



(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION

(13) **A1**

(86) **Date de dépôt PCT/PCT Filing Date:** 2022/03/25
(87) **Date publication PCT/PCT Publication Date:** 2022/09/29
(85) **Entrée phase nationale/National Entry:** 2023/09/21
(86) **N° demande PCT/PCT Application No.:** US 2022/022053
(87) **N° publication PCT/PCT Publication No.:** 2022/204576
(30) **Priorité/Priority:** 2021/03/25 (US63/166,188)

(51) **Cl.Int./Int.Cl.** **C12N 1/14** (2006.01),
C12N 15/57 (2006.01), **C12N 9/50** (2006.01),
C12N 9/62 (2006.01)
(71) **Demandeur/Applicant:**
BIO-CAT, INC., US
(72) **Inventeurs/Inventors:**
GARVEY, SEAN MICHAEL, US;
TINKER, KELLY MARIE, US;
GUICE, JUSTIN LAMONT, US;
LITTLE JR., ROBERT DANIEL, US;
SCHULER, CHRISTOPHER EDWARD, US
(74) **Agent:** SMART & BIGGAR LP

(54) **Titre : MELANGES DE PROTEASES FONGIQUES ET LEURS UTILISATIONS**
(54) **Title: FUNGAL PROTEASE MIXTURES AND USES THEREOF**

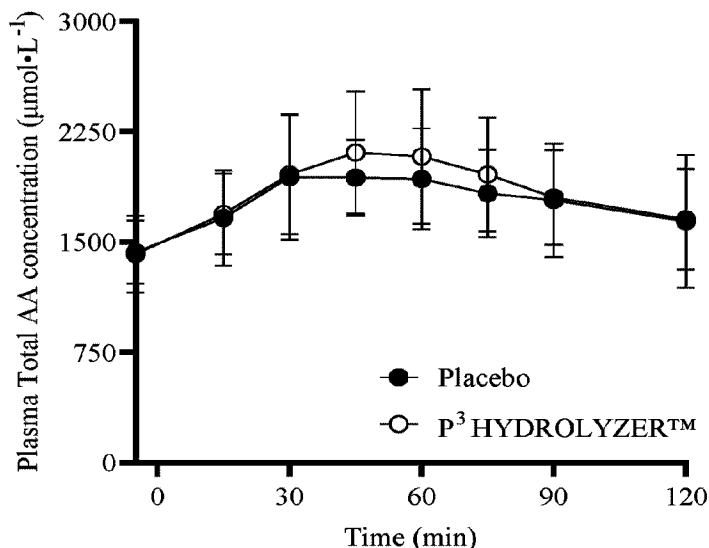


FIG. 17D

(57) **Abrégé/Abstract:**

Fungal protease compositions, and more particularly, proteolytic enzyme mixtures comprising a plurality of *Aspergillus* proteases are provided. The disclosure also relates to protein hydrolysates, food and beverage products and dietary supplements produced using these proteolytic enzyme mixtures, and methods of making and using the same.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property

Organization

International Bureau

(43) International Publication Date

29 September 2022 (29.09.2022)



(10) International Publication Number

WO 2022/204576 A1

(51) International Patent Classification:

C12N 1/14 (2006.01)

C12N 9/62 (2006.01)

C12N 9/50 (2006.01)

C12N 15/57 (2006.01)

(21) International Application Number:

PCT/US2022/022053

(22) International Filing Date:

25 March 2022 (25.03.2022)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/166,188

25 March 2021 (25.03.2021)

US

(71) Applicant: **BIO-CAT, INC.** [US/US]; 9117 Three Notch Road, Troy, VA 22974 (US).

(72) Inventors: **GARVEY, Sean, Michael**; 80 Stonegate Terrace, Apt. 301, Zion Crossroads, VA 22942 (US). **TINKER, Kelly, Marie**; P.O. Box 863, Columbia, VA 23038 (US). **GUICE, Justin, Lamont**; 2940 Finsbury Pl, Fairfax, VA 22031 (US). **LITTLE JR., Robert, Daniel**; 2108 Timber Pointe Rd, Charlottesville, VA 22911 (US). **SCHULER, Christopher, Edward**; 3026 Newcomb Mountain Ln, Charlottesville, VA 22903 (US).

(74) Agent: **SPIEGLER, Alexander, H.** et al.; 1717 K. Street, N.W., Washington, DC 20006 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: FUNGAL PROTEASE MIXTURES AND USES THEREOF

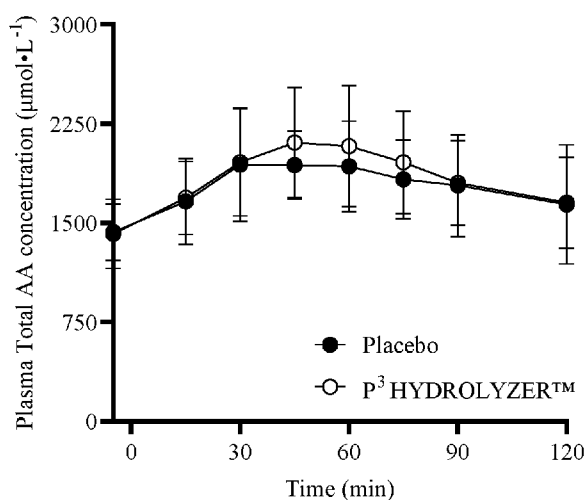


FIG. 17D

(57) Abstract: Fungal protease compositions, and more particularly, proteolytic enzyme mixtures comprising a plurality of *Aspergillus* proteases are provided. The disclosure also relates to protein hydrolysates, food and beverage products and dietary supplements produced using these proteolytic enzyme mixtures, and methods of making and using the same.

[Continued on next page]



WO 2022/204576 A1

WO 2022/204576 A1

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

FUNGAL PROTEASE MIXTURES AND USES THEREOF

Cross-Reference to Related Applications

[0001] This application claims benefit of U.S. Provisional Patent Application No. 63/166,188, filed March 25, 2021, which is incorporated by reference herein in its entirety.

Technical Field

[0002] Novel fungal protease compositions, and more particularly, proteolytic enzyme mixtures comprising a plurality of *Aspergillus* proteases are provided. The disclosure further relates to dietary supplements, foods, and beverage products containing these proteolytic enzyme mixtures or hydrolysates produced using these proteolytic enzyme mixtures, and methods of making and using the same.

Background

[0003] Protein is an essential dietary macronutrient that provides humans and animals with the amino acid building blocks for cells to synthesize hundreds of thousands of proteins. Intracellular proteins such as the actins and myosins in skeletal muscle support healthy cellular function and organismal physiology. Proteins are long chains of amino acids (or “AAs”) that are connected to each other by peptide bonds. At least 20 different amino acids can be encoded by a gene to direct the synthesis of a protein. Of these amino acids, 11 are non-essential amino acids that can be synthesized by human cells. The remaining 9 essential amino acids (or “EAAs”) are not made by human cells and must be supplied from the diet. EAAs include leucine, isoleucine, valine, histidine, lysine, methionine, phenylalanine, threonine, tryptophan. Of these 9 EAAs, leucine, isoleucine, and valine are also branched chain amino acids (or “BCAAs”), which are especially associated with skeletal muscle maintenance and growth. Dietary protein is hydrolyzed in the human gastrointestinal tract to produce peptides and free amino acids that are absorbed in to circulation and distributed to all cells of the body. Protein hydrolysis in the stomach is mediated by both hydrochloric acid and an endogenous protease

called pepsin. Proteases are generally characterized as exopeptidases or endopeptidases depending on whether they cleave peptide bonds of the terminal amino acid or between internal amino acids of a peptide. Proteases may also be specific to a particular amino acid (or sequence of amino acids) on the substrate protein (or peptide) or nonspecific. Proteases are proteolytic enzymes that hydrolyze the peptide bonds between amino acids to release shorter peptides and free amino acids. In addition to gastric pepsin, the pancreas and small intestine also generate proteolytic enzymes that contribute to protein digestion and amino acid liberation.

[0004] Exogenous, oral enzyme supplementation is a candidate approach to optimize protein digestion and absorption of amino acids, in particular EAAs and BCAAs. Proteolytic enzymes accelerate the conversion of protein and peptides to amino acids. Dietary supplements containing proteolytic enzyme mixtures have been marketed to promote amino acid and BCAA liberation from dietary protein. Proteolytic enzyme mixtures known in the art include Aminogen[®], a proteolytic enzyme preparation derived from *A. oryzae* and *A. niger* which comprises at least 2 proteolytic components as described in Oben, J., et al., “An Open Label Study to Determine the Effects of an Oral Proteolytic Enzyme System on Whey Protein Concentrate Metabolism in Healthy Males.” *Journal of the International Society of Sports Nutrition*. 2008. 5, 10, the entire contents of which is incorporated herein by reference. Proteolytic enzyme mixtures known in the art also include ProHydrolase[®], a proteolytic enzyme preparation derived from *Bacillus subtilis* and *Ananas comosus* stem (i.e., pineapple) which comprises at least 2 proteolytic components as described in International Patent Application No. PCT/US2013/026657 (WO2014130007), and in Townsend, J. R., et al., “The Effect of ProHydrolase[®] on the Amino Acid and Intramuscular Anabolic Signaling Response to Resistance Exercise in Trained Males.” *Sports (Basel)*. 2020. 8(2), 13, the entire contents of each of which are incorporated herein by reference.

[0005] The proper selection of an enzyme (or enzymes in a mixture) for oral dietary supplement use is important because proteolytic activity is affected by pH. Generally, the human stomach shows a pH value between 1.5 and 5.0, and the small intestine shows a pH value between 6.0 and 8.0. A proteolytic enzyme mixture may preferably contain enzymes that are optimally active across the entire pH gradient from stomach to small intestine. A proteolytic enzyme mixture may also preferably contain a balance of exopeptidase and endopeptidase activities.

[0006] Raw protein sources may be hydrolyzed to produce peptides and free amino acids using naturally occurring or recombinant proteolytic enzymes or by chemical decomposition. This hydrolysate may then be used for various purposes, e.g., as a seasoning for improved taste, food additive or dietary supplement for nutrition, or as a precursor or component of another protein-related product. Enzymatic digestion may proceed using a single proteolytic enzyme (e.g., a single, nonspecific exopeptidase that may gradually digest a given polypeptide or oligopeptide). Alternatively, digestion may involve the use of a mixture of proteases that display different proteolytic activity profiles (i.e., exopeptidase, endopeptidase, and combinations thereof). Proteolytic enzyme mixtures known in the art include Flavourzyme[®], a proteolytic enzyme preparation derived from *A. oryzae* which comprises at least 5 proteolytic components as described in International Patent Application No. PCT/DK1994/000165 (WO1994/025580), and in Merz, M., et al., “Flavourzyme, an Enzyme Preparation with Industrial Relevance: Automated Nine-step Purification and Partial Characterization of Eight Enzymes.” *Journal of Agricultural and Food Chemistry*. 2015. 63(23), 5682-5693, the contents of each of which is incorporated herein by reference. The proper selection of an enzyme (or enzymes in a mixture) for production of a hydrolysate is important because the characteristics and properties of the hydrolysate will vary depending on the type and degree of proteolysis. For example, incomplete digestion may generate a hydrolysate enriched in oligopeptides or

free amino acids which create a bitter taste or chalky mouthfeel, resulting in a product unsuitable for certain purposes (e.g., an additive for food products). Other properties of proteolytic enzymes, such as stability, efficiency, cost, and compatibility with other common solvents and reagents are also relevant to the selection of a proteolytic enzyme or mixture of enzymes for hydrolysate production. These limitations constrain the commercial or industrial use of particular enzymes and combinations thereof.

Summary of Various Embodiments

[0007] In a general aspect, the present disclosure relates to combinations of proteases obtained from members of the genus *Aspergillus*, such as *A. oryzae* or *A. melleus*, which are capable of digesting protein from various sources. In some exemplary aspects, the proteolytic enzyme mixtures described herein may be used as ingredients in dietary supplements, protein powders, or foods to promote protein digestion, to promote post-prandial plasma amino acid levels, or both, following consumption of a dietary supplement, food, beverage, or meal. In some exemplary aspects, the proteolytic enzyme mixture described herein may be used to produce a hydrolysate containing free EAAs and free BCAAs. In some exemplary aspects, the proteolytic enzyme mixtures described herein may be used to produce a hydrolysate that is more easily digested, more easily absorbed, or both, by the gastrointestinal system of a human or animal. In some exemplary aspects, the proteolytic enzyme mixtures described herein may be also used to produce a hydrolysate that has improved flavor and/or mouthfeel compared to a hydrolysate prepared using currently available enzymes that often produce bitter and/or chalky hydrolysates. Moreover, the mixtures of enzymes described herein are stable and maintain activity over a broad range of temperatures and pH levels, providing additional options for commercial and industrial applications.

[0008] In some aspects, the proteolytic enzyme mixtures comprise 1) a Fungal Protease A (a 42 kDa protease with exo- and endo-protease activity obtained from *A. oryzae*; CAS No.

9025-49-4; IUBMB Enzyme Commission (EC) No. 3.4.23.18), 2) Fungal Protease AM (a 34 kDa protease with peptidase activity obtained from *A. melleus*; CAS No. 9074-07-1; EC No. 3.4.11.-), and 3) a Fungal Protease A2 (a 35 kDa fungal neutral protease obtained from *A. oryzae*; CAS No. 9025-49-4; EC No. 3.4.24.-), when analyzed by polyacrylamide gel electrophoresis. In other aspects, the proteolytic enzyme mixture comprises SEQ ID NOs: 1, 2, and 3.

