ ID NO: 948		
1021/1022	N139C/T156V	+++
1023/1024	N139C/H143A/N145E/Q243L	+++
1025/1026	N145E/S157G	+++
1027/1028	N145E/S346T	+++
1029/1030	N145E/G262A/S312I/V328L/T345R/S346T	+++
1031/1032	N145E/G262A	++
1033/1034	S312I/S342G	++
1035/1036	H143A/Q243L	+++
1037/1038	N139C/T345R	++
1039/1040	S342G	++
1041/1042	H143A/N145E/G262S/S342G	+++
1043/1044	N139L/H143A/T169S	+++
1045/1046	N139C/H143A/N145E/S312I	+++
1047/1048	T169S	++
1049/1050	N139L/N145E/G262A/S312I/V328L/S342G/T345R/S346T	++
1051/1052	N139C/V328L	+++
1053/1054	N139L/Q243L	++
1055/1056	N139L/H143A/V328L	+++
1057/1058	N139L	++
1059/1060	N139C/H143A/Q243L	+++
1061/1062	N139C/N145E	+++
1063/1064	N145E/S312I	++
1065/1066	N145E/T169S	++
1067/1068	N139C/H143A/S157G/S312I	+++
1069/1070	V84M/N139C/H143A	++
1071/1072	N145E/M269Q	++
1073/1074	H143A/N145E/S157G/M269Q/S312I/V328L	+++
1075/1076	H143A/N145E/M269Q	+++
1077/1078	S157G	++
1079/1080	N139L/H143A/S312I	+++
All activities were determined relative to the reference polypeptide of SEQ ID NO: 948. Levels of increased activity are defined as follows: "+" 1.0 to 1.2, "++" > 1.2, "+++" > 2		

Table 5.2: Protease activity relative to SEQ ID NO: 948							
SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 948)	pH 3.9 and Pepsin Challenge Improvement Relative to SEQ ID NO: 948	pH 3.8 and Pepsin Challenge Improvement Relative to SEQ ID NO: 948	pH 4.0 and Pepsin Challenge Improvement Relative to SEQ ID NO: 948	pH 7.5 Unchallenged Activity Improvement Relative to SEQ ID NO: 948	pH 6.5 Unchallenged Activity Improvement Relative to SEQ ID NO: 948	Heat Challenge Improvement Relative to SEQ ID NO: 948
1081/1082	I256L	+			+	+	+
1083/1084	S411T	++		+	+	+	+
1085/1086	V273F	+					
1087/1088	G409E			+	+		+
1089/1090	S268G	+		+	+		+
1091/1092	D172Q				+		
1093/1094	G409R	++		+	+	+	+
1095/1096	H401L	+		+	+	+	
1097/1098	T281C	+		+	+	+	+
1099/1100	G410W				+		+
1101/1102	A253V	+		+	+	+	+
1103/1104	A406M			+	+	+	+
1105/1106	V273T	++		++	+	+	++
1107/1108	A406R			+	+		+
1109/1110	I256M	+		++	+	+	++
1111/1112	G410I	+		++	+	+	+
1113/1114	V273M	+		+			
1115/1116	V273L	++		++	+	+	++
1117/1118	H143A/N145E/Q243L/V328 L	+++	++++		+	+	+

Table 5.2: Protease activity relative to SEQ ID NO: 948									
1119/1120	N145E	++	+++		+	+	+	++	++
1121/1122	N139C/H143A/N145E/V328L/S342G/T345R	+++	++++		+	+	+	++	++
1123/1124	N139L/N145E	++	+++					+	+
1125/1126	H143A/V328L/S342G/T345R	+++	++++		+	++	++	++	++
1127/1128	N145E/S342G/T345R	+++	++++		+	++	++	++	++
1129/1130	H143A	++	++++						
1131/1132	N139C/H143A	++	++++					+	+
1133/1134	N139C/N145E/V328L/S342G/T345R	+++	++++		+	++	++	++	++
1135/1136	H143A/N145E/T169S/S312I/V328L/T345R/S346T	+++	++++		+	++	++	++	++
1137/1138	H143A/Q243L/V328L/S342G/T345R/S346T	+++	++++		+	+	+	++	++
1139/1140	N139C/H143A/S157G/T169S/V328L/S346T	+++	++++		+	+	+	++	++
1141/1142	H143A/N145E/T156V/S312I/V328L	++	++++		+			+	+
1143/1144	N139C/N145E/S157G/S312I/V328L	+++	++++		+	+	+	++	++
1145/1146	H143A/V328L/S342G/T345R/S346T	+++	++++		+	++	++	++	++
1147/1148	N139C/H143A/V328L	++	++++					++	++
1149/1150	H143A/N145E	++	++++		+	+	+		
1151/1152	H143A/N145E/S312I/S342G/T345R	+++	++++		+	+	+		
1153/1154	H143A/N145E/V328L	+++	++++		+	+	+	+	+
All activities were determined relative to the reference polypeptide of SEQ ID NO: 948. Levels of increased activity are defined as follows: "+" 0,9 to 1,2, "++" > 1,2, "+++">> 2, "++++"> 3,5									

Example 6

Screening Results of Protease Variants derived from SEQ ID NO: 1126

[0363] The engineered protease variant of SEQ ID NO: 1126 was selected as the backbone for the construction of additional protease variants. Previously identified mutations were recombined on this backbone and additional mutagenesis was performed. Variants were screened using the casein activity assay at pH 6.5 after a 1 hour pre-incubation at pH 3.35 in the presence of pepsin, as described in Example 2. Analysis of the data relative to SEQ ID NO: 1126 is listed in **Table 6.1**.

Table 6.1: Protease activity relative to SEQ ID NO: 1126		
SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1126)	pH 3.35 and pepsin challenge improvement relative to SEQ ID NO: 1126
1155/1156	Q279Y	+
1157/1158	D250T	++
1159/1160	A154C	+
1161/1162	S214Y	+
1163/1164	A249S	+
1165/1166	T275A	+
1167/1168	H137A	+++
1169/1170	S161D	+
1171/1172	N180H	+
1173/1174	N174L	++
1175/1176	N139K	++
1177/1178	D254C	++
1179/1180	N145E	++
1181/1182	A278V	+
1183/1184	V136M	++
1185/1186	A154L/G413D	++
1187/1188	S294V	+
1189/1190	A154R	+
1191/1192	S237A	+
1193/1194	Q274G	+
1195/1196	H137N	+++
1197/1198	G264P	+
1199/1200	Q274T	++
1201/1202	A278N	+
1203/1204	S214A	+
1205/1206	N185G	+
1207/1208	S214V	++
1209/1210	Q279T	+
1211/1212	V277G	+

Table 6.1: Protease activity relative to SEQ ID NO: 1126		
1213/1214	G264I	+
1215/1216	S293A	++
1217/1218	S233L	+
1219/1220	A278Y	++
1221/1222	H173F	++
1223/1224	Q274L	+
1225/1226	S312C	+++
1227/1228	Q279L	+
1229/1230	S302G	++
1231/1232	L238Q	+
1233/1234	S294W	++
1235/1236	G135A	++
1237/1238	S221E	+
1239/1240	D290S	+
1241/1242	A278S	++
1243/1244	G263Q	++
1245/1246	G263H	+
1247/1248	A278L	++
1249/1250	G263E	++
1251/1252	A154L	++
1253/1254	N139M	++
1255/1256	H137S	++
1257/1258	S233G	+
1259/1260	N139F	++
1261/1262	Q267I	+
1263/1264	S221L	+
1265/1266	H173S	+
1267/1268	S302P	+
1269/1270	S221V	+
1271/1272	Y239M	+
1273/1274	D290G	+
1275/1276	K163H	+
1277/1278	A292V	+
1279/1280	L246V	+
1281/1282	S214N	+
1283/1284	Q243S	+
1285/1286	S233I	++
1287/1288	S235Q	++
1289/1290	N145D	++
1291/1292	Q274V	++
1293/1294	Q279M	++
1295/1296	N185S	+
1297/1298	Q279K	++

Table 6.1: Protease activity relative to SEQ ID NO: 1126		
1299/1300	N145W	+++
1301/1302	D290E	+
1303/1304	S214P	++
1305/1306	T156V	++
1307/1308	T156C	++
1309/1310	T223S	+
1311/1312	A278V/G413D	++
1313/1314	D250C	+
1315/1316	Q267S	++
1317/1318	Y297F	+
1319/1320	S221Q	++
1321/1322	N194D	+
1323/1324	I251T	+
1325/1326	A253V/S411T	++
1327/1328	N145P/S157R/A253I/S268G/V273T/T281V/S312Q/S346T/S411T	+
1329/1330	N139C/S346A	++
1331/1332	A253V	+
1333/1334	S346T/S411T	++
1335/1336	A253V/S346T	++
1337/1338	N139C/S346T	++
1339/1340	S312Q/S346T	++
1341/1342	V273T/S312Q	++
1343/1344	A253I/T281V	++
1345/1346	S157R/A253V/V273T/S312Q/S346T/S411T	+++
1347/1348	N139C/S157K/A253V/S268G/V273F/T281V/S312Q/S346A	++
1349/1350	A253V/V273T/S411T	++
1351/1352	N139C/A253I/S268F/V273T/T281V	+++
1353/1354	N139C/S157R/S411T	+++
1355/1356	S157G	++
1357/1358	V273T	++
1359/1360	N139C/A253V/S268G/V273F/T281V/S312Q/S411T	+++
1361/1362	S157G/A253V/S411T	+++
1363/1364	N139C/N145E/A253V/S346T	+++
1365/1366	N139C/S157K/A253V/V273T/S312Q	+++
1367/1368	N139C/S157G/S268G/V273T/S312Q/S346T	+++
1369/1370	S157G/V273T/S312Q/S346T	+++
1371/1372	N139C/S411T	+++
1373/1374	N139C/A253V/S268G/V273F/T281C/S312I/S346T/S411T	+++
1375/1376	S157K/V273F/S346T/S411T	+++
1377/1378	N139C/N145E/S157G/V162I/A253V/V273F/T281V/S312Q	++
1379/1380	N139C/A253V/V273T/T281C/S312Q	+++
1381/1382	S157G/A253V/S268F/V273F/T281V/S312Q	+++
1383/1384	N139C/A253I/S268F	++

Table 6.1: Protease activity relative to SEQ ID NO: 1126		
1385/1386	N139C/S157R/S312Q	++
1387/1388	A253V/V273T/T281C/S346T	+++
1389/1390	S157K/A253V/S312I/S346T/S411T	+++
1391/1392	S157R/V273T/S312Q/S346T/S411T	+++
1393/1394	N139C/N145E/S157K/A253V/S268G/T281C/S312Q	+++
1395/1396	N139C/V273T/S312Q/S346T	+++
1397/1398	S157K/A253V/S268F/V273T/S312I/S346T	++
1399/1400	N139C/S268G/S346T	++
1401/1402	S157K	++
1403/1404	S268G/V273F/S312Q/S346T	+++
1405/1406	N139C/S157K/A253V/S268F/V273F/S312Q	+++
1407/1408	S157K/V273T/S312Q/S346T	+++
1409/1410	N139C/S157G/A253V	+++
1411/1412	N139C	++
1413/1414	N139C/A253V/T281V	+++
1415/1416	N139C/S157G/A253V/S268G/V273T	+++
1417/1418	A253V/S312Q/S411T	++
1419/1420	N139C/S268G/V273T	+++
1421/1422	A253I	+
All activities were determined relative to the reference polypeptide of SEQ ID NO: 1126. Levels of increased activity are defined as follows: "+" 0.9 to 1.2, "++" > 1.2, "+++" > 1.8		

Example 7

Screening Results of Protease Variants derived from SEQ ID NO: 1368

[0364] The engineered protease variant of SEQ ID NO: 1368 was used as the backbone for the construction of additional protease variants. Previously identified mutations were recombined and additional mutagenesis was performed. Variants were screened using the casein activity assay at pH 6.5 after a 1 hour pre-incubation at pH 3.2 in the presence of pepsin, as described in Example 2. Select variants were additionally screened using the casein activity assay at pH 6.5 after a 1 hour pre-incubation at pH 3.14 in the presence of pepsin, as described in Example 2. Other select variants were additionally screened using the casein activity assay at pH 6.5 with no prior challenge, as described in Example 2. Analysis of the data relative to SEQ ID NO: 1368 is listed in Table 7.1.