[0009] In other general aspects, the proteolytic enzyme mixture may be used as an ingredient in protein supplements and oral nutritional supplements, wherein the proteolytic enzyme mixture is prepared as a powder and may be dry-blended with protein in a manufacturing process that yields a protein or oral nutritional supplement.

[0010] In other general aspects, the disclosure provides methods of administering a dietary supplement comprising the disclosed proteolytic enzyme mixtures to increase amino acids, EAA and/or BCAA absorption following consumption of a dietary supplement, beverage, food, or meal.

[0011] In other general aspects, the disclosure provides methods of preparing a protein hydrolysate from various protein sources using the disclosed proteolytic enzyme mixtures, and in particular, protein hydrolysates containing amino acids, EAAs, and/or BCAAs.

[0012] In still other general aspects, food products, additives, dietary supplements and beverages comprising the protein hydrolysates described herein are provided.

[0013] Methods of using the disclosed dietary supplements containing the proteolytic enzyme mixture or protein hydrolysates are also provided, including methods of increasing exercise performance, decreasing muscle breakdown during exercise, improving recovery from exercise, or combinations thereof, by administering a dietary supplement containing the proteolytic enzyme mixture or protein hydrolysate prepared described herein, alone or as part of a food product, dietary supplement, or beverage.

[0014] Additional aspects will be readily apparent to one of skill in light of the totality of the disclosure.

Brief Description of the Drawings

[0015] **FIG. 1A** is a graph illustrating the relative activity of Fungal Protease A across various pH levels.

[0016] **FIG. 1B** is a graph illustrating the relative activity of Fungal Protease A across various temperature levels.

[0017] **FIG. 2A** is a graph illustrating the relative activity of Fungal Protease AM across various pH levels.

[0018] **FIG. 2B** is a graph illustrating the relative activity of Fungal Protease AM across various temperature levels.

[0019] **FIG. 3A** is a graph illustrating the relative activity of Fungal Protease A2 across various pH levels.

[0020] **FIG. 3B** is a graph illustrating the relative activity of Fungal Protease A2 across various temperature levels.

[0021] **FIG. 3C** is a graph illustrating the residual activity of Fungal Protease A2 across various pH levels.

[0022] **FIG. 3D** is a graph illustrating the residual activity of Fungal Protease A2 across various temperature levels.

[0023] **FIG. 4** is a graph illustrating the relative activity of **OPTIZIOMETM** P³ HYDROLYZERTM (also referred to as P³ HYDROLYZERTM), Aminogen[®], and ProHydrolase[®], across a range of pH levels.

[0024] **FIG. 5A** is a graph illustrating the relative activity of **OPTIZIOMETM** P³ HYDROLYZERTM, across a range of pH levels.

[0025] **FIG. 5B** is a graph illustrating the relative activity of **OPTIZIOME™ P³ HYDROLYZER™** across various temperature levels.

[0026] **FIG. 6** is a bar chart that illustrates a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER™ based on the release of 20 different amino acids following simulated gastric digestion of whey protein for 60 minutes.

[0027] **FIG. 7** is a bar chart that illustrates a comparative analysis of the proteolytic activities of ProHydrolase[®] (60 minutes), P³ HYDROLYZER™ (15 minutes), and Aminogen[®] (60 minutes) based on the release of 20 different amino acids following simulated gastric digestion of whey protein.

[0028] **FIG. 8** is a bar chart that illustrates a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER™, based on the release of 3 different BCAAs following simulated gastric digestion of whey, soy, pea, and rice proteins for 60 minutes.

[0029] **FIGs. 9A-9D** are charts illustrating a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER™, based on the release of total amino acids (Fig. 9A), EAAs (Fig. 9B), BCAAs (Fig. 9C), and leucine (Fig. 9D) following simulated gastric digestion of whey protein for 60 minutes.

[0030] **FIGs. 10A-10D** are charts illustrating a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER™, based on the release of total amino acids (Fig. 10A), EAAs (Fig. 10B), BCAAs (Fig. 10C), and leucine (Fig. 10D) following simulated gastric digestion of soy protein for 60 minutes.

[0031] **FIGs. 11A-11D** are charts illustrating a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER™, based on the release of total amino acids (Fig. 11A), EAAs (Fig. 11B), BCAAs (Fig. 11C), and leucine (Fig. 11D) following simulated gastric digestion of pea protein for 60 minutes.

[0032] **FIGs. 12A-12D** are charts illustrating a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER[™], based on the release of total amino acids (Fig. 12A), EAAs (Fig. 12B), BCAAs (Fig. 12C), and leucine (Fig. 12D) following simulated gastric digestion of rice protein for 60 minutes.

[0033] **FIGs 13A-D** are charts illustrating a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER[™], showing differences in the concentrations of leucine (Fig. 13A), BCAAs (Fig. 13B), EAAs (Fig. 13C), and total amino acids (Fig. 13D) produced from whey protein following simulated salivary-gastric digestion 122 minutes per the INFOGEST protocol. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

[0034] **FIGs 14A-D** are charts illustrating a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER[™], showing differences in the concentrations of leucine (Fig. 14A), BCAAs (Fig. 14B), EAAs (Fig. 14C), and total amino acids (Fig. 14D) produced from pea protein following simulated salivary-gastric digestion for 122 minutes per the INFOGEST protocol. ****, $p < 0.0001$.

[0035] **FIGs 15A-D** are charts illustrating a comparative analysis of the proteolytic activities of Aminogen[®], ProHydrolase[®], and P³ HYDROLYZER[™], showing differences in the concentrations of leucine (Fig. 15A), BCAAs (Fig. 15B), EAAs (Fig. 15C), and total amino acids (Fig. 15D) produced from soy protein following simulated salivary-gastric digestion for 122 minutes per the INFOGEST protocol. ***, $p < 0.001$; ****, $p < 0.0001$.

[0036] **FIG. 16** is a graph illustrating the results of a flavor preference test comparing assessors' preference for whey protein shakes treated with either P³ HYDROLYZER[™] or ProHydrolase[®].

[0037] **FIG. 17A-D** are graphs illustrating the plasma concentrations of leucine (Fig. 17A), BCAAs (Fig. 17B), EAAs (Fig. 17C), and total amino acids (Fig. 17D) across the first 2 hours

of a clinical aminoacidemia trial before and following consumption of a pea protein shake with placebo or P³ HYDROLYZER™. Each circle represents a blood draw for amino acid analysis. Black circle, placebo; white circle, P³ HYDROLYZER™ (n = 24 subjects, cross-over design).

[0038] FIG. 18A-B are charts illustrating the total area under the curve (AUC) plasma concentrations of EAAs (Fig. 18A) and total amino acids (Fig. 18B) across the first 2 hours of a clinical aminoacidemia trial before and following consumption of a pea protein shake with placebo or P³ HYDROLYZER™ (n = 24 subjects, cross-over design).

Detailed Description of Various Embodiments

[0039] The present disclosure relates to proteolytic enzyme mixtures comprising a plurality of fungal proteases obtained from members of the genus *Aspergillus* (e.g., from *A. oryzae* and *A. melleus*). These proteolytic enzyme mixtures may be administered to a subject as a dietary supplement (e.g., to improve protein digestion or the absorption of amino acids, EAAs, and/or BCAAs). They may also be used to produce a protein hydrolysate containing amino acids, EAAs and/or BCAAs, which may also possess additional beneficial properties (e.g., less bitterness, improved flavor and/or mouthfeel) compared to protein hydrolysate produced using currently available proteases and proteolytic enzyme mixtures. Additionally, methods of using these proteolytic enzyme mixtures, and various products (e.g., foods, beverages, dietary supplements) and other vehicles for administering the proteolytic enzyme mixtures or resulting protein hydrolysate are also provided. Methods of producing protein hydrolysates enriched with amino acids, EAAs, and/or BCAAs from a single protein source (e.g., without the need for supplementation with additional amino acids, EAAs and/or BCAAs from another source) are also provided.

[0040] Proteins are high molecular weight polymers composed of multiple amino acids linked by peptide bonds. These bonds must be cleaved in order for protein to be absorbed and utilized by a human or other organism, with such cleavage typically being performed by

endogenous proteolytic enzymes of the gastrointestinal tract that separate the polypeptides into its constituent free amino acids. Amino acids may be classified as essential or non-essential for any given organism, depending on whether an organism is capable of synthesizing the given amino acid. For a dietary regimen to be considered adequate for the support of normal physiological functions, it should contain all essential amino acids in the appropriate levels and in proper proportions. For humans, the nine essential amino acids are leucine, isoleucine, valine, methionine, tryptophan, phenylalanine, threonine, lysine and histidine.

[0041] Three of the essential amino acids (valine, leucine and isoleucine) have aliphatic side-chains with a branch, i.e., a central carbon atom bound to three or more carbon atoms. These BCAAs are particularly notable because these amino acids are an important nutritional factor for proper muscle physiology and metabolism. Reports further indicate that athletic and exercise performance and recovery may be improved by BCAA supplements (*See e.g., Glynn, E. L., et al., "Excess Leucine Intake Enhances Muscle Anabolic Signaling but Not Net Protein Anabolism in Young Men and Women." The Journal of Nutrition. 2010. 140(11), 1970-1976; Sharp, C. P. M., et al., "Amino Acid Supplements and Recovery from High-Intensity Resistance Training." Journal of Strength and Conditioning Research. 2010. 24(4), 1125-1130; Fouré, A. & Bendahan, D. Is Branched-chain Amino Acids Supplementation an Efficient Nutritional Strategy to Alleviate Skeletal Muscle Damage? A Systematic Review. Nutrients. 2017. 9(10), 1047). In older adults, who are at risk of severe muscle wasting, BCAA supplementation in combination with exercise has also been shown to enhance performance and muscle strength (*See e.g., Ikeda, T., et al. Effects and Feasibility of Exercise Therapy Combined with Branched-chain Amino Acid Supplementation on Muscle Strengthening in Frail and Pre-Frail Elderly People Requiring Long-term Care: A Crossover Trial. Applied Physiology, Nutrition, and Metabolism. 2016. 41(4), 438-445).* The remaining non-essential*

amino acids provide a source of metabolizable nitrogen required for the biosynthesis of proteins, purines, nucleic acids, and other metabolites.

[0042] In view of the above, there has been commercial interest in dietary supplements and food additives that contain EAAs and BCAAs (e.g., protein powders and energy drinks directed to athletes). These supplements are typically prepared by fermentation of a raw protein source (e.g., whey protein), or by digestion of a raw protein source using a proteolytic enzyme or combination of enzymes and then supplementing the end product with BCAAs or other EAAs obtained from a second process or source. The manufacturing of such products is therefore complicated by the fact that amino acids must typically be obtained from multiple sources and mixed together to obtain a product which has the desired profile and ratios (e.g., enriched in BCAAs and/or EAAs).

[0043] The present disclosure provides proteolytic enzyme mixtures that simplify this process by generating protein hydrolysates already enriched in EAAs, and more particularly BCAAs. Use of these proteolytic enzyme mixtures reduces the complexity and manufacturing costs associated with having to obtain amino acids from different sources. Moreover, protein hydrolysates produced using these proteolytic enzyme mixtures have been found to have an improved taste, texture (e.g., mouthfeel) and solubility profiles compared to hydrolysates produced using known proteolytic enzymes and combinations thereof.

[0044] Protein hydrolysates produced using the present methods are therefore well-suited for use in commercial food products, dietary supplements, additives, and beverages. These food products and other vehicles may in turn be used by consumers, and athletes in particular, to provide nutrition, as well as athletic and/or exercise benefits.

Proteolytic Enzyme Mixtures Comprising A Plurality Of *Aspergillus* Proteases

[0045] In one general aspect, the present disclosure provides a proteolytic enzyme mixture comprising a plurality of fungal proteases obtained from members of the genus *Aspergillus*

(e.g., a combination of at least three proteases obtained from *A. oryzae* or *A. melleus*). One or more of these enzymes may possess exopeptidase, endopeptidase and/or peptidase activity, alone or operating in combination with other enzymes in the mixture. In some exemplary aspects, the mixture has proteolytic activity across a pH range spanning from 3.0 to 9.0, or any range of integer values therein. In some embodiments, the relative activity of the proteolytic enzyme mixture will be > 40% across a pH range of 4.0 to 9.0, > 60% across a pH range of 5.0 to 9.0, > 80% across a pH range of 5.7 to 6.3, and/or > 90% across a pH range of 5.8 to 6.2, measured at 60°C. The relative activity may also be > 40% across a temperature range of 20 to 80 °C, > 60% across a temperature range of 40 to 80 °C, > 80% across a temperature range of 55 to 70 °C, and/or > 90% across a temperature range of 56 to 63 °C, measured at pH 6.0. The proteolytic enzyme mixture may be capable of digesting a raw protein source (e.g., plant protein, whey protein) and producing a protein hydrolysate enriched in EAAs and/or BCAAs when applied to a food product or dietary supplement comprising a protein (e.g., a protein shake containing whey isolate as the protein) at a minimum of 1,000 to 150,000 hemoglobin unit tyrosine base (HUT) units per gram of the protein. As used herein, references to HUT units of the enzymes and enzyme mixtures described herein are calculated using the standard protocol labeled “PROTEOLYTIC ACTIVITY, FUNGAL (HUT)” in the Food Chemicals Codex (FCC), 12. Ed. (published Mar. 1, 2020), the contents of which is hereby incorporated by reference in its entirety. Briefly, one HUT unit (HUT/g) is defined as the amount of enzyme that produces a hydrolysate from bovine hemoglobin at pH 4.7 whose absorbance at 275 nanometers is the same as that of a solution containing 1.10 µg/mL of tyrosine in 0.006 N hydrochloric acid. Increased HUT correlates with increased proteolytic activity of a protease or proteolytic mixture.

[0046] In some aspects, the proteolytic enzyme mixture may alternatively be applied to a food product or dietary supplement at a minimum of 1,000 to 150,000 HUT units per gram of

total protein in the composition (e.g., at a minimum of 10,000; 20,000; 30,000; 40,000; 50,000; 60,000; 70,000; 80,000; 90,000; 100,000; 110,000; 120,000; 130,000; 140,000; or 150,000 HUT/g, or alternatively within a range bounded by any of these values). In some exemplary aspects, the proteolytic enzyme mixture comprises a three-enzyme blend and may produce a hydrolysate with amino acid, EAA, and/or BCAA enrichment at a concentration several-fold larger than the amino acid, EAA, and/or BCAA concentrations of a hydrolysate prepared by digestion with only one or two of the three enzymes in the proteolytic enzyme mixture. As such, the proteolytic enzyme mixture may be used to improve the absorption of amino acids (e.g., EAAs, BCAAs) by a human or animal that consumes a treated food product or dietary supplement comprising the proteolytic enzyme mixture, by increasing the amount of free amino acids, EAAs and/or BCAAs released during digestion.

[0047] For example, in some aspects, the proteolytic enzyme mixture may comprise “**OPTIZIOME™ P³ HYDROLYZER™**” (also referred to as “P³” or “P³ HYDROLYZER™” herein), a mixture of three fungal proteases distributed by BIO-CAT, Inc.: 1) Fungal Protease A (a 42 kDa protease with exo- and endo-protease activity obtained from *A. oryzae*; CAS No. 9025-49-4; IUBMB Enzyme Commission (EC) No. 3.4.23.18), present at 25,000 HUT units/g, 2) Fungal Protease AM (a 34 kDa protease with peptidase activity obtained from *A. melleus*; CAS No. 9074-07-1; EC No. 3.4.11.-), present at 2,500 HUT units per gram, and 3) Fungal Protease A2 (a 35 kDa fungal neutral protease obtained from *A. oryzae*; CAS No. 9025-49-4; EC No. 3.4.24.-), present at 100,000 HUT units per gram. The molecular weight of the enzymes is analyzed by polyacrylamide gel electrophoresis. As described herein, this combination has been shown to release amino acids, EAAs, and BCAAs across a wide range of pH and temperature levels (e.g., pH 3 to 9, and a temperature of 20-80°C) while maintaining high activity.

[0048] In other aspects, the proteolytic enzyme mixture comprises a plurality of fungal proteases from the genus *Aspergillus*, wherein the mixture comprises SEQ ID NO: 1, a protease with peptidase activity obtained from *A. melleus* (CAS No. 9074-07-1; EC No. 3.4.11.-), and a fungal neutral protease obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.24.-). In another aspect, the mixture comprises SEQ ID NO: 1, SEQ ID NO: 2, and a fungal neutral protease obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.24.-). In another aspect, the mixture comprises SEQ ID NO: 1, a protease with peptidase activity obtained from *A. melleus* (EC No. 3.4.11.-), and SEQ ID NO: 3.

[0049] In another aspect, the mixture comprises a protease with exo- and endo-protease activity obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.23.18), SEQ ID NO: 2, and a fungal neutral protease obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.24.-). In another aspect, the mixture comprises a protease with exo- and endo-protease activity obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.23.18), SEQ ID NO: 2, and SEQ ID NO: 3.

[0050] In another aspect, the mixture comprises a protease with exo- and endo-protease activity obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.23.18), a protease with peptidase activity obtained from *A. melleus* (CAS No. 9074-07-1; EC No. 3.4.11.-), and SEQ ID NO: 3.

[0051] In other aspects, the proteolytic enzyme mixture comprises a plurality of fungal proteases from the genus *Aspergillus*, wherein the mixture comprises SEQ ID NO: 1, a 34 kDa protease with peptidase activity obtained from *A. melleus* (CAS No. 9074-07-1; EC No. 3.4.11.-), and a 35 kDa fungal neutral protease obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.24.-), wherein the molecular weight of the proteases are analyzed by polyacrylamide gel electrophoresis. In another aspect, the mixture comprises SEQ ID NO: 1, SEQ ID NO: 2, and a 35 kDa fungal neutral protease obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.24.-) when analyzed by polyacrylamide gel electrophoresis. In another aspect,

the mixture comprises SEQ ID NO: 1, a 34 kDa protease with peptidase activity obtained from *A. melleus* (CAS No. 9074-07-1; EC No. 3.4.11.-) when analyzed by polyacrylamide gel electrophoresis, and SEQ ID NO: 3.

[0052] In another aspect, the mixture comprises a 42 kDa protease with exo- and endo-protease activity obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.23.18), SEQ ID NO: 2, and a 35 kDa fungal neutral protease obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.24.-), wherein the molecular weight of the proteases are analyzed by polyacrylamide gel electrophoresis. In another aspect, the mixture comprises a 42 kDa protease with exo- and endo-protease activity obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.23.18) when analyzed by polyacrylamide gel electrophoresis, SEQ ID NO: 2, and SEQ ID NO: 3.

[0053] In another aspect, the mixture comprises a 42 kDa protease with exo- and endo-protease activity obtained from *A. oryzae* (CAS No. 9025-49-4; EC No. 3.4.23.18), a 34 kDa protease with peptidase activity obtained from *A. melleus* (CAS No. 9074-07-1; EC No. 3.4.11.-), and SEQ ID NO: 3, wherein the molecular weight of the proteases are analyzed by polyacrylamide gel electrophoresis.

[0054] In other aspects, the proteolytic enzyme mixture comprises a plurality of fungal proteases from the genus *Aspergillus*, wherein the mixture comprises SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3.

[0055] In some aspects, the proteolytic enzyme mixture comprises a plurality of fungal proteases from the genus *Aspergillus*, wherein the mixture comprises SEQ ID NO: 1, SEQ ID NO: 2, and/or SEQ ID NO: 3, or a fragment or variant of any of these enzymes. For example, in some aspects, the proteolytic enzyme mixture comprises at least one variant enzyme which shares at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% full-length sequence identity with any one of SEQ ID NOs: 1-3, and which retains one or more of the enzymatic activities of SEQ ID NOs: 1-3. For example, a polypeptide sequence may differ from any one of SEQ ID NOs: 1-3

by the presence of one or more conservative or non-conservative substitutions which do not impact the catalytic or other activity of the enzyme. As used herein, the term “sequence identity” refers to the degree to which two polypeptide sequences are identical (i.e., on a residue-by-residue basis) over the window of comparison. The percentage of sequence identity is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which identical residues 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 (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

[0056] In some aspects, the proteolytic enzyme mixture comprises a plurality of fungal proteases from the genus *Aspergillus*, wherein the mixture comprises at least one enzyme which shares at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% full-length sequence identity with a portion of the sequence of SEQ ID NOs: 1-3 (e.g., with the region spanning positions 78-404 of SEQ ID NO: 1 or with the region spanning positions 246-634 of SEQ ID NO: 3, which are predicted to represent the mature forms of the enzymes represented by these SEQ ID NOs). In some aspects, the proteolytic enzyme mixture comprises a plurality of fungal proteases from the genus *Aspergillus*, wherein the mixture comprises at least one enzyme which shares at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% full-length sequence identity with the region spanning positions 21-404 of SEQ ID NO: 1 or with the region spanning positions 19-634 of SEQ ID NO: 3.

[0057] **FIGs. 1A-1B** provide graphs that illustrate the relative activity levels of Fungal Protease A across various temperature and pH levels. **FIGs. 2A-2B** provide graphs that illustrate the relative activity levels of Fungal Protease AM across various temperature and pH levels. **FIGs. 3A-3D** provide graphs that illustrate the relative and residual activity levels of Fungal Protease A2 across various temperature and pH levels. **FIG. 4** is a graph that illustrates

the relative activity levels of P³ HYDROLYZER™, ProHydrolase® and Aminogen® across various pH levels. As shown by this figure, P³ HYDROLYZER™ displays peak activity at approximately pH 6. However, it retains activity across a broad range of pH values spanning pH 3 to pH 8, a range of pH common across the gastric and intestinal compartments of the mammalian digestive tract. The activity profile of P³ HYDROLYZER™ under various pH and temperature levels is further illustrates by **FIGs. 5A-5B**, which graph the activity level of P³ HYDROLYZER™ from pH 3 to 9 and across a span of 20-80 °C.

[0058] While the assay results described herein pertain to P³ HYDROLYZER™, it is understood that in some alternative aspects, a proteolytic enzyme mixture according to the disclosure may include at least two of the enzymes in P³ HYDROLYZER™ or comprise at least two of SEQ ID NOs: 1, 2, or 3 (e.g., SEQ ID NOs: 1 and 2, 1 and 3, or 2 and 3). It is further understood that the amounts or ratios of the enzymes in the proteolytic enzyme mixture may be varied to produce a mixture having enhanced or reduced activity levels. For example, Fungal Protease A, Fungal Protease AM, and Fungal Protease A2 may be combined at a ratio of approximately 10:1:40, 10:1:20, 10:1:75, 3:1:10, as measured in HUT activity units, or any other ratio which provides a desired activity level as measured in HUT units. In some aspects, the ratio of any of the individual components may vary by ±5, 10, 15, 20, 25, 30, 35, 40, 45, or 50% from any of the foregoing examples or ratios described herein (e.g., the ratio may be 5-15:0.5-1.5:10-30, as measured in HUT activity units).

[0059] In practice, P³ HYDROLYZER™ will typically be used at 1,000-2,000 HUT unit per gram of protein being digested (e.g., approximately 1,300 HUT units). However, it is understood that this amount will be varied subject to routine optimization for a given application (e.g., additional HUTs/g may be necessary at a lower incubation temperature for a given protein). Additional enzymes (e.g., proteases), coenzymes, cofactors, solvents, salts, etc.,

may be added to any of the protease enzyme mixtures disclosed herein as desired to improve or modify the digestion process as necessary or desired for a particular implementation.

[0060] The proteolytic enzyme mixtures described herein may be in a dehydrated, powdered, granular or freeze-dried form. In other aspects, the enzyme mixture is cell-free.

Dietary Supplements Comprising Proteolytic Enzyme Mixtures

[0061] In some aspects, proteolytic enzyme mixtures described herein may be formulated as dietary supplements, protein supplements, and/or nutritional supplement compositions that may be administered to a subject to provide one or more benefits (e.g., to increase protein digestion and/or the absorption of amino acids, EAAs, or BCAAs, or to improve the subject's muscle health). As used herein, the term "dietary supplement" refers to a manufactured product taken by mouth that comprises one or more "dietary ingredients" intended to supplement the diet (i.e., food) of a subject. Exemplary dietary ingredients include proteins, amino acids, carbohydrates, fat, vitamins, minerals, metabolites, probiotics, enzymes, herbs and botanicals. Unlike medicaments, dietary supplements are not intended to treat, diagnose, prevent, or cure diseases. A dietary supplement may comprise, e.g., a proteolytic enzyme mixture as described herein and at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 wt. % of a dietary ingredient or a mixture of dietary ingredients. In some aspects, the dietary ingredient portion comprises a wt. % within a range bounded by any of these values (e.g., 10-20 wt. %). A "protein supplement" is a type of dietary supplement comprising at least 10 dry wt.% protein, wherein the amount of protein in the composition is greater than that of either carbohydrate or fat. A "nutritional supplement" is a type dietary supplement comprising protein, carbohydrates, and fat.

[0062] In accordance with certain embodiments disclosed herein, the proteolytic enzyme mixtures contains at least 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.10%, 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%, 26%, 27%, 28%, 29%, or 30% by weight solids

of a protease preparation from *Aspergillus oryzae*, including at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, and at least 90% by weight solids of a protease preparation from *Aspergillus oryzae*. In accordance with certain of the preceding embodiments, the proteolytic enzyme mixture contains 30% to 100% total HUT activity from the *Aspergillus oryzae* protease preparation, including from 45% to 100%, 50% to 100%, 70% to 100%, 80% to 100%, and 90% to 100% total HUT activity of the proteolytic enzyme mixture.

[0063] In some aspects, dietary supplements, protein supplements, and/or nutritional supplement compositions comprising a proteolytic enzyme mixture according to the disclosure may be suitable for oral administration. Oral administration, as defined herein, includes any form of administration in which the composition including the proteolytic enzyme mixture passes through the esophagus of the subject. For example, oral administration typically refers to oral consumption, but may also include administration through nasogastric intubation, in which a tube is run from the nose to the stomach of the subject to administer the composition. Oral administration is a form of enteral administration (i.e., administration through the digestive tract). Other forms of enteral administration suitable for use with the methods disclosed herein include administration through a gastric or jejunal tube. In accordance with the embodiments described herein, suitable forms of the composition for enteral administration to the subject include caplets, tablets, pills, capsules, chewable tablets, quick dissolve tablets, effervescent tablets, solutions, suspensions, emulsions, multi-layer tablets, bi-layer tablets, soft gelatin capsules, hard gelatin capsules, lozenges, chewable lozenges, beads, granules, particles, microparticles, dispersible granules, sachets, and combinations thereof.