Table 7.1: Protease activity relative to SEQ ID NO: 1368				
SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1368)	pH 3.2 and pepsin challenge improvement relative to SEQ ID NO: 1368	pH 3.14 and pepsin challenge improvement relative to SEQ ID NO: 1368	Unchallenged activity improvement relative to SEQ ID NO: 1368
1423/1424	D31G	+		

Table 7.1: Protease activity relative to SEQ ID NO: 1368

1425/1426	S318R	+		
1427/1428	S296R	+		
1429/1430	S296M	+		
1431/1432	D252P	+		
1433/1434	S303A	+		
1435/1436	A253C	+		
1437/1438	G413C	+		
1439/1440	E386P	+		
1441/1442	G413A	+		
1443/1444	Q312R	++		
1445/1446	S235R	+		
1447/1448	G412P	+		
1449/1450	G342A	+		
1451/1452	G413S	+		
1453/1454	S302P	+		
1455/1456	I371L	+		
1457/1458	V405L	+		
1459/1460	Q312A	+		
1461/1462	S389P	+		
1463/1464	S318P	+		
1465/1466	T391S	+		
1467/1468	G412T	+		
1469/1470	A358S	+		
1471/1472	S389C	+		
1473/1474	S235V	++		
1475/1476	T391L	+		
1477/1478	C139N/T273V/D311T/L328V/ V372S			++
1479/1480	D311T			+
1481/1482	Q312S	+		+
1483/1484	V372S	+		
1485/1486	C139N/A143H/G157S/L160S/ G268S/T273V/D311T/V315T			++
1487/1488	A143H			+
1489/1490	C139N/A143H			++
1491/1492	C139N/L160S/Q312S/V372S			++
1493/1494	A143H/T273V/L328V			++
1495/1496	T346S	+		+
1497/1498	G135A/C139N/L160S/G268S/ Q312S/G342S/T346S			++
1499/1500	C139N/V141N/T273V			++
1501/1502	G135A/V141N/A143H/G268S /T273V/Q312S/V372S			++
1503/1504	C139N/V141N/A143H/D311T			++

Table 7.1: Protease activity relative to SEQ ID NO: 1368				
1505/1506	C139N/G157S/G268S/L328V/T346S/V372S			++
1507/1508	T53A/C139N/V141N/A143H/T273V/V372S			++
1509/1510	C139N			++
1511/1512	C139N/V141N/A143H/T273V/Q312S			++
1513/1514	C139L	+	+	
1515/1516	H137A/C139N/S221Q/S233L/G413D	++	++	
1517/1518	S233L	+	+	
1519/1520	S221Q/Q279K	+	++	
1521/1522	H137N/C139N/S233L/Q279M	++	+++	
1523/1524	S221Q	+	++	
1525/1526	C139L/S214V	+	++	
1527/1528	H137N/C139N/T156V	++	+++	
1529/1530	C139L/S214V/S221Q	++	+++	
1531/1532	S214V/S233L	+	++	
1533/1534	H137A/C139L/S221Q/S233L/Q279K	+	+++	
1535/1536	H137A/C139L/S221Q	++	+++	
1537/1538	H137N/C139L	++	+++	
1539/1540	H137N/C139L/Q279K	++	+++	
1541/1542	H137A/C139N	+	++	
1543/1544	H137N	++	+++	
1545/1546	H137N/C139L/S221Q	++	+++	
1547/1548	H137N/C139L/S214P/Q279M	++	+++	
1549/1550	N266Y	+	+	
1551/1552	C139L/S221Q	+	++	
1553/1554	H137N/S221Q	++	+++	
1555/1556	H137N/C139N	++	+++	
1557/1558	H137N/T156V/S214V/Q312C	++	++	
1559/1560	H137N/S221Q/S233L	++	+++	
1561/1562	H137N/C139N/T156V/S221Q	++	+++	
1563/1564	H137A/C139L/S214V	++	+++	
1565/1566	G413D	+	+	
1567/1568	Q279K	+	+	
1569/1570	Q279M	+	+	
1571/1572	H137A/C139L/S233L	++	++	
1573/1574	H137N/C139N/S214V/S233L	++	+++	
1575/1576	S221Q/G413D	++	+	
1577/1578	H137N/S214V/S233L	++	+++	
1579/1580	H137A/C139L/S214P	++	+++	
1581/1582	H137N/C139N/S221Q/S233L/Q279M	++	+++	

Table 7.1: Protease activity relative to SEQ ID NO: 1368				
1583/1584	H137N/T156V	++	+++	
1585/1586	H137N/C139L/S221Q/S233I	+	+++	
1587/1588	S214V/S221Q	++	++	
1589/1590	H137N/S221Q/G413D	++	+++	
1591/1592	H137A/C139L/S221Q/S233L/Q279M	++	+++	
1593/1594	S214V	+	++	
1595/1596	H137A/C139L	++	+++	
1597/1598	H137N/S233L	++	+++	
1599/1600	H137A	+	+++	
1601/1602	H137N/G413D	++	+++	
1603/1604	H137N/S221Q/Q279K	++	+++	
1605/1606	H137N/C139N/T156V/S214V/S233L/G413D	+	+++	
1607/1608	H137N/C139L/T156V/S214V	+	+++	
All activities were determined relative to the reference polypeptide of SEQ ID NO: 1368. Levels of increased activity are defined as follows: "+" 0.9 to 1.2, "++" > 1.2, "+++" > 1.8				

Example 8

Screening Results of Protease Variants derived from SEQ ID NO: 1548

[0365] The engineered protease variant of SEQ ID NO: 1548 was used as the backbone for the construction of additional protease variants. Previously identified mutations were recombined on this backbone. Variants were screened using the casein activity assay at pH 6.5 with no prior challenge and after a 1 hour pre-incubation at pH 2.8 in the presence of pepsin, as described in Example 2. Analysis of the data relative to SEQ ID NO: 1548 is listed in Table 8.1.

Table 8.1:: Protease activity relative to SEQ ID NO: 1548			
SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1548)	Unchallenged activity improvement relative to SEQ ID NO: 1548	pH 2.8 and pepsin challenge improvement relative to SEQ ID NO: 1548
1609/1610	N145E/T273L/V372S	++	++
1611/1612	N145E/I256M/T273L	++	+
1613/1614	S221Q/Q243L/T273L/L328V/V372S	++	++
1615/1616	V372S	+	+
1617/1618	N145E/S221Q/M279L/V372S/A406R	+	++
1619/1620	T273L/L328V		++
1621/1622	N145E/T169S/T273L/T346S/A406R	++	
1623/1624	I256M/T273L	+	+
1625/1626	N145E/S221Q/T273L/L328V/T346S/A406R	+	++

Table 8.1:: Protease activity relative to SEQ ID NO: 1548			
1627/1628	Q243L/T273L		+
1629/1630	N145E/P214V/I256M/T273L/M279L/L328V/V372S	+	+
1631/1632	T169S/S221Q/L328V/V372S/A406R	++	
1633/1634	V372S/A406R	++	+
1635/1636	T169S/L328V/V372S/A406R	++	
1637/1638	S221Q/V372S	++	+
1639/1640	N145E/S221Q/T273L/L328V/V372S	+	++
1641/1642	P214V/Q243L/T273L/L328V	+	+
1643/1644	N145E/S221Q	+	+
1645/1646	P214V/I256M/T273L/T346S/V372S	+	++
1647/1648	S221Q/A406R	+	++
1649/1650	T169S/V372S	++	
1651/1652	N145E/P214V/S221Q/T273L		++
1653/1654	N145E/S221Q/T346S/V372S	+	++
1655/1656	Q243L/T273L/L328V/V372S/A406R	++	++
1657/1658	N145E/T169S/T273L/L328V/T346S	++	
1659/1660	L328V	+	+
1661/1662	T169S/T273L/V372S	++	
1663/1664	N145E/S221Q/L328V	++	++
1665/1666	T169S/P214V/T273L	++	
1667/1668	S221Q/T273L/L328V	+	++
1669/1670	S221Q	++	++
1671/1672	N145E/L328V	+	++
1673/1674	P214N/T346S	+	
1675/1676	Q312R	+	++
1677/1678	Y212S	+	
1679/1680	M279K	+	+
1681/1682	Y212S/Q312R	++	
1683/1684	N145E	+	+
1685/1686	A179S/T346S	+	
1687/1688	P214N	++	
1689/1690	T346S	+	+
1691/1692	V315R/V372Y		+
1693/1694	V372Y	+	
1695/1696	Q375A	+	+
1697/1698	G264F	+	
1699/1700	A179S	+	
1701/1702	N185T	+	+
1703/1704	Q220R/V372Y	+	
1705/1706	S324D	+	
1707/1708	N145E/S221Q/T273L/L328V/V372S	+	+
1709/1710	N145E/S221Q/T273L/L328V/V372S	+	+

Table 8.1:: Protease activity relative to SEQ ID NO: 1548

All activities were determined relative to the reference polypeptide of SEQ ID NO: 1548. Levels of increased activity are defined as follows: "+" 0.9 to 1.2, "++" > 1.2

[0366] SEQ ID NO: 1639/1640 was codon optimized for improved expression in E. coli, resulting in variations in the polynucleotide sequence and represented by SEQ ID NO: 1707 and SEQ ID NO: 1709.

Example 9

Screening Results of Protease Variants derived from SEQ ID NO: 4

[0367] SEQ ID NO: 4 was utilized as the backbone for the construction of novel variants. Variants were generated through saturation mutagenesis and by introducing truncations at the C terminus. Select variants were screened in triplicate using the casein activity assay at pH = 7.5, as described in Example 2. Select variants were screened in triplicate using the casein activity assay at pH = 7.5 after a one hour pre-incubation at pH = 4.5 in the presence of pepsin, as described in Example 2. Other select variants were screened in triplicate using the casein activity assay at pH = 7.5 with a one-hour heat challenge at 63 °C, as described in Example 2. Analysis of the average data relative to SEQ ID NO: 4 are listed in Table 9.1.

Table 9.1 Protease activity relative to SEQ ID NO: 4

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 4)	Unchallenged FIOP Relative to SEQ ID NO: 4	Gastric Challenge FIOP Relative to SEQ ID NO: 4	Thermo challenge FIOP Relative to SEQ ID NO: 4
1711/1712	A135G	+	+	+
1713/1714	A135S	+	+	
1715/1716	A135V	+	+	+
1717/1718	A135L	+		
1719/1720	A135R	+	+	
1721/1722	A135E	++		+
1723/1724	A135P	+		
1725/1726	A135H	+	++	
1727/1728	A135C	+		
1729/1730	A135T	+	+	
1731/1732	A135Y	+		
1733/1734	A135W	+		
1735/1736	A135M	+		
1737/1738	A135N	+	+	
1739/1740	H137S	+		
1741/1742	H137A	+		

Table 9.1 Protease activity relative to SEQ ID NO: 4				
1743/1744	H137N	+	++	
1745/1746	H137D	+		
1747/1748	N139R	+	+	+
1749/1750	N139E	+		
1751/1752	N139F	+		
1753/1754	N139L	+	++	++
1755/1756	N139K	+	+	++
1757/1758	N139D	+		
1759/1760	N139H	+		
1761/1762	N139I	+		
1763/1764	N139S	+		
1765/1766	N141T	+	++	++
1767/1768	N141S	+	++	++
1769/1770	N141V	+	+++	+++
1771/1772	N141L	+	+	++
1773/1774	N141R	+	++	++
1775/1776	N141M	+	++	++
1777/1778	N141G	+		
1779/1780	N141Y	+	++	++
1781/1782	N141I	+	++	++
1783/1784	N141C	+	+++	++
1785/1786	N141F	+	++	++
1787/1788	N141A	+	+++	+++
1789/1790	N141D	+		+
1791/1792	N141E	+	++	++
1793/1794	N141H	+	+	+
1795/1796	H143T	+		
1797/1798	H143C	+		
1799/1800	H143Q	++	+++	
1801/1802	H143A	++	+++	
1803/1804	H143D		+	
1805/1806	H143S	+	+++	
1807/1808	H143N	+	+++	++
1809/1810	N145Q	+	++	+
1811/1812	N145T	+		+
1813/1814	N145V	+		
1815/1816	N145H	+		
1817/1818	N145L	+		
1819/1820	N145E	+	++	+
1821/1822	N145R	++	+	
1823/1824	N145D	+		
1825/1826	N145R	+	+	
1827/1828	N145A	+		++

Table 9.1 Protease activity relative to SEQ ID NO: 4				
1829/1830	N145F	+		
1831/1832	N145S	+		+
1833/1834	N145G	+		+
1835/1836	N145I	+		
1837/1838	N145K	++		+
1839/1840	N145M	++		
1841/1842	N145C	+		+
1843/1844	N145W	+		
1845/1846	S157A	+	+	+
1847/1848	S157E	+		
1849/1850	S157P	+	+	+
1851/1852	S157V	+	+++	++
1853/1854	S157T	+	++	++
1855/1856	S157N	+		+
1857/1858	S157R	+	++	+
1859/1860	S157G	+	+	+
1861/1862	S157L	+		+
1863/1864	S157W	+		
1865/1866	S157K	+	+	+
1867/1868	S157C	+		+
1869/1870	S157D	+		
1871/1872	S157Q	+		+
1873/1874	S157M	+		+
1875/1876	S157H	+		+
1877/1878	S157I	+	++	++
1879/1880	S157F	+		
1881/1882	S160R	+		+
1883/1884	S160V	+	+	++
1885/1886	S160C	+	++	+
1887/1888	S160Q	+	+	+
1889/1890	S160A	+	+	+
1891/1892	S160P	+	+	++
1893/1894	S160L	+	++	++
1895/1896	S160F	+	++	+
1897/1898	S160T	++	+	++
1899/1900	S160D	+		+
1901/1902	S160Y	+		
1903/1904	S160W	++		
1905/1906	S160E	+	+	+
1907/1908	S160K	+	+	+
1909/1910	S160N	+		+
1911/1912	S160M	+	++	++
1913/1914	S214G	+		

Table 9.1 Protease activity relative to SEQ ID NO: 4				
1915/1916	S214M	++	+++	++
1917/1918	S214L	+	++	++
1919/1920	S214Q	+	+	++
1921/1922	S214T	++	+	+
1923/1924	S214P	+	++	++
1925/1926	S214R	++	+	+
1927/1928	S214D	+	++	+
1929/1930	S214F	+	+	+
1931/1932	S214K	+	++	+
1933/1934	S214A	+	+	++
1935/1936	S214V	++	++	+++
1937/1938	S214I	+	+++	++
1939/1940	S214E	+	+	+
1941/1942	S214H	+		+
1943/1944	S214Y	++	+	++
1945/1946	S214C	+	+++	++
1947/1948	S214W	+		+
1949/1950	S221L	++	++	++
1951/1952	S221T	+	+	++
1953/1954	S221I	++	++	+++
1955/1956	S221R	+		++
1957/1958	S221D	+	+	++
1959/1960	S221A	++	+	+
1961/1962	S221C	++	++	++
1963/1964	S221V	+	+	++
1965/1966	S221F	++		+
1967/1968	S221G	+		
1969/1970	S221P	+		
1971/1972	S221K	++	+	++
1973/1974	S221Y	++		++
1975/1976	S221E	+	++	++
1977/1978	S221Q	+	+	++
1979/1980	S221M	++	++	++
1981/1982	S221H	++		++
1983/1984	S221W	+		+
1985/1986	S268V	+	+	
1987/1988	S268Y	++	+	+
1989/1990	S268A	+	+	+
1991/1992	S268Q	+	+	++
1993/1994	S268P	+	+	+
1995/1996	S268G	+	+	+
1997/1998	S268T	+		
1999/2000	S268H	+		+

Table 9.1 Protease activity relative to SEQ ID NO: 4				
2001/2002	S268I	+	++	
2003/2004	S268F	+		
2005/2006	S268N	+		++
2007/2008	V273S	+	++	+
2009/2010	V273C	+	+	+++
2011/2012	V273A	+	+	
2013/2014	V273L	+	+	++
2015/2016	V273F		+	
2017/2018	V273T	++	+	+++
2019/2020	V273M	+	+	
2021/2022	Q279R	+	+	+
2023/2024	Q279E	+	++	+
2025/2026	Q279F	++	+	+
2027/2028	Q279G	+	+	+
2029/2030	Q279T	+	++	+
2031/2032	Q279M	+	+	++
2033/2034	Q279L	+	+	++
2035/2036	Q279S	+	+	+
2037/2038	Q279A	+	+	
2039/2040	Q279K	+	++	+
2041/2042	Q279V	+	++	+
2043/2044	Q279W	+		
2045/2046	Q279Y	++	++	++
2047/2048	Q279H	+		+
2049/2050	T311S	+		+
2051/2052	T311D	+	++	+++
2053/2054	T311Q	+		+
2055/2056	T311M	+		
2057/2058	T311K	+		
2059/2060	T311G	+	+	
2061/2062	T311A	++		
2063/2064	T311E	+		+++
2065/2066	S312V	+	+	+
2067/2068	S312D	+		
2069/2070	S312G	+		
2071/2072	S312R	+	++	++
2073/2074	S312W	+	+	
2075/2076	S312M	++	++	+
2077/2078	S312L	+		
2079/2080	S312N	+		
2081/2082	S312E	+	++	++
2083/2084	S312A	+	+	++
2085/2086	S312T	+		

Table 9.1 Protease activity relative to SEQ ID NO: 4				
2087/2088	S312Y	+	+	+
2089/2090	S312P	+		
2091/2092	S312H	+	+	+
2093/2094	S312Q	+	++	++
2095/2096	S312K	+	++	+++
2097/2098	S312C	+	+	+
2099/2100	S312I	+	++	++
2101/2102	T315E	+		+
2103/2104	T315S	+		
2105/2106	T315L	+		
2107/2108	T315R	++		
2109/2110	T315G	+		
2111/2112	T315A	++		
2113/2114	T315M	+		
2115/2116	T315Y	+		
2117/2118	T315K	+		
2119/2120	T315Q	+		+
2121/2122	T315D	+		
2123/2124	T315W	+		
2125/2126	T315C	++		
2127/2128	T315V	+	+	+
2129/2130	T315I	++		+
2131/2132	T315H	+		
2133/2134	T315F	+		
2135/2136	S342F	+		
2137/2138	S342G	+	++	++
2139/2140	S342R	++		++
2141/2142	S342Q	+		+
2143/2144	S342E	+		+
2145/2146	S342V	+		
2147/2148	S342T	+		
2149/2150	S342C	++	+	++
2151/2152	S342N	++	+	++
2153/2154	S342I	+		
2155/2156	S342P	+		
2157/2158	S342M	+		+
2159/2160	S342A	+	++	++
2161/2162	S342W	+		
2163/2164	S342K	+	++	++
2165/2166	S342D	+		
2167/2168	S342Y	+		
2169/2170	T345G	+		
2171/2172	T345R	+	++	+

Table 9.1 Protease activity relative to SEQ ID NO: 4				
2173/2174	T345L	+		
2175/2176	T345V	+	+	
2177/2178	T345A	+	+	+
2179/2180	T345M	+		++
2181/2182	T345W	+		
2183/2184	T345I		+	
2185/2186	T345S	++		
2187/2188	T345E	+		
2189/2190	T345Y	+		
2191/2192	T345D	+		
2193/2194	T345Q	+	+	
2195/2196	T345F	+		
2197/2198	T345C		+	+
2199/2200	T345K		++	++
2201/2202	S346T	+	++	++
2203/2204	S346Q	+		
2205/2206	S346V	+	+	++
2207/2208	S346R	++		
2209/2210	S346P	++		
2211/2212	S346L	+		+
2213/2214	S346D	+		
2215/2216	S346W	+	+	++
2217/2218	S346G	+		
2219/2220	S346A	++		
2221/2222	S346C	+	+	+
2223/2224	S346M	+		+
2225/2226	S346F		+	++
2227/2228	S346N	+		
2229/2230	S346Y	++		++
2231/2232	S346K	+		
2233/2234	A402*	+		+
2235/2236	G409*	+		+
2237/2238	G410*	+	+	+
2239/2240	G412*	+	+	+
2241/2242	G413*	+	+	++
Levels of increased activity were determined relative to the reference polypeptide of SEQ ID NO: 1710 and are defined as follows: "+" > 1, "++" > 1.1, "+++" > 1.3				

[0368] While the invention has been described with reference to the specific embodiments, various changes can be made and equivalents can be substituted to adapt to a particular situation, material,

composition of matter, process, process step or steps, thereby achieving benefits of the invention without departing from the scope of what is claimed.