[0064] In some aspects, dietary supplement, protein supplement, and/or nutritional supplement compositions may be formulated consisting of or consisting essentially of a proteolytic enzyme mixture according to the disclosure. In other aspects, the proteolytic enzyme mixture is formulated into a protein supplement. Such protein supplements disclosed

herein are useful to provide supplemental sources of protein, including providing the subjects one or more benefits as described herein. In other embodiments, the proteolytic enzyme mixture is formulated in to a nutritional supplement. Such nutritional supplements disclosed herein are useful to provide supplemental sources of nutrition, including providing the subjects one or more benefits as described herein.

[0065] In accordance with certain methods of the embodiments disclosed herein, the dietary supplements, protein supplements, and nutritional supplement compositions may be provided as needed to deliver the desired level of proteolytic enzyme mixture, e.g., by providing at least one serving per day to achieve the desired effect. In some aspects, the dietary supplements, protein supplements, and nutritional supplement compositions maybe administered at one serving per day, two servings per day, three servings per day, four servings per day, etc., as needed to achieve a desired effect. Typically, the compositions disclosed herein are administered in at least one serving per day or at least two servings per day.

[0066] In some aspects, the proteolytic enzyme mixture is administered as a dietary supplement in capsule form before, during, or following consumption of a protein supplement. The final dose per serving of the proteolytic enzyme mixture may be between 5,000 and 300,000 HUT, 10,000 and 100,000 HUT, and 25,000 and 75,000 HUT. Table 1 shows an example formulation, from which a 250 mg capsule would be expected to deliver ~ 31,000 HUT. Table 2 shows an alternative exemplary formulation for a 300 mg capsule.

INGREDIENTS	AMOUNT (mg/1,000 mg)	ACTIVITY (HUT/g)
Maltodextrin	687.3	n/a
Fungal Protease A2 (<i>Aspergillus oryzae</i>)	256.4	100,000
Fungal Protease A (<i>Aspergillus oryzae</i>)	34.4	25,000
Fungal Protease AM (<i>Aspergillus melleus</i>)	21.9	2,500

Table 1. An exemplary formulation of a dietary supplement in capsule form comprising a proteolytic enzyme mixture according to the disclosure.

INGREDIENTS	AMOUNT (mg)	ACTIVITY (HUT/g)
Maltodextrin	171.8	n/a
Fungal Protease A2 (<i>Aspergillus oryzae</i>)	64.1	25,000
Microcrystalline Cellulose	25	n/a
Fungal Protease A (<i>Aspergillus oryzae</i>)	8.6	6,250
Fungal Protease AM (<i>Aspergillus melleus</i>)	5.5	625
Silicon Dioxide	2.3	n/a
Magnesium Stearate	1.2	n/a

Table 2. A second exemplary formulation of a dietary supplement in capsule form comprising a proteolytic enzyme mixture according to the disclosure.

[0067] In some aspects of the disclosure, the proteolytic enzyme mixture is administered as a dietary supplement in the form of a powder sachet or stick pack reconstituted in 4 to 6 ounces of water before, during, or following consumption of a meal. The final dose per serving of the proteolytic enzyme mixture may be between 5,000 and 300,000 HUT, 10,000 and 100,000 HUT, and 25,000 and 75,000 HUT. Table 3 shows an example formulation, from which a 4.5 g stick pack would be expected to deliver ~ 31,000 HUT:

INGREDIENTS	AMOUNT PER SERVING (g)
Hydrolyzed Collagen	4.0
Maltodextrin	0.376
Fungal Protease A2 (<i>Aspergillus oryzae</i>)	0.064
Stevia Leaf Extract	0.030
Strawberry Flavor	0.015
Fungal Protease A (<i>Aspergillus oryzae</i>)	0.009
Fungal Protease AM (<i>Aspergillus melleus</i>)	0.006

Table 3. An exemplary formulation of a dietary supplement in powder sachet or stick pack form comprising a protein mixture according to the disclosure.

[0068] In some aspects of the disclosure, the proteolytic enzyme mixture is formulated as a protein supplement. The final dose per serving of the proteolytic enzyme mixture is between 5,000 and 300,000 HUT, 10,000 and 100,000 HUT, and 25,000 and 75,000 HUT. Tables 4 and

5 show example protein powder formulations, from which a serving would be expected to deliver ~ 31,000 HUT:

INGREDIENTS	AMOUNT PER SERVING (g)
Whey protein concentrate	25
Cocoa powder	2.7
Maltodextrin	1
Natural Flavors	0.6
Xanthan Gum	0.1
Carageenan	0.1
Salt	0.1
Fungal Protease A2 (<i>Aspergillus oryzae</i>)	0.064
Sucralose	0.030
Acesulfame potassium	0.025
Fungal Protease A (<i>Aspergillus oryzae</i>)	0.009
Fungal Protease AM (<i>Aspergillus melleus</i>)	0.006

Table 4. An exemplary formulation of a protein supplement comprising a protein mixture according to the disclosure.

INGREDIENTS	AMOUNT PER SERVING (g)
Pea Protein Isolate	11
Rice Protein	9
Coconut Oil	2.0
Tapioca Maltodextrin	1
Natural Vanilla Flavors	0.6
Guar Gum	0.1
Carageenan	0.1
Sea Salt	0.1
Stevia Leaf Extract	0.08
Fungal Protease A2 (<i>Aspergillus oryzae</i>)	0.064
Fungal Protease A (<i>Aspergillus oryzae</i>)	0.009
Fungal Protease AM (<i>Aspergillus melleus</i>)	0.006

Table 5. A second exemplary formulation of a protein supplement comprising a protein mixture according to the disclosure.

[0069] Any source of protein may be used so long as it is suitable for protein supplement or nutritional supplement compositions and is otherwise compatible with any other selected ingredients or features in the protein supplement or nutritional supplement compositions. For

example, the source of protein may include, but is not limited to, intact, hydrolyzed, and partially hydrolyzed protein, which may be derived from any known or otherwise suitable source such as milk (e.g., casein, whey), animal (e.g., meat, fish, egg), cereal (e.g., rice, corn, oat, wheat), vegetable (e.g., pea, soy, hemp, potato), pulses (chick pea, mung bean, fava bean), fungi, bacteria, insect and combinations thereof. The source of protein may also include a mixture of amino acids known for use in protein supplements or a combination of such amino acids with the intact, hydrolyzed, and partially hydrolyzed proteins described herein. The amino acids may be naturally occurring or synthetic amino acids. The amino acids may include branched chain amino acids, essential amino acids, non-essential amino acids, or combination thereof.

[0070] Examples of suitable sources of protein for use in the protein supplements and nutritional supplements disclosed herein include, but are not limited to, whey protein concentrates, whey protein isolates, whey protein hydrolysates, acid caseins, sodium caseinates, calcium caseinates, potassium caseinates, casein hydrolysates, milk protein concentrates, milk protein isolates, milk protein hydrolysates, nonfat dry milk, condensed skim milk, pea protein isolates, pea protein hydrolysates, soy protein concentrates, soy protein isolates, soy protein hydrolysates, pea protein concentrates, collagen proteins, potato proteins, rice proteins, insect proteins, earthworm proteins, fungal (e.g., mushroom) proteins, proteins expressed by microorganisms (e.g., bacteria and algae), and the like, as well as combinations thereof. The nutritional supplement compositions can include any individual source of protein or a combination of two or more the various sources of protein listed above or otherwise encompassed by the general inventive concepts.

[0071] A variety of dairy protein and plant protein sources may be utilized for the protein system of the protein supplement or nutritional supplement described herein. An exemplary dairy protein suitable for use in the nutritional supplement powder described herein is Avonlac[®]

282, a whey protein concentrate, available from Glanbia Nutritionals (Kilkenny, Ireland). An exemplary plant protein suitable for use in the nutritional supplement powder described herein is NUTRALYS® S85F, a pea protein isolate, available from Roquette Frères (Lestrem, France).

[0072] In some aspects of the disclosure, the proteolytic enzyme mixture is formulated in to a nutritional supplement. The final dose per serving of the proteolytic enzyme mixture may be between 5,000 and 300,000 HUT, 10,000 and 100,000 HUT, and 25,000 and 75,000 HUT. Table 6 shows an example nutritional formulation, from which a serving would be expected to deliver ~ 31,000 HUT:

INGREDIENTS	AMOUNT PER SERVING (g)
Corn Maltodextrin	20.0
Sugar	10.0
Milk protein concentrate	10.0
Soybean Oil	5.0
Soy Protein Isolate	5.0
Canola Oil	3.0
Vitamin and Mineral Blend	1.5
Whey Protein Concentrate	1.0
Guar Gum	1.0
Carageenan	1.0
Salt	0.8
Natural Flavors	0.6
Fungal Protease A2 (<i>Aspergillus oryzae</i>)	0.064
Sucralose	0.030
Acesulfame potassium	0.025
Fungal Protease A (<i>Aspergillus oryzae</i>)	0.009
Fungal Protease AM (<i>Aspergillus melleus</i>)	0.006
Alpha-galactosidase	0.003

Table 6. An exemplary formulation of a nutritional supplement comprising a protein mixture according to the disclosure.

[0073] The dietary supplements, protein supplements and nutritional supplements described herein may be administered to a subject in order to increase protein digestion and/or to improve the absorption of amino acids, EAAs, and/or BCAAs. In other aspects, these compositions may be administered to a subject to improve muscle health. In each case, such methods comprise

administering at least one serving per day of a composition comprising a proteolytic enzyme mixture according to the disclosure. In some aspects, such methods comprise administering 10 mg to 1,000 mg of proteases per serving, or approximately 5,000 HUT to 300,000 HUT per serving, to the subject.

Protein Hydrolysate Compositions and Methods of Preparation

[0074] Proteolytic enzyme mixtures described herein may be used to produce protein hydrolysates enriched in essential amino acids and/or BCAAs compared to protein hydrolysates produced by other protease enzymes and mixtures known in the art. Such hydrolysates may be produced from any raw protein source capable of digestion by a selected proteolytic enzyme mixture, including plant proteins (e.g., soy, hemp, rice, whey or pea protein), animal proteins (e.g., beef, chicken, or pork) and microbial proteins. Non-traditional protein sources such as insect protein (e.g., cricket protein) may also be used, as may proteins expressed from a recombinant organism (e.g., protein synthesized by a genetically-modified yeast culture).

[0075] As described above, proteolytic enzyme mixtures according to the disclosure may be used, in some embodiments, to produce hydrolysates having desirable properties such as enriched levels of essential amino acids or BCAAs. In some exemplary aspects, hydrolysates produced as described herein may have free leucine, isoleucine and/or valine levels which are several-fold higher than the levels of these free amino acids in protein hydrolyzed by any of the individual enzymes in the proteolytic enzyme mixture or by currently available proteases and mixtures. In some embodiments, such hydrolysates may be produced as a one-step process without supplementation from a secondary amino acid source (e.g., the initial hydrolysate may have a several-fold increase in one or more of these amino acids, avoiding the need for supplementation with additional BCAAs). In some exemplary aspects, hydrolysates produced as described herein may contain at least 10, 20, 30 or 40 mg/L of valine, at least 10, 20, 30 or

40 mg/L of isoleucine, and/or at least 10, 20, 30 or 40 mg/L of leucine. In some instances, the concentration of leucine in such hydrolysates may be further enriched to a level of at least 50, 100 or 150 mg/L.

[0076] In addition to providing higher concentrations of free BCAAs, hydrolysates produced according to the disclosure have also been found to display improved organoleptic properties. As noted above, hydrolysates produced according to methods known in the art often have a chalky mouthfeel and/or bitter taste. However, as described by Example 4 and illustrated by **FIG. 16**, protein hydrolysates prepared using P³ HYDROLYZER™ were preferred by a panel of assessors compared to untreated whey protein shake or a hydrolysate digested with ProHydrolase®.

Food Products, Ingredients or Additives, Dietary Supplements and Beverages Comprising Protein Hydrolysates

[0077] Protein hydrolysates produced according to the present disclosure may be used as food products, dietary supplements, as an ingredient or additive for a food product, in beverages, or in any other vehicle suitable for administration to or ingestion by a person or animal. In particular, hydrolysates according to the disclosure enriched in essential amino acids and/or BCAAs may be particularly desirable as food products, beverages or dietary supplements intended for athletes and subjects interested in improving exercise performance.

[0078] In some exemplary aspects, a protein hydrolysate may be prepared from a protein source (e.g., plant, animal or microbial-sourced raw protein) using any of the protease enzyme mixtures or methods of production described herein. The resulting hydrolysate may be optionally processed, such as by heat-inactivating the protease enzymes used to perform the digestion, chemically treating the mixture, and/or by filtering the mixture. The hydrolysate may also be optionally converted into a form more convenient for transport or storage (e.g., by drying, dehydrating or freeze-drying the hydrolysate). The hydrolysate may, subject to any

such optional processing, be added to a food product, dietary supplement, beverage or any other vehicle suitable for administration to a human or animal, as indicated above.

[0079] In some exemplary aspects, the hydrolysate is dried or dehydrated to form a protein powder enriched in essential amino acids and/or BCAAs. In other exemplary aspects, the hydrolysate is added to a food product such as a meal replacement or energy bar or beverage. The hydrolysate may be added to a vehicle as a powder or in liquid form, as is preferred for a given application.

Methods of Using Protein Hydrolysates

[0080] Protein hydrolysates may be provided or administered to a human or animal in need of additional nutrition and/or to promote or provide a beneficial physiological effect. Protein hydrolysates enriched in essential amino acids and/or BCAAs are particularly useful as these classes of amino acid are associated with proper nutrition, muscle physiology and metabolism. As a result, protein hydrolysates produced according to the methods described herein may be used as a dietary supplement or as part of a treatment for humans or animals in order to improve nutrition or to improve athletic or exercise performance.