[0369] For all purposes, each and every publication and patent document cited in this disclosure is incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an indication that any such document is pertinent prior art, nor does it constitute an admission as to its contents or date.

CLAIMS

What is claimed is:

1. An engineered protease polypeptide, or a biologically active fragment thereof, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or to a reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.
2. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or to the reference sequence corresponding to SEQ ID NO: 4 or 628, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4, or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.
3. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.
4. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

5. The engineered protease polypeptide of any one of claims 1-4, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 11, 31, 42, 45, 50, 53, 84, 99, 100, 126, 128, 129, 130, 131, 132, 134, 135, 136, 137, 138, 139, 140, 141, 143, 145, 151, 154, 156, 157, 159, 160, 161, 162, 163, 169, 172, 173, 174, 179, 180, 184, 185, 186, 187, 188, 190, 191, 192, 193, 194, 198, 199, 212, 214, 220, 221, 222, 223, 225, 231, 232, 233, 235, 237, 238, 239, 240, 242, 243, 245, 246, 249, 250, 251, 252, 253, 254, 256, 258, 262, 263, 264, 265, 266, 267, 268, 269, 271, 273, 274, 275, 277, 278, 279, 280, 281, 283, 285, 290, 292, 293, 294, 296, 297, 300, 302, 303, 311, 312, 313, 314, 315, 316, 318, 324, 328, 336, 339, 341, 342, 343, 345, 346, 355, 358, 360, 364, 367, 368, 369, 370, 371, 372, 373, 374, 375, 377, 381, 382, 384, 386, 389, 391, 392, 401, 402, 405, 406, 409, 410, 411, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

6. The engineered protease polypeptide of any one of claims 1-5, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution 11K, 31G, 42W, 45Y, 50R, 53A, 84M, 99V, 100V, 126T, 128G/I/K/L/P/R/S/T/V, 129E/F/H/I/K/L/R/S/T/V, 130A/F/G/N/V, 131E/P/R/T/V/Y, 132A/C/D/E/G/P/R/V/Y, 134A/C/D/E/G/I/L/M/N/P/S/T/V/W/Y, 135C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 136C/G/I/M, 137A/D/N/S, 138Q, 139C/D/E/F/H/I/K/L/M/R/S, 140L, 141A/C/D/E/F/G/H/I/L/M/Q/R/S/T/V/W/Y, 143A/C/D/N/Q/S/T, 145A/C/D/E/F/G/H/I/K/L/P/Q/R/S/T/V/W, 151D/Q, 154C/D/L/R, 156C/V, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W, 159G, 160A/C/D/E/F/K/L/M/N/P/R/Q/T/V/W/Y, 161D/E/G/L/R, 162I, 163H/L, 169S, 172Q, 173F/S, 174L, 179K/S, 180H/L/M, 184A/D/G/L/M/Q/R, 185A/D/E/F/G/L/M/P/Q/R/S/T/V, 186A/R/S/T/Y, 187A, 188A/C/D/F/G/L/M/S/T/W, 190S, 191R, 192C/D/M/N, 193T, 194A/D/L/T, 198G, 199C/K/L, 212S, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 220K/L/R, 221A/C/D/E/F/G/H/I/K/L/M/P/Q/R/T/V/W/Y, 222G, 223S, 225V, 231H/V, 232S, 233G/I/L, 235Q/R/V, 237A/G, 238Q, 239L/M, 240A/L, 242E/S, 243E/L/M/R/S/T, 245L/V, 246I/V, 249G/M/S, 250A/C/F/L/N/T, 251D/S, 252P, 253C/I/V, 254C/E, 256L/M, 258W, 262A/S, 263E/H/P/Q/R/S, 264A/C/F/I/L/N/P/R/T/V, 265C/G/R, 266H/T/Y, 267A/G/H/I/L/M/R/S/T/V/W, 268A/F/G/H/I/N/P/Q/T/V/Y, 269Q/T, 271A, 273A/C/F/L/M/S/T, 274A/G/K/L/T/V/W, 275A/V, 277D/G, 278L/N/S/V/Y, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 280D/K/S/T, 281C/V, 283M, 285S, 290E/G/S, 292V, 293A, 294V/W, 296M/R, 297F, 300R/V, 302G/P, 303A/V, 311A/E/D/G/K/M/Q/S, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 313A/Q/S/T, 314G, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/V/W/Y, 316K, 318N/P/R, 324A/D/E/I/R/V/W/Y, 328L/M, 336F, 339S/W, 341G, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/T/V/W/Y, 343S, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/T/V/W/Y, 355A, 358S, 360S, 364A/V, 367V, 368G/T, 369I/V/W, 370C/E/F/G/I/K/L/P/Q/R/S/V, 371L, 372A/C/F/L/R/V/Y,

373A/C/E/F/M/S/Y, 374E/G/L/R/S/W/Y, 375A/E/I/L/M/S/T/V, 377H, 381N, 382G/R/S/T, 384C, 386P/W, 389C/P, 391L/S, 392Y, 401L, 402G/*, 405L/Q, 406C/M/R/W, 409E/R/*, 410C/I/W/*, 411L/R/T/V, 412P/T/*, or 413A/C/D/S/*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

7. The engineered protease polypeptide of any one of claims 1-5, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 157, 160, 214, 268, 273, 279, 311, 312, 315, 328, 342, 345, 346, or 372, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

8. The engineered protease polypeptide of any one of claims 1-5 and 7, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution 135C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 137A/D/N/S, 139C/D/E/F/H/I/K/L/M/R/S, 141A/C/D/E/F/G/H/I/L/M/Q/R/S/T/V/W/Y, 143A/C/D/N/Q/S/T, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W, 160A/C/D/E/F/K/L/M/N/P/R/Q/T/V/W/Y, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 268A/F/G/H/I/N/P/Q/T/V/Y, 273A/C/F/L/M/S/T, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 311A/E/D/G/K/M/Q/S, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/V/W/Y, 328L/M, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/T/V/W/Y, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/T/V/W/Y, or 372A/C/F/L/R/V/Y, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

9. The engineered protease polypeptide of any one of claims 1-5, 7, and 8, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution 135G, 137N, 139L/C, 141V, 143A, 157G, 160L, 214P, 268G, 273T, 279M, 311D, 312Q, 315V, 328L, 342G, 345R, 346T, or 372V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

10. The engineered protease polypeptide of any one of claims 1-5, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set at amino acid positions 135/141/160/311/315/372, 143/328/342/345, 139/157/268/273/312/346, or 137/139/214/279, wherein the amino acid positions are relative to the reference sequence

corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

11. The engineered protease polypeptide of any one of claims 1-5 and 10, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set 135G/141V/160L/311D/315V/372V, 143A/328L/342G/345R, 139C/157G/268G/273T/312Q/346T, or 137N/139L/214P/279M, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

12. The engineered protease polypeptide of any one of claims 1-5 and 10, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set A135G/N141V/S160L/T311D/T315V/S372V, H143A/V328L/S342G/T345R, N139C/S157G/S268G/V273T/S312Q/S346T, or H137N/C139L/S214P/Q279M, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

13. The engineered protease polypeptide of any one of claims 1-5, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 185, 134, 129, 135, 184, 132, 186, 193, 263, 370, 45/134, 199, 368, 161, 141, 267, 179, 264, 160, 138, 131, 372, 151, 274, 128, 339, 313, 374, 314, 191, 324, 315, 375, 136, 220, 194, 231, 277, 369, 251, 180, 163, 343, 264/279, 279, 232, 141/300, 367, 266, 188, 130, 318, 265, 341, 190, 145, 126/192, 11/220, 192, 370/392, 99/278, 265/311, 84/159/265/279/311/370, 311/316, 342/370, 265/311/370, 192/311/316, 141/154/192, 265/311/316/342, 279/311/316, 141/265/279/311/342, 141/192/311/316/370, 141/265/311, 198/279, 392, 342/370/392, 141/198/265, 265/392, 184/267, 342, 312, 100/251, 141/220, 311/316/370, 99, 278, 405, 311/342/370, 141/198, 311/342, 141/311, 279/311/377/392, 186/198/311/342/370/392, 141/392, 311/370/392, 141/311/392, 311/370, 311/316/392, 265/311/392, 141/192, 311, 141/265/311/392, 192/311/370/392, 198/265/311/316/370, 141/186/265/311, or 141/198/265/311/370, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

14. The engineered protease polypeptide of any one of claims 1-5 and 13, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 185F, 134I, 129T, 135C, 184A, 129R, 132Y, 186R, 193T, 263P, 370C, 45Y/Q134W, 185M, 199K, 368T, 161E, 141T, 267L, 179S, 185V, 264L, 199C, 160M, 138Q, 131Y, 184D, 372R, 134L, 370R, 370I, 134E, 368G, 151D, 274K, 134D, 134V, 128I, 339S, 313A, 131E, 185P, 374W, 314G, 191R, 128V, 132E, 324R, 315M, 132V, 375L, 375T, 129L, 132P, 184M, 136G, 186A, 135S,

220L, 134P, 132A, 141M, 135I, 194D, 185Q, 263H, 274L, 231V, 315R, 375S, 135T, 185G, 135R, 277D, 128P, 132R, 369I, 264C, 315H, 251S, 136I, 160P, 375I, 180M, 369V, 251D, 264A, 163L, 231H, 343S, 264R/279R, 274A, 279Y, 131P, 232S, 220R, 315Q, 186T, 324V, 313S, 132D, 141R/300V, 324I, 367V, 135V, 370L, 132G, 267G, 131T, 266T, 179K, 372A, 372F, 185T, 324D, 135K, 188A, 141D, 374L, 185D, 130N, 370V, 161R, 315I, 315L, 318N, 188C, 180L, 372Y, 135P, 375E, 324A, 129K, 134M, 184G, 185A, 129H, 188D, 130F, 265C, 141W, 324W, 370E, 184R, 134A, 161L, 134T, 370G, 375A, 128G, 130V, 134N, 341G, 190S, 370P, 145R, 279H, 279S, 160Q, 370K, 126T/G192C, 374E, 128K, 160C, 186S, 11K/Q220K, 134W, 129V, 128L, 151Q, 375M, 134C, 374R, 160T, 279T, 264F, 132C, 129F, 264V, 129I, 184Q, 192M, 374S, 370F, 267A, 369W, 199L, 145M, 194A, 185S, 265R, 129S, 185R, 188W, 161G, 370G/392Y, 99V/278N, 265G/311D, 84M/159G/265G/279K/311D/370G, 311D/316K, 342N/370G, 265G/311D/370G, 192D/311D/316K, 141Q/154D/192D, 265G/311D/316K/342N, 279K/311D/316K, 141Q/265G/279K/311D/342N, 141Q/192D/311D/316K/370G, 141Q, 141Q/265G/311D, 198G/279K, 392Y, 342N/370G/392Y, 141Q/198G/265G, 265G/392Y, or 265G, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

15. The engineered protease polypeptide of any one of claims 1-5 and 13, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 135L, 194L, 128T, 134S, 313T, 184L/267L, 185E, 342G, 374Y, 141R, 186Y, 312R, 33Q, 315V, 374G, 128S, 136A, 128R, 370Q, 267V, 188M, 188F, 263S, 188S, 339W, 100V/251S, 131V, 188T, 141L, 134Y, 267M, 264N, 134G, 185L, 370S, 267W, 279M, 267R, 264T, 279L, 263R, 136C, 145E, 188G, 130A, 192N, 188L, 312I, 129E, 315E, 145A, 267H, 372V, 130G, 267T, 274W, 136M, 372C, 194T, 375V, 135G, 267I, 141L/220R, 324E, 160L, 141S, 372L, 135Y, 141V, 141A, 131R, 135E, 324Y, 311D/316K/370G, 99V, 278N, 405Q, 311D/342N/370G, 141Q/198G, 311D/342N, 141Q/311D, 279K/311D/377H/392Y, 186Y/198G/311D/342N/370G/392Y, 141Q/392Y, 311D/370G/392Y, 141Q/311D/392Y, 311D/370G, 311D/316K/392Y, 265G/311D/392Y, 141Q/192D, 311D, 141Q/265G/311D/392Y, 192D/311D/370G/392Y, 198G/265G/311D/316K/370G, 141Q/186Y/265G/311D, or 141Q/198G/265G/311D/370G, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

16. The engineered protease polypeptide of any one of claims 1-5, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 145, 145, 157, 160, 214, 221, 268, 273, 279, 311, 312, 315, 315, 342, 345, 346, 402, 409, 410, 412, or 413, or combinations thereof, wherein the amino acid positions

are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

17. The engineered protease polypeptide of any one of claims 1-5 and 16, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135G, 135S, 135V, 135L, 135R, 135E, 135P, 135H, 135C, 135T, 135Y, 135W, 135M, 135N, 137S, 137A, 137N, 137D, 139R, 139E, 139F, 139L, 139K, 139D, 139H, 139I, 139S, 141T, 141S, 141V, 141L, 141R, 141M, 141G, 141Y, 141I, 141C, 141F, 141A, 141D, 141E, 141H, 143T, 143C, 143Q, 143A, 143D, 143S, 143N, 145Q, 145T, 145V, 145H, 145L, 145E, 145R, 145D, 145R, 145A, 145F, 145S, 145G, 145I, 145K, 145M, 145C, 145W, 157A, 157E, 157P, 157V, 157T, 157N, 157R, 157G, 157L, 157W, 157K, 157C, 157D, 157Q, 157M, 157H, 157I, 157F, 160R, 160V, 160C, 160Q, 160A, 160P, 160L, 160F, 160T, 160D, 160Y, 160W, 160E, 160K, 160N, 160M, 214G, 214M, 214L, 214Q, 214T, 214P, 214R, 214D, 214F, 214K, 214A, 214V, 214I, 214E, 214H, 214Y, 214C, 214W, 221L, 221T, 221I, 221R, 221D, 221A, 221C, 221V, 221F, 221G, 221P, 221K, 221Y, 221E, 221Q, 221M, 221H, 221W, 268V, 268Y, 268A, 268Q, 268P, 268G, 268T, 268H, 268I, 268F, 268N, 273S, 273C, 273A, 273L, 273F, 273T, 273M, 279R, 279E, 279F, 279G, 279T, 279M, 279L, 279S, 279A, 279K, 279V, 279W, 279Y, 279H, 311S, 311D, 311Q, 311M, 311K, 311G, 311A, 311E, 312V, 312D, 312G, 312R, 312W, 312M, 312L, 312N, 312E, 312A, 312T, 312Y, 312P, 312H, 312Q, 312K, 312C, 312I, 315E, 315S, 315L, 315R, 315G, 315A, 315M, 315Y, 315K, 315Q, 315D, 315W, 315C, 315V, 315I, 315H, 315F, 342F, 342G, 342R, 342Q, 342E, 342V, 342T, 342C, 342N, 342I, 342P, 342M, 342A, 342W, 342K, 342D, 342Y, 345G, 345R, 345L, 345V, 345A, 345M, 345W, 345I, 345S, 345E, 345Y, 345D, 345Q, 345F, 345C, 345K, 346T, 346Q, 346V, 346R, 346P, 346L, 346D, 346W, 346G, 346A, 346C, 346M, 346F, 346N, 346Y, 346K, 402*, 409*, 410*, 412*, or 413*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

18. The engineered protease polypeptide of any one of claims 1-5, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 242, 157, 250, 373, 243, 336, 187, 240, 280, 271, 237, 386, 382, 328, 42, 391, 381, 275, 249, 239, 384, 139, 364, 346, 389, 254, 246, 345, 360, 303, 300, 269, 135/141/372, 311/315/372, 136/141/311, 141/188, 135/136, 135/141/315, 372, 135/141/160/267/372, 135/136/141/160/185/188/267/311/315, 160/185, 135/141/188/279/311, 135/136/141, 135/136/141/372, 135/141/160/185/267/279, 135/141/160/267, 141/188/311/372, 160/185/188/279/311, 136/141/279, 135/136/141/160/185/188, 141/372, 135/136/141/311, 185/311/315/372, 135/141/188, 136/185, 135/141, 135/136/141/279/315/372, 135/311/315, 141, 311/372, 188/311, 135/141/188/372, 141/160/279, 313/392, 342/392, 279/392, 128, 198/342, 313,

128/312, 50, 145/263, 313/342, 279/312, 312/392, 279/342, 128/342, 342, 263, 143, 262, 156, 169, 143/237, 136/160/185/267/311/372, 135/160/311/372, 135/141/311/315, 141/311/315, 136/141/160/185/188/311/315/372, 135/141/311/315/372, 135/141/160/185/311/315, 135/141/267/311/315/372, 135/136/141/160/311/315, 135/136/141/279, 135/141/267/279/311/315, 135/141/160, 135/141/160/311/315/372, 135/141/160/311/315, 135/136/141/188/311, 141/160/311, 135/141/160/279/311/315/372, 141/160/185/279/311/372, 135/136/141/160/315/372, 135/136/160/279/311/372, 128/279/312/342, 128/198/312/342, 263/342, 145/263/279/312/342/392, or 128/145/198/312/313/392, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

19. The engineered protease polypeptide of any one of claims 1-5 and 18, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 242E, 157V, 250N, 373F, 243R, 336F, 187A, 240A, 280K, 271A, 237G, 386W, 382G, 280S, 373Y, 328M, 157R, 157A, 42W, 243E, 382S, 391L, 381N, 243M, 275V, 157I, 373S, 157T, 280D, 249G, 239L, 384C, 139M, 240L, 243T, 250L, 250A, 382T, 364A, 346V, 373M, 389P, 373C, 382R, 373E, 254E, 246I, 250F, 280T, 373A, 139K, 345I, 360S, 275A, 249M, 364V, 303V, 300R, 239M, 269T, 135G/141Q/372L, 311D/315V/372L, 136M/141V/311D, 141V/188M, 135E/136M, 136M/141Q/311D, 135E/141V/315V, 372V, 135E/141V/160L/267I/372V, 135G/136M/141V/160L/185E/188M/267I/311D/315V, 135G/136M, 160L/185E, 135E/141V/188M/279M/311D, 135G/136M/141Q, 135G/136M/141Q/372L, 135G/141V/160L/185E/267I/279M, 135G/141V/160L/267I, 141Q/188M/311D/372V, 160L/185E/188M/279M/311D, 136M/141V/279M, 135G/136M/141V/160L/185E/188L, 141Q/372V, 135E/136M/141Q/311D, 185E/311D/315V/372V, 135G/141V/188M, 136M/185E, 135E/141Q, 135E/136M/141Q/279M/315V/372L, 135G/311D/315V, 141V, 135G/141V, 311D/372L, 188M/311D, 135E/141V/188L/372L, 141V/160L/279M, 313Q/392Y, 342G/392Y, 279L/392Y, 128T, 198G/342G, 313Q, 128T/312I, 50R, 145E/263S, 313Q/342G, 279L/312I, 312I/392Y, 279K/342G, 279K/392Y, 128T/342G, 342G, or 263S, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

20. The engineered protease polypeptide of claim 1-5 and 18, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 139C, 345R, 243L, 143A, 249S, 262S, 139R, 269Q, 328L, 157G, 156V, 242S, 139L, 262A, 169S, 346T, 143N/237A, 136M/160L/185E/267I/311D/372L, 135E/160L/311D/372L, 135G/141V/311D/315V, 141V/311D/315V, 136M/141V/160L/185E/188M/311D/315V/372V, 135G/141V/311D/315V/372L, 135G/141Q/160L/185E/311D/315V,

135G/141V/267I/311D/315V/372V, 135G/136M/141V/160L/311D/315V, 135G/136M/141V/279M, 135G/141Q/267I/279M/311D/315V, 135E/141V/311D/315V/372V, 135E/141Q/160L, 135G/141Q/311D/315V, 135G/141V/160L/311D/315V/372V, 135G/141V/160L/311D/315V, 135G/141Q/267I/311D/315V/372L, 135G/136M/141V/188M/311D, 141V/160L/311D, 135E/141V/160L/279M/311D/315V/372L, 141V/160L/185E/279M/311D/372V, 135E/141V/311D/315V, 135G/136M/141V/160L/315V/372V, 135E/141V/160L, 135E/136M/160L/279M/311D/372V, 128T/279K/312I/342G, 128T/198G/312I/342G, 263S/342G, 145E/263S/279L/312I/342G/392Y, or 128T/145E/198G/312I/313Q/392Y, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

21. The engineered protease polypeptide of claim 1, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at an amino acid position set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

22. The engineered protease polypeptide of claim 1, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

23. The engineered protease polypeptide of claim 1, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

24. The engineered protease polypeptide of claim 1, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set of an engineered set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

25. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to an amino acid sequence comprising at least a substitution or substitution set of an

engineered protease polypeptide variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or relative to the reference sequence corresponding to SEQ ID NO: 4 or 628.

26. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

27. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710.

28. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

29. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

30. The engineered protease polypeptide of claim 28 or 29, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 11, 31, 42, 45, 50, 53, 84, 99, 100, 126, 128, 129, 130, 131, 132, 134, 135, 136, 137, 138,

139, 140, 141, 143, 145, 151, 154, 156, 157, 159, 160, 161, 162, 163, 169, 172, 173, 174, 179, 180, 184, 185, 186, 187, 188, 190, 191, 192, 193, 194, 198, 199, 212, 214, 220, 221, 222, 223, 225, 231, 232, 233, 235, 237, 238, 239, 240, 242, 243, 245, 246, 249, 250, 251, 252, 253, 254, 256, 258, 262, 263, 264, 265, 266, 267, 268, 269, 271, 273, 274, 275, 277, 278, 279, 280, 281, 283, 285, 290, 292, 293, 294, 296, 297, 300, 302, 303, 311, 312, 313, 314, 315, 316, 318, 324, 328, 336, 339, 341, 342, 343, 345, 346, 355, 358, 360, 364, 367, 368, 369, 370, 371, 372, 373, 374, 375, 377, 381, 382, 384, 386, 389, 391, 392, 401, 402, 405, 406, 409, 410, 411, 412, or 413, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

31. The engineered protease polypeptide of any one of claims 28-30, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 11K, 31G, 42W, 45Y, 50R, 53A, 84M, 99V, 100V, 126T, 128G/I/K/L/P/R/S/T/V, 129E/F/H/I/K/L/R/S/T/V, 130A/F/G/N/V, 131E/P/R/T/V/Y, 132A/C/D/E/G/P/R/V/Y, 134A/C/D/E/G/I/L/M/N/P/S/T/V/W/Y, 135A/C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 136C/G/I/M, 137A/D/N/S, 138Q, 139C/D/E/F/H/I/K/L/M/N/R/S, 140L, 141A/C/D/E/F/G/H/I/L/M/N/Q/R/S/T/V/W/Y, 143A/C/D/H/N/Q/S/T, 145A/C/D/E/F/G/H/I/K/L/P/Q/R/S/T/V/W, 151D/Q, 154C/D/L/R, 156C/V, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/S/T/V/W, 159G, 160A/C/D/E/F/K/L/M/N/P/R/Q/S/T/V/W/Y, 161D/E/G/L/R, 162I, 163H/L, 169S, 172Q, 173F/S, 174L, 179K/S, 180H/L/M, 184A/D/G/L/M/Q/R, 185A/D/E/F/G/L/M/P/Q/R/S/T/V, 186A/R/S/T/Y, 187A, 188A/C/D/F/G/L/M/S/T/W, 190S, 191R, 192C/D/M/N, 193T, 194A/D/L/T, 198G, 199C/K/L, 212S, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 220K/L/R, 221A/C/D/E/F/G/H/I/K/L/M/P/Q/R/T/V/W/Y, 222G, 223S, 225V, 231H/V, 232S, 233G/I/L, 235Q/R/V, 237A/G, 238Q, 239L/M, 240A/L, 242E/S, 243E/L/M/R/S/T, 245L/V, 246I/V, 249G/M/S, 250A/C/F/L/N/T, 251D/S/T, 252P, 253C/I/V, 254C/E, 256L/M, 258W, 262A/S, 263E/H/P/Q/R/S, 264A/C/F/I/L/N/P/R/T/V, 265C/G/R, 266H/T/Y, 267A/G/H/I/L/M/R/S/T/V/W, 268A/F/G/H/I/N/P/Q/S/T/V/Y, 269Q/T, 271A, 273A/C/F/L/M/S/T/V, 274A/G/K/L/T/V/W, 275A/V, 277D/G, 278L/N/S/V/Y, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 280D/K/S/T, 281C/V, 283M, 285S, 290E/G/S, 292V, 293A, 294V/W, 296M/R, 297F, 300R/V, 302G/P, 303A/V, 311A/E/D/G/K/M/Q/S/T, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/S/T/V/W/Y, 313A/Q/S/T, 314G, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/T/V/W/Y, 316K, 318N/P/R, 324A/D/E/I/R/V/W/Y, 328L/M/V, 336F, 339S/W, 341G, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/S/T/V/W/Y, 343S, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/S/T/V/W/Y, 355A, 358S, 360S, 364A/V, 367V, 368G/T, 369I/V/W, 370C/E/F/G/I/K/L/P/Q/R/S/V, 371L, 372A/C/F/L/R/S/V/Y, 373A/C/E/F/M/S/Y, 374E/G/L/R/S/W/Y, 375A/E/I/L/M/S/T/V, 377H, 381N,

382G/R/S/T, 384C, 386P/W, 389C/P, 391L/S, 392Y, 401L, 402G/*, 405L/Q, 406C/M/R/W, 409E/R/*, 410C/I/W/*, 411L/R/T/V, 412P/T/*, or 413A/C/D/S/*, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

32. The engineered protease polypeptide of any one of claims 28-30, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at amino acid position 135, 137, 139, 141, 143, 157, 160, 214, 268, 273, 279, 311, 312, 315, 328, 342, 345, 346, or 372, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

33. The engineered protease polypeptide of any one of claims 28-30 and 32, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135A/C/E/G/H/I/K/L/M/N/P/R/S/T/V/W/Y, 137A/D/N/S, 139C/D/E/F/H/I/K/L/M/N/R/S, 141A/C/D/E/F/G/H/I/L/M/N/Q/R/S/T/V/W/Y, 143A/C/D/H/N/Q/S/T, 157A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/S/T/V/W, 160A/C/D/E/F/K/L/M/N/P/R/Q/S/T/V/W/Y, 214A/C/D/E/F/G/H/I/K/L/M/N/P/Q/R/T/V/W/Y, 268A/F/G/H/I/N/P/Q/S/T/V/Y, 273A/C/F/L/M/S/T/V, 279A/E/F/G/H/K/L/M/R/S/T/Y/V/W, 311A/E/D/G/K/M/Q/S/T, 312A/C/D/E/G/H/I/K/L/M/N/P/Q/R/S/T/V/W/Y, 315A/C/D/E/F/G/H/I/K/L/M/Q/R/S/T/V/W/Y, 328L/M/V, 342A/C/D/E/F/G/I/K/M/N/P/R/Q/S/T/V/W/Y, 345A/C/D/E/F/G/I/K/L/M/Q/R/S/V/W/Y, 346A/C/D/F/G/K/L/M/N/P/Q/R/S/T/V/W/Y, or 372A/C/F/L/R/S/V/Y, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

34. The engineered protease polypeptide of any one of claims 28-30 and 32, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or amino acid residue 135G, 137N, 139L/C, 141V, 143A, 157G, 160L, 214P, 268G, 273T, 279M, 311D, 312Q, 315V, 328L, 342G, 345R, 346T, or 372V, or combinations thereof, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, 1126, 1368, or 1548, or to the reference sequence corresponding to SEQ ID NO: 948, 1126, 1368, or 1548.

35. The engineered protease polypeptide of claim 28 or 29, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or to the reference sequence

corresponding to SEQ ID NO: 948, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

36. The engineered protease polypeptide of claim 28 or 29, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 950-1154, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 950-1154, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or relative to the reference sequence corresponding to SEQ ID NO: 948.

37. The engineered protease polypeptide of claim 35 or 36, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 411, 402, 285, 245, 266, 355, 258, 222, 140, 268, 225, 283, 406, 410, 143/145/243/312, 139/143/145/157/312, 139/157/345, 139/269, 156/157/342/346, 139/143, 269, 139/243/328, 269/328, 143/145/169, 328, 143/145/262, 145/262/312/328, 139/156/157, 139/145/312, 312, 139, 139/312, 139/156, 139/143/145/243, 145/157, 145/346, 145/262/312/328/345/346, 145/262, 312/342, 143/243, 139/345, 342, 143/145/262/342, 139/143/169, 139/143/145/312, 169, 139/145/262/312/328/342/345/346, 139/328, 139/243, 139/143/328, 139/143/243, 139/145, 145/312, 145/169, 139/143/157/312, 84/139/143, 145/269, 143/145/157/269/312/328, 143/145/269, 157, 139/143/312, 256, 273, 409, 172, 401, 281, 253, 143/145/243/328, 145, 139/143/145/328/342/345, 143/328/342/345, 145/342/345, 143, 139/145/328/342/345, 143/145/169/312/328/345/346, 143/243/328/342/345/346, 139/143/157/169/328/346, 143/145/156/312/328, 139/145/157/312/328, 143/328/342/345/346, 143/145, 143/145/312/342/345, or 143/145/328, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or to the reference sequence corresponding to SEQ ID NO: 948.

38. The engineered protease polypeptide of any one of claims 35-37, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 411V, 411R, 402G, 285S, 245V, 266H, 355A, 258W, 222G, 140L, 268T, 411L, 225V, 245L, 283M, 406W, 410C, 406C, 143A/145E/243L/312I, 139L/143A/145E/157G/312I, 139C/157G/345R, 139C/269Q, 156V/157G/342G/346T, 139L/143A, 269Q, 139L/243L/328L, 269Q/328L, 143A/145E/169S, 328L, 143A/145E/262S, 145E/262S/312I/328L, 139L/156V/157G, 139C/145E/312I, 312I, 139C, 139C/312I, 139C/156V, 139C/143A/145E/243L, 145E/157G, 145E/346T, 145E/262A/312I/328L/345R/346T, 145E/262A, 312I/342G, 143A/243L, 139C/345R, 342G, 143A/145E/262S/342G, 139L/143A/169S, 139C/143A/145E/312I, 169S,

139L/145E/262A/312I/328L/342G/345R/346T, 139C/328L, 139L/243L, 139L/143A/328L, 139L, 139C/143A/243L, 139C/145E, 145E/312I, 145E/169S, 139C/143A/157G/312I, 84M/139C/143A, 145E/269Q, 143A/145E/157G/269Q/312I/328L, 143A/145E/269Q, 157G, or 139L/143A/312I, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or to the reference sequence corresponding to SEQ ID NO: 948.

39. The engineered protease polypeptide of any one of claims 35-37, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 256L, 411T, 273F, 409E, 268G, 172Q, 409R, 401L, 281C, 410W, 253V, 406M, 273T, 406R, 256M, 410I, 273M, 273L, 143A/145E/243L/328L, 145E, 139C/143A/145E/328L/342G/345R, 139L/145E, 143A/328L/342G/345R, 145E/342G/345R, 143A, 139C/143A, 139C/145E/328L/342G/345R, 143A/145E/169S/312I/328L/345R/346T, 143A/243L/328L/342G/345R/346T, 139C/143A/157G/169S/328L/346T, 143A/145E/156V/312I/328L, 139C/145E/157G/312I/328L, 143A/328L/342G/345R/346T, 139C/143A/328L, 143A/145E, 143A/145E/312I/342G/345R, or 143A/145E/328L, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 948, or to the reference sequence corresponding to SEQ ID NO: 948.