[0081] In some exemplary aspects, a protein hydrolysate as described herein may be administered to a subject in need thereof once, on a periodic basis or as part of any other regimen suitable to provide the subject with sufficient levels of one or more essential amino acids and/or BCAAs (e.g., to provide a desirable trait or reach a selected threshold associated with a desirable physiological state). The hydrolysate may be provided or administered as a food product, additive or ingredient to a food product, dietary supplement, beverage, or any other vehicle suitable which allows a subject to ingest or otherwise absorb amino acids in the hydrolysate.

[0082] Protein hydrolysates prepared using P³ HYDROLYZER™ in accordance with any of the exemplary aspects above may be provided to a human or animal to promote nutrition or

improved athletic or exercise performance, particularly hydrolysates enriched in BCAAs. It is understood that any such hydrolysates may be provided to a human or animal in need thereof as part of a food product, dietary supplement or beverage and may be provided in any amount necessary to provide a desirable function or outcome, with such amounts being the product of routine optimization depending on the nature of the individual or animal receiving the hydrolysate and/or the composition of the hydrolysate.

[0083] All statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

[0084] All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

Examples

[0085] The following examples demonstrate the performance of an exemplary protease mixture comprising Fungal Protease (*A. oryzae*), Fungal Protease AM (*A. melleus*), and Fungal

Protease A2 (*A. oryzae*) at a ratio of 10:1:40. Each of these enzyme ingredients was obtained from BIO-CAT, Inc (Troy, Virginia, USA).

Example 1: Comparative Amino Acid Releasing Performance of P³ HYDROLYZER™ in a 60 Minute Simulation of Gastric Digestion of Whey Protein

[0086] The primary objective of these in vitro experiments was to determine the effects of P³ HYDROLYZER™ in releasing amino acids from dietary proteins in a gastric digestion simulation, with a comparison to two additional protease ingredient products. To start, the effects of P³ HYDROLYZER™ on amino acid release from whey protein powder (NAKED Nutrition®; Coral Gables, FL, USA) was compared to Aminogen® and ProHydrolase®, two commercially available protease mixtures. Three experimental treatment groups, in addition to one control with no supplemental enzymes, were tested: 1) a blend of proteases and maltodextrin that meets at least the minimum protease concentration in the Aminogen® product; 2) the ProHydrolase® blend of proteases; and 3) a blend of P³ HYDROLYZER™ and maltodextrin. Three grams of whey protein (approximately 1/10 serving size) were dissolved in 20 mL deionized water for each experimental enzyme treatment and control. Enzymes were weighed on tared weigh paper and then transferred to 20 mL test tubes using deionized water. Enzymes were prepared as follows: 1) 0.250 grams of a protease blend and maltodextrin that meets at least the minimum protease concentration in Aminogen® per 10 mL, 2) 0.250 grams ProHydrolase® per 5 mL, and 3) 0.125 grams of a protease blend and maltodextrin reflective of the final protease concentration in P³ HYDROLYZER™ per 10 mL. All treatments reflect an equivalent recommended dose of the enzymes in Aminogen®, ProHydrolase®, and P³ HYDROLYZER™. One mL of each enzyme solution (approximately 1/10 recommended dose) was added to a separate beaker of dissolved protein. One mL deionized water (instead of enzyme) was also added to a separate beaker of dissolved protein (control). 2.5 mL simulated, acidic gastric solution (0.015 g mucin, 0.0125 grams pepsin 1:10,000, electrolytes, pH 1.9) was added to each beaker, including the control. This simulated gastric solution has been previously

described (*See Donhowe, E., et al., “Characterization and In Vitro Bioavailability of β -Carotene: Effects of Microencapsulation Method and Food Matrix.” *LWT - Food Science and Technology*. 2014. 57, 42-48, which is hereby incorporated by reference in its entirety*). Importantly, the simulated gastric solution contains porcine pepsin, which is an endogenous, naturally occurring mammalian protease that is released in to the stomach during food or beverage consumption. This porcine pepsin in this in vitro experiment simulates the activity of human gastric pepsin. The experimental samples now containing protein, experimental enzymes (with the exception of the control), and simulated gastric fluid with pepsin were then placed on a stir plate in a 37 °C water bath for 60 minutes with agitation by magnetic stir bar. After the full 60 minute incubation, beaker contents—now called “gastric digestas”—were transferred to 50 mL centrifuge tubes and enzymatic activity was halted by placing tubes in a 90 °C water bath for 10 minutes. Heat-killed gastric digestas were stored at 4 °C until high performance liquid chromatography (HPLC) analysis of all 20 essential and non-essential amino acids using o-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC). The results reported here are the average of amino acid values measured from three gastric simulations carried out on three separate days ($n = 3$). A second experiment was also performed using the same compositions and parameters, except that the simulated gastric digestion incubation time was reduced to 15 minutes to better understand early activity of P³ HYDROLZYER™ during gastric digestion.

[0087] Amino acids were measured by HPLC (Agilent 1100 Series HPLC, Agilent Technologies, Inc.; Santa Clara, CA, USA) with fluorescence detection. Combining OPA and FMOC enables fast pre-column derivatization of amino acids for chromatographic analysis. The HPLC reaction mixture was buffered at a pH of 10.2, which allows direct derivatization of acid hydrolyzed protein/peptide samples. Primary amino acids contain both a basic amino group and an acidic carboxyl group. Proline is the only proteinogenic amino acid that is a

secondary amino acid, i.e., the nitrogen atom is attached both to the α -carbon and to a chain of three carbons that together form a five-membered ring. Only primary amino acids react with OPA, so FMOC is needed for the detection of proline. The primary amino acids were reacted first with OPA using 3-mercaptopropionic acid (3-MPA). The secondary are then derivatized using FMOC. The incorporation of 3-MPA into the indoles decreases their hydrophobicity, and as a result, the OPA derivatives elute chromatographically before the FMOC derivatives. Excess FMOC and its degradation products elute after the last of the secondary amino acids and do not interfere with the analysis.

[0088] The results of these experiments are summarized by **FIGs. 6-7**. As demonstrated by these experiments, P³ HYDROLYZER™ displayed superior performance at both time points (i.e., after a 15 minute and 60 minute digestion), releasing > 2-fold greater levels of several amino acids from whey protein compared to Aminogen® and ProHydrolase®. **FIG. 7** illustrates that only 15 minutes of digestion with P³ HYDROLYZER™ released several amino acids at greater levels than the full 60 minutes of digestion with Aminogen® or ProHydrolase®. Physiologically, it takes approximately 0.5 to 4 hours, depending on the amount of food or beverage consumed, to reach maximum postprandial total plasma amino acid concentrations after protein consumption. As evidenced by these data, P³ HYDROLYZER™ may be applied to (or ingested concurrently with) a food product, beverage product, or dietary supplement containing protein to expedite the digestive process and to increase the amount of free amino acids that are available for absorption during and following digestion. Under in vivo conditions, it is expected that this increase in the amount of free amino acids will result in increased uptake by gut cells, higher blood amino acid concentrations, and increased absorption by tissues and organs such as skeletal muscle, which requires amino acids for the synthesis of new muscle proteins and muscle growth.

Example 2: Comparative Amino Acid Releasing Performance of P³ HYDROLYZER™ in a 60 Minute Simulation of Gastric Digestion of Whey, Soy, Pea, and Rice Proteins

[0089] A similar experiment was performed to evaluate the use of P³ HYDROLYZER™ to release BCAAs from several additional protein sources (i.e., whey, soy, pea and rice protein), compared to a control sample with protein and only porcine pepsin, and protein digested with ProHydrolase® and Aminogen®. One dairy protein source and three plant protein sources were evaluated for their amino acid release profile under experimental enzymatic treatment: 1) whey protein powder (NAKED Nutrition®, Coral Gables, FL, USA), 2) soy protein isolate powder (Hard Eight Nutrition LLC, Hendersonville, NV, USA), 3) pea protein isolate powder (Hard Eight Nutrition LLC, Hendersonville, NV, USA), and 4) brown rice powder (Raw Power®, Coeur d'Alene, ID, USA).

[0090] For each protein source, 3 treatment groups in addition to no enzyme control were tested: 1) a blend of proteases and maltodextrin that meets at least the minimum protease concentration in the Aminogen® product; 2) the ProHydrolase® blend of proteases; and 3) a blend of P³ HYDROLYZER™ and maltodextrin. The test compositions with experimental enzymes, protein, and simulated gastric fluid (containing porcine pepsin) were prepared, digested for 60 minutes, and analyzed as described in Example 1. The results of this experiment are summarized by **FIG. 8**, which provides a graph showing the concentration of BCAAs released by each enzyme-protein substrate pairing. As illustrated by **FIG. 8**, the gastric digestas treated with P³ HYDROLYZER™ are remarkably enriched in BCAAs compared to the digestas treated with ProHydrolase® and Aminogen®.

[0091] The effects of P³ HYDROLYZER™ on total amino acid, EAA, and leucine release profiles, in addition to the BCAA concentrations reported previously in Example 2, were also determined. The results of these additional measurements are summarized by **FIGs. 9-12**, which provide graphs showing the concentration of AAs, EAAs, BCAAs and leucine released from whey, soy, pea, and rice protein sources by P³ HYDROLYZER™, compared to two competitor protease mixtures and a control (protein and pepsin only). As illustrated by **FIGs.**

9-12, the gastric digestas treated with P³ HYDROLYZER™ across all 4 protein sources are remarkably enriched in total amino acids, EAAs, BCAAs and leucine, as compared to the gastric digestas treated with Aminogen® and ProHydrolase®.

[0092] To better characterize the superior performance of P³ HYDROLYZER™, a statistical analysis was performed using R® version 3.6.2 (R Core Team, 2020). Graphs were produced using the ggplot2 package (Wickham, 2016). A one-way ANOVA analysis was performed to determine significance and an F–statistic of $p < 0.05$ was considered statistically significant. Tukey’s Honestly Significant Difference adjustment was used for multiple comparisons using a 95% family-wise confidence level. Each individual amino acid, total EAAs, total BCAAs, and total amino acids were analyzed.

[0093] P³ HYDROLYZER™ treatment of whey protein promoted greater amino acid liberation compared to competitor protease products, including greater liberation of total amino acids, EAAs BCAAs and leucine (**FIG. 9**, Table 7; all comparisons to P³ HYDROLYZER™ were statistically significant). These data in Table 7 represent values from averaging the amino acid liberation across 3 separate experiments performed on 3 separate days. Adjusted values are the result of subtracting the control values from the experimental enzyme treatment values to establish normalized baseline values adjusted to controls. Adjusted values are compared vs. P³ HYDROLYZER™ to determine fold-change and percent increases. The “Average” value represents the average of the two competitor enzyme ingredient products, Aminogen® and ProHydrolase®. The “Fold Change” value represents the average of the two competitor enzyme ingredient products, rounded down to the nearest whole number. “Unadjusted” values do not subtract the control from the experimental enzyme treatments.

	Measured Value (mg/g)	Adjusted Values (mg/g)	P ³ vs Comp	P ³ vs Comp Average	Fold Change	P ³ vs Comp Unadjusted	Unadjusted average	Unadjusted fold change
Essential amino acids:								
Control	0.89					2257%		22x
Comp A	2.01	1.12	1714%			1000%		

WO 2022/204576						PCT/US2022/022053		
Comp B	3.44	2.55	753%	1234%	12x	584%	792%	7x
P ³	20.09	19.2						
Branched chain amino acids:								
Control	0.31					2545%		25x
Comp A	0.51	0.2	3790%			1547%		
Comp B	0.76	0.45	1684%	2737%	27x	1038%	1293%	12x
P ³	7.89	7.58						
Total amino acids:								
Control	3.1					1023%		10x
Comp A	6.2	3.1	923%			511%		
Comp B	8.6	5.5	520%	721%	7x	369%	440%	4x
P ³	31.7	28.6						
Leucine:								
Control	0.15					3247%		32x
Comp A	0.30	0.15	3147%			1623%		
Comp B	0.44	0.29	1628%	2387%	23x	1107%	1365%	13x
P ³	4.87	4.72						

Table 7. Amino acid release from whey protein in a 60 minute simulation of gastric digestion (Comp, competitor; Comp A, Aminogen[®]; Comp B, ProHydrolase[®]).

[0094] P³ HYDROLYZER[™] treatment of soy protein (**FIG. 10**, Table 8), pea protein (**FIG. 11**, Table 9), and rice protein (**FIG. 12**, Table 10) also promoted greater amino acid liberation compared to competitor protease products, including greater liberation of total amino acids, EAA, BCAA, and leucine (all comparisons to P³ HYDROLYZER[™] were statistically significant, $p < 0.05$). Altogether, P³ HYDROLYZER[™] treatment showed an average of 3-fold greater amino acid liberation and 9-fold greater liberation of BCAA across all three plant protein sources, compared to two top competitors (Table 11).

[0095] In this example, whey, soy, pea and rice protein were assayed as protein sources for digestion. However, it is understood that other raw protein sources obtained from plants, fungi, bacteria or animals may also be digested using the protease enzyme mixtures disclosed herein. Similarly, the incubation time and temperature parameters described above may vary as necessary for a given application, while remaining in accordance with the present disclosure.