40. The engineered protease polypeptide of claim 28 or 29, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or to the reference sequence corresponding to SEQ ID NO: 1126, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

41. The engineered protease polypeptide of claim 28 or 29, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1156-1422, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1156-1422, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

42. The engineered protease polypeptide of claim 40 or 41, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 279, 250, 154, 214, 249, 275, 137, 161, 180, 174, 139, 254, 145, 278, 136,

154/413, 294, 237, 274, 264, 185, 277, 293, 233, 173, 312, 302, 238, 135, 221, 290, 263, 267, 239, 163, 292, 246, 243, 235, 156, 223, 278/413, 297, 194, 251, 253/411, 145/157/253/268/273/281/312/346/411, 139/346, 253, 346/411, 253/346, 312/346, 273/312, 253/281, 157/253/273/312/346/411, 139/157/253/268/273/281/312/346, 253/273/411, 139/253/268/273/281, 139/157/411, 157, 273, 139/253/268/273/281/312/411, 157/253/411, 139/145/253/346, 139/157/253/273/312, 139/157/268/273/312/346, 157/273/312/346, 139/411, 139/253/268/273/281/312/346/411, 157/273/346/411, 139/145/157/162/253/273/281/312, 139/253/273/281/312, 157/253/268/273/281/312, 139/253/268, 139/157/312, 253/273/281/346, 157/253/312/346/411, 157/273/312/346/411, 139/145/157/253/268/281/312, 139/273/312/346, 157/253/268/273/312/346, 139/268/346, 268/273/312/346, 139/157/253/268/273/312, 139/157/253, 139/253/281, 139/157/253/268/273, 253/312/411, or 139/268/273, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or to the reference sequence corresponding to SEQ ID NO: 1126.

43. The engineered protease polypeptide of any one of claims 40-42, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 279Y, 250T, 154C, 214Y, 249S, 275A, 137A, 161D, 180H, 174L, 139K, 254C, 145E, 278V, 136M, 154L/413D, 294V, 154R, 237A, 274G, 137N, 264P, 274T, 278N, 214A, 185G, 214V, 279T, 277G, 264I, 293A, 233L, 278Y, 173F, 274L, 312C, 279L, 302G, 238Q, 294W, 135A, 221E, 290S, 278S, 263Q, 263H, 278L, 263E, 154L, 139M, 137S, 233G, 139F, 267I, 221L, 173S, 302P, 221V, 239M, 290G, 163H, 292V, 246V, 214N, 243S, 233I, 235Q, 145D, 274V, 279M, 185S, 279K, 145W, 290E, 214P, 156V, 156C, 223S, 278V/413D, 250C, 267S, 297F, 221Q, 194D, 251T, 253V/411T, 145P/157R/253I/268G/273T/281V/312Q/346T/411T, 139C/346A, 253V, 346T/411T, 253V/346T, 139C/346T, 312Q/346T, 273T/312Q, 253I/281V, 157R/253V/273T/312Q/346T/411T, 139C/157K/253V/268G/273F/281V/312Q/346A, 253V/273T/411T, 139C/253I/268F/273T/281V, 139C/157R/411T, 157G, 273T, 139C/253V/268G/273F/281V/312Q/411T, 157G/253V/411T, 139C/145E/253V/346T, 139C/157K/253V/273T/312Q, 139C/157G/268G/273T/312Q/346T, 157G/273T/312Q/346T, 139C/411T, 139C/253V/268G/273F/281C/312I/346T/411T, 157K/273F/346T/411T, 139C/145E/157G/162I/253V/273F/281V/312Q, 139C/253V/273T/281C/312Q, 157G/253V/268F/273F/281V/312Q, 139C/253I/268F, 139C/157R/312Q, 253V/273T/281C/346T, 157K/253V/312I/346T/411T, 157R/273T/312Q/346T/411T, 139C/145E/157K/253V/268G/281C/312Q, 139C/273T/312Q/346T, 157K/253V/268F/273T/312I/346T, 139C/268G/346T, 157K, 268G/273F/312Q/346T, 139C/157K/253V/268F/273F/312Q, 157K/273T/312Q/346T, 139C/157G/253V, 139C, 139C/253V/281V, 139C/157G/253V/268G/273T, 253V/312Q/411T, 139C/268G/273T, or 253I, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1126, or relative to the reference sequence corresponding to SEQ ID NO: 1126.

44. The engineered protease polypeptide of claim 28 or 29, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or to the reference sequence corresponding to SEQ ID NO: 1368, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

45. The engineered protease polypeptide of claim 28 or 29, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1424-1608, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1424-1608, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

46. The engineered protease polypeptide of claim 44 or 45, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 31, 318, 296, 252, 303, 253, 413, 386, 312, 235, 412, 342, 302, 371, 405, 389, 391, 358, 139/273/311/328/372, 311, 372, 139/143/157/160/268/273/311/315, 143, 139/143, 139/160/312/372, 143/273/328, 346, 135/139/160/268/312/342/346, 139/141/273, 135/141/143/268/273/312/372, 139/141/143/311, 139/157/268/328/346/372, 53/139/141/143/273/372, 139, 139/141/143/273/312, 137/139/221/233/413, 233, 221/279, 137/139/233/279, 221, 139/214, 137/139/156, 139/214/221, 214/233, 137/139/221/233/279, 137/139/221, 137/139, 137/139/279, 137, 137/139/214/279, 266, 139/221, 137/221, 137/156/214/312, 137/221/233, 137/139/156/221, 137/139/214, 279, 137/139/233, 137/139/214/233, 221/413, 137/214/233, 137/156, 137/139/221/233, 214/221, 137/221/413, 214, 137/233, 137/413, 137/221/279, 137/139/156/214/233/413, or 137/139/156/214, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

47. The engineered protease polypeptide of any one of claims 44-46, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 31G, 318R, 296R, 296M, 252P, 303A, 253C, 413C, 386P, 413A, 312R, 235R, 412P, 342A, 413S, 302P, 371L, 405L, 312A, 389P, 318P, 391S, 412T, 358S, 389C, 235V, 391L, 139N/273V/311T/328V/372S, 311T, 312S, 372S, 139N/143H/157S/160S/268S/273V/311T/315T, 143H, 139N/143H, 139N/160S/312S/372S, 143H/273V/328V, 346S.

135A/139N/160S/268S/312S/342S/346S, 139N/141N/273V, 135A/141N/143H/268S/273V/312S/372S, 139N/141N/143H/311T, 139N/157S/268S/328V/346S/372S, 53A/139N/141N/143H/273V/372S, 139N, 139N/141N/143H/273V/312S, 139L, 137A/139N/221Q/233L/413D, 233L, 221Q/279K, 137N/139N/233L/279M, 221Q, 139L/214V, 137N/139N/156V, 139L/214V/221Q, 214V/233L, 137A/139L/221Q/233L/279K, 137A/139L/221Q, 137N/139L, 137N/139L/279K, 137A/139N, 137N, 137N/139L/221Q, 137N/139L/214P/279M, 266Y, 139L/221Q, 137N/221Q, 137N/139N, 137N/156V/214V/312C, 137N/221Q/233L, 137N/139N/156V/221Q, 137A/139L/214V, 413D, 279K, 279M, 137A/139L/233L, 137N/139N/214V/233L, 221Q/413D, 137N/214V/233L, 137A/139L/214P, 137N/139N/221Q/233L/279M, 137N/156V, 137N/139L/221Q/233L, 214V/221Q, 137N/221Q/413D, 137A/139L/221Q/233L/279M, 214V, 137A/139L, 137N/233L, 137A, 137N/413D, 137N/221Q/279K, 137N/139N/156V/214V/233L/413D, or 137N/139L/156V/214V, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1368, or relative to the reference sequence corresponding to SEQ ID NO: 1368.

48. The engineered protease polypeptide of claim 28 or 29, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or to the reference sequence corresponding to SEQ ID NO: 1548, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

49. The engineered protease polypeptide of claim 28 or 29, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 1610-1710, or to a reference sequence corresponding to an even-numbered SEQ ID NO. of SEQ ID NOs: 1610-1710, wherein the amino acid sequence comprises one or more substitutions relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

50. The engineered protease polypeptide of claim 48 or 49, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) 145/273/372, 145/256/273, 221/243/273/328/372, 372, 145/221/279/372/406, 273/328, 145/169/273/346/406, 256/273, 145/221/273/328/346/406, 243/273, 145/214/256/273/279/328/372, 169/221/328/372/406, 372/406, 169/328/372/406, 221/372, 145/221/273/328/372, 214/243/273/328, 145/221, 214/256/273/346/372, 221/406, 169/372,

145/214/221/273, 145/221/346/372, 243/273/328/372/406, 145/169/273/328/346, 328, 169/273/372, 145/221/328, 169/214/273, 221/273/328, 221, 145/328, 214/346, 312, 212, 279, 212/312, 145, 179/346, 214, 346, 315/372, 375, 264, 179, 185, 220/372, or 324, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

51. The engineered protease polypeptide of any one of claims 48-50, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set 145E/273L/372S, 145E/256M/273L, 221Q/243L/273L/328V/372S, 372S, 145E/221Q/279L/372S/406R, 273L/328V, 145E/169S/273L/346S/406R, 256M/273L, 145E/221Q/273L/328V/346S/406R, 243L/273L, 145E/214V/256M/273L/279L/328V/372S, 169S/221Q/328V/372S/406R, 372S/406R, 169S/328V/372S/406R, 221Q/372S, 145E/221Q/273L/328V/372S, 214V/243L/273L/328V, 145E/221Q, 214V/256M/273L/346S/372S, 221Q/406R, 169S/372S, 145E/214V/221Q/273L, 145E/221Q/346S/372S, 243L/273L/328V/372S/406R, 145E/169S/273L/328V/346S, 328V, 169S/273L/372S, 145E/221Q/328V, 169S/214V/273L, 221Q/273L/328V, 221Q, 145E/328V, 214N/346S, 312R, 212S, 279K, 212S/312R, 145E, 179S/346S, 214N, 346S, 315R/372Y, 372Y, 375A, 264F, 179S, 185T, 220R/372Y, 324D, 145E/221Q/273L/328V/372S, or 145E/221Q/273L/328V/372S, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: 1548, or relative to the reference sequence corresponding to SEQ ID NO: 1548.

52. The engineered protease polypeptide of claim 1, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution at an amino acid position set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

53. The engineered protease polypeptide of claim 1, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

54. The engineered protease polypeptide of claim 1, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set at amino acid position(s) set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid

positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

55. The engineered protease polypeptide of claim 1, wherein the amino acid sequence of the engineered protease polypeptide comprises at least a substitution or substitution set of an engineered protease polypeptide variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

56. The engineered protease polypeptide of claim 1, comprising an amino acid sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference sequence corresponding to an amino acid sequence comprising at least a substitution or substitution set of an engineered protease polypeptide variant set forth in **Tables 3.1, 3.2, 4.1, 4.2, 5.1, 5.2, 6.1, 7.1, 8.1, and 9.1**, wherein the amino acid positions are relative to the reference sequence corresponding to residues 135-413 of SEQ ID NO: SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or relative to the reference sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

57. The engineered protease polypeptide of claim 1, comprising an amino acid sequence comprising residues 135-413 of an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, or an amino acid sequence comprising an even-numbered SEQ ID NO. of SEQ ID NOs: 6-1710, wherein optionally the amino acid sequence comprises 1, 2, 3, 4, 5, 6, 7, 8, 9 or up to 10 substitutions.

58. The engineered protease polypeptide of claim 1, comprising an amino acid sequence comprising residues 135-413 of SEQ ID NO: 628, 948, 1126, 1368, 1548, 1640, or 1710, or an amino acid sequence comprising SEQ ID NO: 628, 948, 1126, 1368, 1548, 1640, or 1710, wherein optionally the amino acid sequence comprises 1, 2, 3, 4, 5, 6, 7, 8, 9 or up to 10 substitutions.