	Measured Value (mg/g)	Adjusted Values (mg/g)	P ³ vs Comp	P ³ vs Comp Average	Fold Change	P ³ vs Comp Unadjusted	Unadjusted average	Unadjusted fold change
Essential amino acids:								
Control	1.5					1667%		16x

Comp A	3.1	1.6	1469%			806%		
Comp B	5.2	3.7	635%	1052%	10x	481%	644%	6x
P ³	25	23.5						
Branched chain amino acids:								
Control	0.59					1820%		18x
Comp A	1.09	0.5	2030%			985%		
Comp B	1.44	0.85	1194%	1612%	16x	746%	866%	8x
P ³	10.74	10.15						
Total amino acids:								
Control	5.2					856%		8x
Comp A	8.1	2.9	1355%			549%		
Comp B	11.8	6.6	595%	975%	9x	377%	463%	4x
P ³	44.5	39.3						
Leucine:								
Control	0.37					1800%		18x
Comp A	0.81	0.44	1430%			822%		
Comp B	0.95	0.58	1084%	1257%	15x	701%	762%	7x
P ³	6.66	6.29						

Table 8. Amino acid release from soy protein in a 60 minute simulation of gastric digestion (Comp, competitor; Comp A, Aminogen[®]; Comp B, ProHydrolase[®]).

	Measured Value (mg/g)	Adjusted Values (mg/g)	P ³ vs Comp	P ³ vs Comp Average	Fold Change	P ³ vs Comp Unadjusted	Unadjusted average	Unadjusted fold change
Essential amino acids:								
Control	1.7					994%		9x
Comp A	2.1	0.4	3800%			805%		
Comp B	5.3	3.6	422%	2111%	21x	319%	562%	5x
P ³	16.9	15.2						
Branched chain amino acids:								
Control	0.74					1232%		12x
Comp A	0.80	0.06	13967%			1140%		
Comp B	1.86	1.12	748%	7357%	73x	490%	815%	8x
P ³	9.12	8.38						
Total amino acids:								
Control	5.4					580%		5x
Comp A	7.1	1.7	1524%			441%		
Comp B	12.4	7	370%	947%	9x	252%	347%	3x
P ³	31.3	25.9						
Leucine:								
Control	0.4					1425%		14x
Comp A	0.5	0.1	5300%			1140%		
Comp B	1.0	0.6	883%	3092%	30x	570%	855%	8x
P ³	5.7	5.3						

Table 9. Amino acid release from soy protein in a 60 minute simulation of gastric digestion (Comp, competitor; Comp A, Aminogen[®]; Comp B, ProHydrolase[®]).

	Measured Value (mg/g)	Adjusted Values (mg/g)	P ³ vs Comp	P ³ vs Comp Average	Fold Change	P ³ vs Comp Unadjusted	Unadjusted average	Unadjusted fold change
Essential amino acids:								
Control	0.7					2043%		20x
Comp A	1.45	0.75	1813%			986%		
Comp B	3.91	3.21	424%	1119%	11x	366%	676%	6x
P ³	14.3	13.6						
Branched chain amino acids:								
Control	0.27					3015%		360x
Comp A	0.48	0.21	3748%			1696%		
Comp B	1.36	1.09	722%	2235%	22x	599%	1147%	11x
P ³	8.14	7.87						
Total amino acids:								
Control	3.0					907%		9x
Comp A	6.7	3.7	654%			406%		
Comp B	10.5	7.5	323%	488%	4x	259%	333%	3x
P ³	27.2	24.2						
Leucine:								
Control	0.14					3564%		35x
Comp A	0.32	0.18	2694%			1559%		
Comp B	0.73	0.59	822%	1758%	17x	684%	1121%	11x
P ³	4.99	4.85						

Table 10. Amino acid release from rice protein in a 60 minute simulation of gastric digestion (Comp A, Aminogen[®]; Comp B, ProHydrolase[®]).

	Essential amino acids	Branched chain amino acids	Total amino acids	Leucine
Soy	644%	866%	463%	762%
Pea	562%	815%	347%	855%
Rice	676%	1147%	333%	1121%
Average	627%	943%	381%	913%

Table 11. Average percent increase in percent greater amino acid liberation from three plant protein sources by P³ HYDROLYZER[™] compared to the average of two competitor products (Aminogen[®] and ProHydrolase[®]) in a 60 minute simulation of gastric digestion.

Example 3: Comparative Amino Acid Releasing Performance of P³ HYDROLYZER[™] in the INFOGEST 122 Minute Simulation of Salivary-Gastric Digestion of Whey, Pea, and Soy Proteins

[0096] The “INFOGEST” simulation of salivary-gastric digestion was used to test the efficacy

of P³ HYDROLYZER[™], Aminogen[®], and ProHydrolase[®] on protein digestion in vitro. Protein substrates included each of 1) Avonlac[®] 282 whey protein concentrate (Glanbia Nutritionals,

Kilkenny, Ireland), 2) soy protein isolate (Hard Eight Nutrition LLC, Hendersonville, NV, USA), and 3) NUTRALYS® S85F pea protein isolate (Roquette Frères; Lestrem, France).

[0097] The INFOGEST protocol has been extensively described elsewhere (*See* Minekus, M., et al., “A Standardised Static In Vitro Digestion Method Suitable for Food - An International Consensus.” *Food and Function*. 2014. 5(6), 1113-1124; Brodkorb, A., et al., “INFOGEST Static In Vitro Simulation of Gastrointestinal Food Digestion.” *Nature Protocols*. 2019. 14(4), 991-1014, each of which is hereby incorporated by reference in its entirety). The INFOGEST protocol models three phases of digestion: salivary, gastric, and intestinal. The salivary and gastric phases were modeled herein.

[0098] The salivary phase proceeded for 2 minutes in a simulated salivary fluid with agitation at 37 °C and neutral pH in the presence of porcine salivary amylase. The gastric phase proceeded by addition of simulated gastric fluid containing porcine pepsin and incubation for 2 hours with agitation at 37 °C at a starting pH of 3. In the experimental groups (“treatments”), a partial dose of experimental enzymes (i.e., P³ HYDROLYZER™, Aminogen®, or ProHydrolase®) based on the partial serving size of the food substrate, was added to the gastric digesta 10 minutes after the start of the gastric phase to mimic the time to dissolution of a vegetarian capsule shell in the human stomach. The control groups contained protein substrate and the endogenous porcine amylase and porcine pepsin in the salivary and gastric phases, respectively, to model human endogenous enzyme activities. Small samples were withdrawn at the end of the 2 hour gastric phase, followed by inactivation of enzymatic activity at 90 °C for 10 minutes. Samples were stored at 4° C until measurement of all 20 amino acids by HPLC (Chemstation, Revision B.04.01 SP1, Agilent Technologies; Santa Clara, CA, USA). The results reported here are the average of amino acid values measured from 3 digestion experiments.

[0099] Statistical analysis was performed using R[®] version 3.6.2 (R Core Team, 2020). Figures were produced using GraphPad Prism version 9.1.2 for Windows (San Diego, California, USA). A one-way ANOVA analysis was performed to determine significance and an F-statistic of $p < 0.05$ was considered statistically significant. Tukey's Honestly Significant Difference adjustment was used for multiple comparisons using a 95% family-wise confidence level. Normality was assessed by Shapiro-Wilk test on residuals. Homoscedasticity was assessed with the Levene's Test of Equality of Variances. No violations of normality or homoscedasticity were observed. Data below the limit of quantitation was imputed at the lowest standard value. Amino acids resolved as "not detected" were imputed with a value of zero for analytical purposes. Each amino acid, total EAAs, total BCAAs, and total amino acids were analyzed. All observations are expressed as mean $\pm$ standard deviation. Figures with asterisks indicate increased levels of significance as follow: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

[0100] P³ HYDROLYZER[™] treatment of whey protein promoted greater amino acid liberation compared to competitor protease products, including greater liberation of leucine, BCAAs, EAAs, and total amino acids leucine (**FIG. 13**, Table 12). All comparisons to P³ HYDROLYZER[™] for leucine and BCAA were significant ($p < 0.05$, **FIGs. 13A & 13B**). Comparisons to P³ HYDROLYZER[™] for EAAs and total amino acids were not statistically significant when compared to ProHydrolase[®], but were significant as compared to control and Aminogen[®] (**FIGs. 13C & 13D**). The data in Table 12 represent values from averaging the amino acid liberation across 3 separate experiments performed on 3 separate days. Adjusted values are the results of subtracting the porcine enzyme-only control values from the experimental enzyme treatments to establish values normalized to control. Adjusted values are compared vs. P³ HYDROLYZER[™] to determine fold-change and percent increases. The "Average" value represents the average of the two competitor enzyme ingredient products,

Aminogen[®] and ProHydrolase[®]. The “Fold Change” value represents the average of the two competitor enzyme ingredient products, rounded down.

	Measured Value (mg/g)	Adjusted Values (mg/g)	% Increase (P ³ vs Comp)	Average % Increase (P ³ vs Comps)	Average Fold Change (P ³ vs)
Total amino acids:					
Control	7.12				
Aminogen [®]	11.15	4.03	256%		
ProHydrolase [®]	14.35	7.23	143%	200%	3
P ³	17.45	10.33			
Essential amino acids:					
Control	6.08				
Aminogen [®]	9.29	3.21	212%		
ProHydrolase [®]	11.00	4.92	139%	176%	2.7
P ³	12.90	6.82			
Branched chain amino acids:					
Control	1.55				
Aminogen [®]	3.02	1.47	186%		
ProHydrolase [®]	3.26	1.71	160%	173%	2.7
P ³	4.29	2.74			
Leucine:					
Control	1.26				
Aminogen [®]	1.95	0.69	339%		
ProHydrolase [®]	2.06	0.80	293%	316%	4.1
P ³	3.60	2.34			

Table 12. Amino acid release from whey protein in the INFOGEST simulation of salivary-gastric digestion.

[0101] P³ HYDROLYZER[™] treatment of pea protein (FIG. 14, Table 13) and soy protein (FIG. 15, Table 14) also promoted greater amino acid liberation compared to competitor protease products, including greater liberation of leucine, BCAAs, EAAs, and total amino acids (all comparisons to P³ HYDROLYZER[™] significant, $p < 0.05$). Altogether, P³ HYDROLYZER[™] treatment showed an average of two-fold greater total amino acid liberation and 3-fold greater liberation of BCAA across the two plant protein sources, compared to two top competitors (Table 15, FIGS. 14B & 15B).

	Measured Value (mg/g)	Adjusted Values (mg/g)	% Increase (P ³ vs Comp)	Average % Increase (P ³ vs Comps)	Average Fold Change (P ³ vs)
Total amino acids:					
Control	4.61				
Aminogen [®]	10.35	5.74	198%		
ProHydrolase [®]	10.09	5.48	208%	203%	3
p ³	15.99	11.38			
Essential amino acids:					
Control	2.17				
Aminogen [®]	6.28	4.11	191%		
ProHydrolase [®]	6.26	4.09	191%	191%	2.9
p ³	10.00	7.83			
Branched chain amino acids:					
Control	0.45				
Aminogen [®]	1.69	1.24	246%		
ProHydrolase [®]	1.26	0.81	377%	311%	4.1
p ³	3.50	3.05			
Leucine:					
Control	0.22				
Aminogen [®]	1.23	1.01	261%		
ProHydrolase [®]	0.82	0.60	440%	351%	4.5
p ³	2.86	2.64			

Table 13. Amino acid release from pea protein in the INFOGEST simulation of salivary-gastric digestion.

	Measured Value (mg/g)	Adjusted Values (mg/g)	% Increase (P ³ vs Comp)	Average % Increase (P ³ vs Comps)	Average Fold Change (P ³ vs)
Total amino acids:					
Control	8.94				
Aminogen [®]	12.40	3.46	247%		
ProHydrolase [®]	12.09	3.15	271%	259%	3.5
p ³	17.48	8.54			
Essential amino acids:					
Control	7.03				
Aminogen [®]	9.24	2.21	249%		
ProHydrolase [®]	9.12	2.09	264%	256%	3.5
p ³	12.54	5.51			
Branched chain amino acids:					
Control	1.95				
Aminogen [®]	2.71	0.76	280%		
ProHydrolase [®]	2.47	0.52	410%	345%	4.4

P ³	4.08	2.13			
Leucine:					
Control	1.44				
Aminogen [®]	1.9	0.46	385%		
ProHydrolase [®]	1.79	0.35	506%	445%	5.4
P ³	3.21	1.77			

Table 14. Amino acid release from soy protein in the INFOGEST simulation of salivary-gastric digestion.

	Total amino acids	Essential amino acids	Branched chain amino acids	Leucine
Pea	103%	91%	211%	251%
Soy	159%	156%	245%	345%
Average	131%	124%	228%	298%

Table 15. Average percent increase in percent greater amino acid liberation from 2 plant protein sources by P³ HYDROLYZER[™] compared to the average of two competitor products (Aminogen[®] and ProHydrolase[®]) in the INFOGEST simulation of salivary-gastric digestion.

Example 4: Evaluation of the Organoleptic Properties of an Exemplary Hydrolysate Produced Using P³ HYDROLYZER[™].

[0102] A sensory test was performed in order to determine the effects of the addition of P³ HYDROLYZER[™] and ProHydrolase[®] on sensory attributes of a whey protein shake. The panel of sensory assessors consisted of thirteen volunteers, and the whey protein shakes used in this test were prepared with whey protein powder from NAKED Nutrition[®] (Coral Gables, FL, USA) and whole milk, as summarized by Table 16.

	Control	P ³ HYDROLYZER [™]	ProHydrolase [®]
Whole milk (mL)	250	250	250
Whey protein (g)	25	25	25
Enzyme (mg)	N/A	250	250

Table 16. Composition of the whey protein shakes for sensory testing.