59. The engineered protease polypeptide of any one of claims 1-58, wherein the amino acid sequence of the engineered protease polypeptide comprises amino acid residues 135-413 or amino acid residues 128-413, wherein the engineered protease polypeptide is proteolytically active or is an active protease.

60. The engineered protease polypeptide of any one of claims 1-58, wherein the engineered protease polypeptide is capable of converting to a proteolytically active polypeptide or is an active protease.

61. The engineered protease polypeptide of claim 59 or 60, wherein the proteolytically active polypeptide or active protease has increased protease activity as compared to a reference protease.

62. The engineered protease polypeptide of claim 59 or 60, wherein the proteolytically active polypeptide or active protease has increased resistance to a gastric protease as compared to a reference protease.

63. The engineered protease polypeptide of claim 59 or 60, wherein the proteolytically active polypeptide or active protease has increased stability and/or activity at acidic pH or neutral pH as compared to a reference protease.

64. The engineered protease polypeptide of claim 59 or 60, wherein the proteolytically active polypeptide or active protease has increased thermostability as compared to a reference protease.

65. The engineered protease polypeptide of claim 59 or 60, wherein the proteolytic active polypeptide or active protease is characterized by an improved property selected from: i) increased protease activity, ii) increased resistance to pepsin, iii) increased stability and/or activity at acidic pH, iv) increased stability and/or activity at neutral pH, or v) increased thermostability, or any combination of i), ii), iii), iv), and v) as compared to a reference protease.

66. The engineered protease polypeptide of any one of claims 61-65, wherein the reference protease has an amino acid sequence corresponding to residues 135-413 of SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548, or an amino acid sequence corresponding to SEQ ID NO: 4, 628, 948, 1126, 1368, or 1548.

67. The engineered protease polypeptide of claim 66, wherein the reference protease has an amino acid sequence corresponding to residues 135-413 of SEQ ID NO: 4 or 628, or an amino acid sequence corresponding to SEQ ID NO: 4 or 628.

68. The engineered protease polypeptide of any one of claims 1-67, further comprising a Big1 domain at or fused to the carboxy terminus of the engineered protease polypeptide.

69. The engineered protease polypeptide of claim 68, wherein the Big1 domain comprises amino acid residues 426 to 522 of SEQ ID NO: 2.

70. The engineered protease polypeptide of any one of claims 1-69, further comprising a heterologous fusion polypeptide.

71. The engineered protease polypeptide of claim 70, wherein the fusion polypeptide comprises a His-tag.

72. The engineered protease polypeptide of any one of claims 1-71, further comprising a signal sequence.

73. The engineered protease polypeptide of claim 72, wherein the signal sequence comprises a bacterial, fungal, or mammalian signal sequence.

74. An engineered protease polypeptide comprising at least a carboxy terminal deletion of SEQ ID NO: 2, wherein the deletion maintains protease activity of the mature form of SEQ ID NO: 2 with the carboxy terminal deletion.

75. The engineered protease polypeptide of claim 74, wherein the carboxy terminal deletion comprises deletion of the Big1 domain.

76. The engineered protease polypeptide of claim 75, wherein the carboxy terminal deletion is up to and including amino acid residue 426, or up to and including amino acid residue 414 of SEQ ID NO: 2.

77. The engineered protease polypeptide of claim 76, further comprising 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 amino acid deletions of the carboxy terminus at amino acid residue 413 of SEQ ID NO: 2, wherein the further amino acid deletion(s) maintains proteolytic activity of the mature form of SEQ ID NO: 2 having the further amino acid deletions.

78. The engineered protease polypeptide of any one of claims 74-77, wherein the mature form has an amino terminus at amino acid residue 128 or 135 of SEQ ID NO: 2.

79. The engineered protease polypeptide of any one of claims 1-78, wherein the engineered protease polypeptide is a purified preparation.

80. A recombinant polynucleotide comprising a polynucleotide sequence encoding an engineered protease polypeptide of any one of claims 1-78.

81. The recombinant polynucleotide of claim 80, comprising a polynucleotide sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 403 to 1239 of SEQ ID NO: 3, 627, 947, 1125, 1367, or 1547, or to a reference polynucleotide corresponding to SEQ ID NO: 3, 627, 947, 1125, 1367, or 1547, wherein the recombinant polynucleotide encodes an engineered protease polypeptide.

82. The recombinant polynucleotide of claim 80, comprising a polynucleotide sequence having at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or more sequence identity to a reference polynucleotide sequence corresponding to nucleotide residues 403 to 1239 of an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-1709, or to a reference polynucleotide corresponding to an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-1709, wherein the recombinant polynucleotide encodes an engineered protease polypeptide.

83. The recombinant polynucleotide of any one of claims 80-82, wherein the polynucleotide sequence is codon optimized.

84. The recombinant polynucleotide of claim 80, comprising a polynucleotide sequence comprising residues 403-1239 of an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-1709, or comprising an odd-numbered SEQ ID NO. of SEQ ID NOs: 5-1709.

85. The recombinant polynucleotide of claim 80, comprising a polynucleotide sequence comprising residues 403-1239 of SEQ ID NO: 627, 947, 1125, 1367, 1547, 1639, or 1709, or a polynucleotide sequence comprising SEQ ID NO: 627, 947, 1125, 1367, 1547, 1639, or 1709.

86. An expression vector comprising a recombinant polynucleotide of any one of claims 80-85.

87. The expression vector of claim 86, further comprising a control sequence operably linked to the recombinant polynucleotide.

88. The expression vector of claim 87, wherein the control sequence comprises at least a promoter.

89. A host cell comprising an expression vector of any one of claims 86-88.

90. The host cell of claim 89, wherein the host cell is a bacterial cell, fungal cell, or mammalian cell.

91. A method of producing an engineered protease polypeptide, comprising culturing a host cell of any one of claims 89-90 under suitable conditions such that the encoded engineered protease is expressed or produced.

92. The method of claim 91, further comprising isolating the expressed or produced engineered protease polypeptide from culture medium and/or cells.

93. The method of claim 91 or 92, further comprising purifying the expressed or produced engineered protease polypeptide.

94. A method of preparing a proteolytically active protease polypeptide comprising incubating an engineered protease polypeptide of any one of claims 1-79 under suitable conditions such that the proteolytically active protease polypeptide or active protease is produced.

95. The method of claim 94, wherein the proteolytically active protease polypeptide has an amino terminus at amino acid residue 128 or 135, wherein the amino acid positions are numbered with respect to SEQ ID NO: 4.

96. The method of claim 94 or 95, wherein suitable conditions is sufficient for activation by autoproteolysis of the engineered protease polypeptide.

97. The method of claim 94 or 95, wherein the engineered protease polypeptide is contacted with a proteolytically active polypeptide or an active protease of any one of claims 1-79.

98. A pharmaceutical composition comprising an engineered protease polypeptide of any one of claims 1-76.

99. The pharmaceutical composition of claim 98, further comprising a pharmaceutically acceptable excipient and/or carrier.

100. The pharmaceutical composition of claim 98 or 99, further comprising an amylase.

101. The pharmaceutical composition of any one of claims 98-100 further comprising a lipase.

102. The pharmaceutical composition of any one of claims 98-101, comprising an effective amount of the engineered protease for treating exocrine pancreatic insufficiency.

103. A composition comprising an engineered protease polypeptide of any one of claims 1-79.

104. The composition of claim 103, wherein the engineered protease polypeptide is admixed with protein-containing food or drink.

105. A method of treating a disease or condition associated with a deficiency in pancreatic enzymes, the method comprising administering to a subject in need thereof an effective amount of an engineered protease polypeptide of any one of claims 1-79 or a pharmaceutical composition of any one of claims 96-100.

106. The method of claim 105, wherein the disease or condition is exocrine pancreatic insufficiency.

107. The method of claim 105 or 106, wherein the engineered protease polypeptide or pharmaceutical composition thereof is administered immediately prior to, concurrently with, or subsequent to consumption of a protein-containing food or drink.

108. The method of any one of claims 105-107, wherein the subject is a human infant or child.

109. The method of any one of claims 105-107, wherein the subject is a human adult.

110. Use of an engineered protease polypeptide of any one of claims 1-79 for treating exocrine pancreatic insufficiency.

111. Use of an engineered protease polypeptide of any one of claims 1-79 in the preparation of a medicament for treating exocrine pancreatic insufficiency.

MADQASQANG	NQDVIEGELI	VSVEPKNGKP	DVSIQSAQSV	TGQEDVLKSK	GFTIVDTLHV
EDSSVKPLVS	KNFNKKVVEK	MGLVYLVQYS	PDQFKSSNIA	KKELRKELMD	LGFKVRYISE
NYKMMALIEGT	ATLQAVHPNQ	NWHYNMIKAP	EAWAITSGSS	SVKVAVLDTG	IDHNHPSLAN
FVNTNLGRSF	VGGNTMDVQG	HGTHVAGTIA	SYGSVSGVMQ	SATLIPVKVL	DNSGSGSLYG
ITQGILYAAD	IDADVINMSL	GGGGYNQSMQ	EAVQTAVAQG	TIVVAATGND	GASSISYPAA
YSSVIAVGSV	TSNRTRSSFS	NYGSGLEVMA	PGSNIYSTYP	NSRYTSLSGT	SMATPHVAGV
AGLIRAANPD	ISVAQARQIL	RDTAQEAGSF	TQYGYGIVDA	HAAVVAASGG	SGGGTTPPPP
SSTDTVTTVS	TNYSYYRGE	TVYMTSTVKD	SNGVAISNAT	VNFKITRPNG	TSVTATAATN
SSGVATYSIG	TNYSTATGTY	RVDATASKSG	YTTSSASTTF	RVRGQTGHHH	HHH

FIG. 1

MADQASQANG	NQDVIEGELI	VSVEPKNGKP	DVSIQSAQSV	TGQEDVLKSK	GFTIVDTLHV
EDSSVKPLVS	KNFNKKVVEK	MGLVYLVQYS	PDQFKSSNIA	KKELRKELMD	LGFKVRYISE
NYKMMALIEGT	ATLQGVHPNQ	VWAYNMIKAP	EAWAITSGSL	SVKVAVLDTG	IDHNHPSLAN
FVNTNLGRSF	VGGNTMDVQG	HGTHVAGTIA	SYGSVSGVMQ	SATLIPVKVL	DNSGSGSLYG
ITQGILYAAD	IDADVINMSL	GGGGYNQSMQ	EAVQTAVAQG	TIVVAATGND	GASSISYPAA
YSSVIAVGSV	DSNRVRSSFS	NYGSGLELMA	PGSNIYSTYP	NGRYRSLSGT	SMATPHVAGV
AGLIRAANPD	IVVAQARQIL	RDTAQEAGSF	TQYGYGIVDA	HAAVVAASGG	SGG

FIG. 2