[0103] Each experimental group was coded with a 3 digit number to keep the study participants blinded. The control samples were numbered 200 to 300, samples treated with P³ HYDROLYZER[™] were numbered 400 to 500, and samples treated with ProHydrolase[®] were numbered 600 to 700. Each assessor evaluated each shake using a rating system based on a 1 to 5 point scale (1, very poor; 2, poor; 3, fair; 4, good; 5, excellent) where a 1 indicated the

very poor taste, texture, or overall flavor perception and a 5 indicated excellent taste, texture, or overall flavor perception. Participants also scored one of the 3 experimental shakes as “Best” and one of the remaining two experimental shakes as “Worse.” The results were ranked, scored and then analyzed to determine the preference of protein shakes without enzyme, with P³ HYDROLYZER™, or with ProHydrolase®.

Control Product Code	Participant ID	Taste	Texture	Overall	Best	Worse
301	J1	4	4	4	X	
304	M3	3	4	4	X	
310	J2	4	5	4	X	
319	S2	3	4	4		
322	D2	4	5	5	X	
330	S1	4	5	4	X	
350	K1	3	5	4	X	
381	M1	3	3	3	X	
236	J3	4	4	4		
240	D1	3	5	5		
291	M2	2	4	3		
293	C1	4	5	4	X	
295	K2	4	4	4	X	
Totals:		45	57	52	9	0
% of Groups:					69.2%	0.0%

Table 17. Sensory testing of whey protein shakes without added enzymes (control).

P ³ HYDROLYZER™ Product Code	Participant ID	Taste	Texture	Overall	Best	Worse
405	M2	4	4	4		
414	S1	4	4	4		
415	K1	2	3	2		X
434	M3	2	3	2		
447	S2	4	5	5	X	
451	J2	4	5	4		
455	D2	5	4	4		
457	J1	2	3	2		X
500	J3	4	4	4		
507	K2	4	3	3		
512	D1	4	3.5	4	X	
527	C1	3	5	4		
549	M3	4	4	4	X	
Totals:		46	50.5	46	3	2
% of Groups:					23.1%	15.4%

Table 18. Sensory testing of whey protein shakes with added P³ HYDROLYZER™.

ProHydrolase® Product Code	Participant ID	Taste	Texture	Overall	Best	Worse
602	K1	2	2	2		
608	J2	3	3	3		X
622	M2	2	2	2		X
628	S2	2	2	2		X
632	S1	3	2	3		X
638	D2	2	1	2		X
658	M3	1	1	1		X
681	J1	3	1	2		
691	J3	2	2	2		
716	K2	2	1	2		X
747	D1	3	3	3		X
750	M2	1	1	1		X
756	C1	2	1	2		X
Totals:		28	22	27	0	10
% of Groups:					0.0%	76.9%

Table 19. Sensory testing of whey protein shakes with added ProHydrolase®.

[0104] As illustrated by these results shown in Tables 17-19 and **FIG. 16**, the participants in this blinded experiment generally preferred the protein shake without enzymes, but also preferred the protein shake treated with P³ HYDROLYZER™ rather than an otherwise identical protein shake treated with ProHydrolase®. While this experiment evaluated flavor

preferences in connection with a beverage produced using whey protein, it is understood that P³ HYDROLYZER™ may be used to produce protein shakes with desirable flavor profiles from various other protein sources (e.g., plant proteins such as soy, rice, pea, chickpea, or wheat protein, mushroom protein, animal protein, dairy protein, egg protein, bacterial protein, and combinations thereof).

Example 5: Clinical Evaluation of the Effects of Protease Supplementation on Post-Prandial Plasma Amino Acid Levels.

[0105] Dietary protein is digested in the stomach and intestines to smaller peptides and 20 individual amino acids which, when absorbed by the gut into circulation and taken up by skeletal muscle, help stimulate muscle protein synthesis (MPS). Amino acids also provide the building blocks for muscle proteins that contribute to muscle growth and increased strength following resistance exercise. Therefore, strategies to efficiently maximize amino acid exposure without protein overconsumption are warranted. Oral enzyme supplementation is a candidate approach to optimize amino acid absorption from dietary protein and protein supplements. Microbial proteases can theoretically speed up the conversion of protein and peptides to amino acids. Protease supplements have been marketed to promote muscle strength by optimizing amino acid absorption, however meaningful and statistically significant clinical evidence is lacking.

[0106] Protease supplementation using the proteolytic enzyme mixture described herein was evaluated in a randomized, double-blind, placebo-controlled, cross-over clinical trial. Eligible participants were randomized into groups given either P³ HYDROLYZER™ (n = 12) or placebo (n = 12) in conjunction with a liquid protein blend for their 1st aminoacidemia trial. A 2nd aminoacidemia trial consisted of the opposite treatment condition. The objective of this study was to determine whether P³ HYDROLYZER™ enzyme supplementation (e.g., with 31,875 HUT protease activity) is an effective dosage to improve the early (0-2 h) and cumulative (0-5h) net area under the total amino acid, EAA, BCAA, and leucine curves after

the ingestion of a protein shake in healthy subjects. Statistically significant results showed that P³ HYDROLYZER™ increased postprandial amino acid concentrations greater than that of the placebo in the acute protein shake challenge aminoacidemia trial. These clinical trial data provide evidence that P³ HYDROLYZER™ supports digestive health, improves protein digestion, and enhances amino acid absorption from dietary protein.

[0107] Twenty-four recreationally active healthy adults volunteered to participate in this study. All participants were recruited and performed two experimental trials in the Nutrition and Exercise Performance Research Group clinical laboratory at the University of Illinois at Urbana-Champaign from April to August 2021. All participants were deemed healthy based on their responses to a routine medical screening questionnaire. All participants were informed about the experimental procedures, the purpose of the study, and potential risks before giving written consent. All trials conformed to standards for the use of human participants in research as outlined in the Helsinki Declaration (Clinicaltrials.gov ID NCT04821557) and were approved by the local Institutional Review Board at the University of Illinois at Urbana-Champaign (IRB approval no. 21545)

[0108] At Visit 1, study participants were asked to report to the testing facility at ~0700 hours after an overnight fast for measurement of body weight, height, and body composition by dual-energy x-ray absorptiometry (DEXA; Horizon W, Hologic Inc., Marlborough, MA, USA). After the preliminary testing session, participants were randomized to ingest 25 grams of pea protein isolate (Roquette NUTRALYS® S85F Pea Protein; 101 kcal, 20g protein, 2.2g fat) with either added P³ HYDROLYZER or placebo in a counterbalanced fashion on their first experimental trial. For allocation of the participants, a computer-generated list of random numbers was used. The study product was coded with a random numerical-alphabetical code and unblinded after all analyses were completed. Participants were instructed to refrain from any strenuous physical exercise 72 hours and alcohol 48 hours prior to the experimental trial.

Participants were provided an identical standardized meal for consumption the evening before both trials (25-30% of energy requirement; 50% of energy from carbohydrate 25% energy from protein and 25% energy from fat). In addition, participants were instructed to maintain the same dietary intake for three days prior to each experimental trial, which was confirmed by the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool (version 2020; National Cancer Institute, Bethesda, MD, USA). Both test days were separated by a 7-d wash-out.

[0109] For both trials, participants reported to the laboratory between 0600 hours and 0800 hours after an overnight fast. Following assessment of blood pressure, a Teflon catheter was inserted into an antecubital vein, and an arterialized baseline blood sample was collected. Immediately after catheter placement and blood sample collection, participants consumed 25 g of pea protein powder dissolved in 300 mL of water with either P³ HYDROLYZER™ or a maltodextrin placebo. The test articles were manufactured in capsule form, i.e., as: 250 mg P³ HYDROLYZER™ formulated to contain no less than 31,875 HUT activity, plus 20 mg microcrystalline cellulose, or placebo with 250 mg maltodextrin and 20 mg microcrystalline cellulose. On the morning of a protein shake challenge test and aminoacidemia trial, a capsule was opened and contents mixed into the shake. Arterialized blood samples were collected in EDTA-containing tubes before (t = -5 min) and after treatment ingestion (t = 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240 and 300 min). Blood samples were centrifuged at 3000 g for 10 min at 4 °C and the plasma was subsequently aliquoted and stored at -80 °C for future analysis. Following an at least 1 week washout, participants repeated the aminoacidemia trial at Visit 2 with the opposite test article.

[0110] Plasma amino acid concentrations were determined as follows: the Amino Acid standard solution (AAS18, Sigma, USA), containing 2.5 µmol/mL each of L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-

lysine·HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L- threonine, L-tyrosine and L-valine, and 1.25 µmol/mL L-cystine and a custom mixture containing 2.5 µmol/mL each of L-tryptophane, L-glutamine, L-asparagine, L-citrulline, L-cysteine were used for the calibration curve. Plasma samples (50 µL) were deproteinized with methanol (940 µL), centrifuged with following supernatant evaporation in vacuum and re-suspended in 1 mL of 0.1% formic acid in water before instrument injection. Ten µL of internal standard (DL-p-Chlorophenylalanine, 1 mg/mL 0.1 M HCL) was added to each sample and standard solution. Samples were analyzed by the Thermo Altis Triple Quadrupole liquid chromatography with tandem mass spectrometry (LC/MS/MS) system. Software TraceFinder 4.1 was used for data acquisition and analysis. The LC separation was performed on a Thermo Accucore Vanquish C18+ column (2.1 x 100mm, 1.5µm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetone) and the flow rate was 0.2 mL/min. The linear gradient was as follows: 0-0.5 min, 0% B; 0.5-3.5 min, 60% B; 3.5-5.5 min, 100% B; 5.5-7.5 min, 0% B. The autosampler and HPLC column chamber were set at 10 °C, 50 °C, respectively. The injection volume was 1 µL. Mass spectra was acquired under positive electrospray ionization (ESI) with the ion spray voltage of 3500 V. Selected reaction monitoring (SRM) used for the amino acid quantitation.

[0111] All data are presented as mean ± standard deviation (SD). A priori power analysis was conducted using GPOWER version 3.1.9.2. Based on previous research, the power analysis showed that a sample size of 20 participants was sufficient to detect differences in postprandial AUC BCAA concentrations between conditions when using a one-tailed t-test ($p < 0.05$, 85% power, $d = 0.62$). Accounting for a dropout rate of 15%, we recruited a total of 24 participants. All data were assessed for normality before analysis via visual inspection of normal Q-Q plots and skewness kurtosis values. Differences in amino acid concentrations were analyzed using linear mixed-effects models with time and group as fixed factors and participant

intercept as a random effect. Bonferroni's post hoc test was used when significant main effects were identified. Differences in plasma AUC amino acid concentrations were analyzed using a paired samples two-tailed t-test. The level of statistical significance was set at $p < 0.05$ for all analyses. All analyses were performed using IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, USA). For the analysis of amino acid concentrations at baseline, 45, and 60 minute time points, normality was assessed by the Shapiro-Wilk test on residuals and by visual confirmation using QQ-plot. Data points were considered outliers and subsequently removed only if their presence prevented normality. Normality was reassessed prior to removing any additional outliers.

[0112] Table 19 reports the subject characteristics at screening. Postprandial leucine, BCAA, EAA, and total amino acid concentrations over time are illustrated in **FIG. 17**. Postprandial EAA and total amino acid concentrations AUC two hours following consumption of the protein shake are illustrated in **FIG. 18**. Postprandial leucine, BCAA, EAA and total amino acid concentrations AUC two hours following consumption of the protein shake, with outputs of statistical analysis, are shown in Table 20. Postprandial leucine, BCAA, EAA and total amino acid concentrations at 45 and 60 minutes, absolute and baseline-adjusted, with outputs of statistical analysis are shown in Table 21.

[0113] Plasma leucine concentrations increased during the two hours after P³ HYDROLYZER™ and placebo ingestion with the protein shake (time effect: $p < 0.001$), with no significant differences between conditions (condition effect: $p = 0.602$, linear mixed-effects model, **FIG. 17A**). Plasma leucine concentration AUC showed a trend for near-significantly higher levels during the first two hours with P³ ingestion, compared to placebo ($p = 0.086$, **FIG. 18**, Table 20). At 60 minutes postprandial, baseline-adjusted plasma leucine concentrations were 13.5% higher with P³ ingestion, compared to placebo ($p = 0.036$, Table 21).

Subject characteristics at screening

Characteristic	Statistic/Category	Overall (n = 24)
Biological sex	Female (n)	12
	Male (n)	12
Age (years)	Mean	27
	SD	4
Weight (kg)	Mean	74.6
	SD	11.2
BMI (kg/m ²)	Mean	24.8
	SD	1.9
BMI group	18.0-24.99 kg/m ²	13
	25.0-29.99 kg/m ²	11
Systolic BP (mmHg)	Mean	119
	SD	12
Diastolic BP (mmHg)	Mean	67
	SD	8
Body fat (%)	Mean	29.2
	SD	9.4
Lean body mass	Mean	50.4
	SD	13.5
Fasting blood glucose (mmol/L)	Mean	4.90
	SD	0.52
Energy intake (kcal/d)	Mean	2189
	SD	595
Relative protein intake (g×kg bw ⁻¹ ×d ⁻¹)	Mean	1.5
	SD	0.6
Carbohydrate intake (g/d)	Mean	236
	SD	69
Fat intake (g/d)	Mean	93
	SD	27

SD, standard deviation; bw, body weight

Table 19. Subject characteristics at screening.

[0114] Postprandial plasma BCAA concentrations increased during the two hours after P³ HYDROLYZER™ and placebo ingestion with the protein shake (time effect: $p < 0.001$), with no differences between conditions (condition effect: $p = 0.724$, linear mixed-effects model, **FIG. 17B**). Plasma BCAA concentrations AUC did not differ significantly between conditions during first two hours ($p = 0.350$, Table 20). At 60 minutes postprandial, baseline-adjusted

plasma BCAA concentrations were 10.1% higher with P³ ingestion, compared to placebo, albeit not significant ($p = 0.135$, Table 21).

Timepoint	Amino Acid Category	Statistic	Placebo	P ³ HYDROLYZER™
0 – 2 hours	Leucine	n	20	20
		Mean, $\mu\text{mol} \cdot 120 \text{ min} \cdot \text{L}^{-1}$ (SD)	18031 (3326)	18566 (3133)
		Difference (P ³ - Placebo)	535.3	
		paired t-test p-value	0.0858	
	BCAA	n	19	19
		Mean, $\mu\text{mol} \cdot 120 \text{ min} \cdot \text{L}^{-1}$ (SD)	46765 (6559)	47433 (6674)
		Difference (P ³ - Placebo)	667.8	
		paired t-test p-value	0.3497	
	EAA	n	21	21
		Mean, $\mu\text{mol} \cdot 120 \text{ min} \cdot \text{L}^{-1}$ (SD)	101420 (14282)	103993 (15028)
		Difference (P ³ - Placebo)	2574	
		paired t-test p-value	0.0379	
	TAA	n	21	21
		Mean, $\mu\text{mol} \cdot 120 \text{ min} \cdot \text{L}^{-1}$ (SD)	208597 (28265)	216999 (32284)
		Difference (P ³ - Placebo)	8402	
		paired t-test p-value	0.0332	

SD, standard deviation

Table 20. Postprandial plasma amino acid concentration total area under the curve (AUC) across the first two hours following consumption of a pea protein shake with placebo or P³ HYDROLYZER™.

[0115] Plasma EAA concentrations increased during the two hours after P³ HYDROLYZER™ and placebo ingestion with the protein shake (time effect: $p < 0.001$), with no differences between conditions (condition effect: $p = 0.125$, linear mixed-effects model, **FIG. 17C**). Postprandial plasma EAA concentration AUC was significantly greater in the P³ HYDROLYZER™ group when compared to placebo during the first two hours ($p = 0.038$, **FIG. 18A**, Table 20). At 60 minutes postprandial, baseline-adjusted plasma EAA concentrations were significantly 21.7% higher with P³ ingestion, compared to placebo ($p = 0.015$, Table 21).

[0116] Plasma total amino acids concentrations increased during the two hours after protein shake consumption ($p < 0.001$), with higher concentrations with P³ HYDROLYZER™ when compared to placebo (condition effect: $p = 0.003$, linear mixed-effects model, **FIG. 17D**). Plasma total amino acid concentration AUC was significantly greater in P³ HYDROLYZER™

when compared to placebo during the first two hours ($p = 0.033$, **FIG. 18B**, Table 20). At 45 and 60 minutes postprandial, baseline-adjusted plasma total amino acid concentrations were significantly 23.5% and 33.0% higher, respectively, with P³ ingestion, compared to placebo ($p = 0.023$ and $p = 0.005$, respectively, Table 21).

Timepoint	Amino Acid Category	Statistic	Placebo	P ³ HYDROLYZER™
45 minutes	Leucine	n	24	24
		Mean, $\mu\text{mol/L}$ (SD)	183.1 (31.75)	188.7 (38.70)
		Δ , $\mu\text{mol/L}$ (P ³ - Placebo)	5.64	
		paired t-test p-value	0.3346	
		Baseline-adjusted comparison ^a	6.5%	
		paired t-test p-value	0.2343	
	BCAA	n	23	23
		Mean, $\mu\text{mol/L}$ (SD)	471.4 (77.03)	482.2 (99.99)
		Δ , $\mu\text{mol/L}$ (P ³ - Placebo)	10.76	
		paired t-test p-value	0.4601	
		Baseline-adjusted comparison ^a	6.5%	
		paired t-test p-value	0.2731	
	EAA	n	23	23
		Mean, $\mu\text{mol/L}$ (SD)	965.7 (128.1)	999.7 (188.1)
		Δ , $\mu\text{mol/L}$ (P ³ - Placebo)	34.03	
		paired t-test p-value	0.1989	
		Baseline-adjusted comparison ^a	12.7%	
		paired t-test p-value	0.1040	
	TAA	n	22	22
		Mean, $\mu\text{mol/L}$ (SD)	1952 (258.5)	2019 (337.7)
		Δ , $\mu\text{mol/L}$ (P ³ - Placebo)	157.3	
		paired t-test p-value	0.0009	
		Baseline-adjusted comparison ^a	23.5%	
		paired t-test p-value	0.0225	
60 minutes	Leucine	n	23	23
		Mean, $\mu\text{mol/L}$ (SD)	175.2 (36.37)	185.4 (39.03)
		Δ , $\mu\text{mol/L}$ (P ³ - Placebo)	10.14	
		paired t-test p-value	0.1056	
		Baseline-adjusted comparison ^a	13.5%	
		paired t-test p-value	0.0361	
	BCAA	n	24	24
		Mean, $\mu\text{mol/L}$ (SD)	466.8 (100.90)	479.9 (96.86)
		Δ , $\mu\text{mol/L}$ (P ³ - Placebo)	13.06	
		paired t-test p-value	0.3525	
		Baseline-adjusted comparison ^a	10.1%	
		paired t-test p-value	0.1351	
	EAA	n	24	24
		Mean, $\mu\text{mol/L}$ (SD)	957.1 (177.6)	1012 (2074.6)
		Δ , $\mu\text{mol/L}$ (P ³ - Placebo)	55.33	
		paired t-test p-value	0.0448	
		Baseline-adjusted comparison ^a	21.7%	
		paired t-test p-value	0.0153	
	TAA	n	24	24
		Mean, $\mu\text{mol/L}$ (SD)	1927 (342.5)	2079 (457.2)
		Δ , $\mu\text{mol/L}$ (P ³ - Placebo)	152.1	

paired t-test p-value	0.017
Baseline-adjusted comparison ^a	33.0%
paired t-test p-value	0.0053

^aAverage baseline amino acid concentration was subtracted from average amino acid concentration at each time point, within each experimental group, and then compared between groups and expressed as a relative percent increase (P3 vs Placebo). n, sample size; SD, standard deviation; Δ, difference

Table 21. Postprandial plasma amino acid concentrations at 45 and 60 minutes following consumption of a pea protein shake with P³ HYDROLYZER™ or placebo.

[0117] This is the first randomized, double-blind, placebo-controlled, cross-over study showing the efficacy of microbial protease supplementation in increasing postprandial plasma amino acid concentrations following protein shake consumption in healthy men and women. The results suggest that P³ HYDROLYZER™ is a well-tolerated and safe strategy to improve dietary protein digestion and increase postprandial plasma concentrations in healthy adults.

[0118] A study according to this exemplary protocol may be used to evaluate the effects of protease supplementation using the proteolytic enzyme mixtures described herein and to provide data that can be used to select optimal amounts and/or administration schedules for the proteolytic enzyme mixtures described herein.

Description of Sequences

SEQ ID No.:	1
Trade name:	Fungal Protease A (BIO-CAT, Inc.)
Source organism:	<i>Aspergillus oryzae</i>
Protein:	Aspergillopepsin-1
Gene:	pepA
UniPROTKB ID:	Q06902
Synonyms:	Acid protease A2, Aspartic protease A2, Aspergillus acid protease Aspergillus acid proteinase, Aspergillus aspartic proteinase, Aspergillopepsin I, Aspergillopepsin O, Aspergillopeptidase A
IUBMB Enzyme Commission (EC) number:	3.4.23.18
Length:	≤ 404 amino acids
Amino acid sequence:	10203040 50 MVILSKVAAV AVGLSTVASA LPTGPSHSPH ARRGTINQI TRQTARVGPK 60708090 100 TASFPPIYSR ALAKYGGTVP AHLKSAVASG HGTVVTSPEP NDIEYLTPVN 110120130140 150 IGGTTLNLDF DTGSADLWVF SEELPKSEQT GHDVYKPSGN ASKIAGASWD 160170180190 200 ISYGDGSSAS GDVYQDTVTV GGVTAQGQAV EAASKISDQF VQDKNNDGLL 210220230240 250 GLAFSSINTV KPKPQTTFD TVKDQLDAPL FAVTLKYHAP GSYDFGFIDK 260270280290 300 SKFTGELAYA DVDDSQGFQW FTADGYSVGK GDAQKAPITG IADTGTTLVM 310320330340 350 LDDEIVDAYY KQVQGAKNDA SAGGYVFPCE TELPEFTVVI GSYNAVIPGK 360370380390 400 HINYAPLQEG SSTCVGGIQS NSGLGLSILG DVFLKSQYVV FDSQGPRLGF AAQA
Molecular mass:	≤ 43 kDa (irrespective of post-translational modifications and including lower molecular weight transcriptional or alternate promoter variants)

SEQ ID No.:	2
Trade Name:	Fungal Protease AM (BIO-CAT, Inc.)
Protein:	Leucine aminopeptidase 1, variant 2
Gene:	LAP1

NCBI Accession:	XP_045945651
IUBMB Enzyme Commission (EC) number:	3.4.11.-
Source organism:	<i>Aspergillus melleus</i>
Length:	≤ 387 amino acids
Amino Acid sequence:	10203040 50 MKVGAAALALG ATASTGVLAA VIPQAPLTNP HIYHNQEKYL IELAPYQTRW 60708090 100 VTEEEKWALK LDGVNFIDVT TERNAGFYPT LHAPSYVRYP SKMEHTDEVT 110120130140 150 TLIKDLSKAN MQHNLEKFTS FHTRYKYSQT GIESATWLYN QVLDVIKSSG 160170180190 200 AAKHGATVDQ FAHPWGQFSV IARVPGKTNK TVVLGAHQDS INLFLPSILA 210220230240 250 APGADDDGSG TVTILEALRG LLQSDPIIKG EAPNTIEFHW YSAEEGGMGLG 260270280290 300 SQAIFSQYKQ DKRDIKAMLQ QDMTGYTQGA LEAGRQEAVG IMVDYVDQGL 310320330340 350 TQFLKDAVTT YCDIGFINTK CGYACSDHTS ASKYGYPAAM ATESEMENS 360370380 KRIHTTDDKI KYLSFDHMLQ HAKLTLGFAY ELAFAPF
Molecular mass:	≤ 43 kDa (irrespective of post-translational modifications and including lower molecular weight transcriptional or alternate promoter variants)

SEQ ID No.:	3
Trade name:	Fungal Protease A2 (BIO-CAT, Inc.)
Source organism:	<i>Aspergillus oryzae</i>
Protein:	Extracellular metalloproteinase NpI
Gene:	NpI
UniPROTKB ID:	Q2U1G7
Synonyms:	Elastinolytic metalloproteinase NpI, Fungalysin NpI, Neutral protease I
IUBMB Enzyme Commission (EC) number:	3.4.24.-
Length:	≤ 634 amino acids
Amino acid sequence:	10203040 50 MRGLLLAGAL GLPLAVLAHP THHAHGLQRR TVDLNSFRLH QAAKYINATE 60708090 100 SSSDVSSSFS PFTEQSYVET ATQLVKNILP DATFRVVKDH YIGSNGVAHV

	110 120 130 140 150 NFRQTAHGLD IDNADFNVNV GKNGKIFSYG HSFYTGKIPD ANPLTKRDYT 160 170 180 190 200 DPVAALRGTN EALQLSITLD QVSTEATEDK ESFNFKGVSG TVSDPKAQLV 210 220 230 240 250 YLVKEDGSLA LTKKVETDID SNWLLTYIDA NTGKDVHGVV DYVAEADYQV 260 270 280 290 300 YAWGINDPTE GPRTVISDPW DSSASAFTWI SDGENNYTTT RGNNGIAQSN 310 320 330 340 350 PTGGSQYLKN YRPDSPDLKF QYPYSLNATP PESYIDASIT QLFYTANTYH 360 370 380 390 400 DLLYTLGFNE EAGNFQYDNN GKGGAGNDYV ILNAQDGSGT NNANFATPPD 410 420 430 440 450 GQPGRMRMYI WTESQPYRDG SFEAGIVIHE YTHGLSNRLT GGPANSRCLN 460 470 480 490 500 ALESggmGEG WGDFMATAIR LKAGDTHSTD YTMGEWAANK KGGIRAYPFS 510 520 530 540 550 TSLETNPLTY TSLNELDEVH AIGAVWANVL YELLWNLIDK HGKNDGPKPE 560 570 580 590 600 FKDGVPTDGK YLAMKLVIDG MALQPCNPNC VQARDAILDA DKALTDGANK 610 620 630 CEIWKAFAKR GLGEGAHEYHA SRRVGSDKVP SDAC
Molecular mass:	≤ 70 kDa (irrespective of post-translational modifications and including lower molecular weight transcriptional or alternate promoter variants)

CLAIMS

1. A proteolytic enzyme mixture comprising a plurality of fungal proteases from members of the genus *Aspergillus*, wherein the plurality of fungal proteases comprises: (a) a 42 kDa protease with exo- and endo-protease activity obtained from *A. oryzae*, (b) a 34 kDa protease with peptidase activity obtained from *A. melleus*, and (c) a 35 kDa fungal neutral protease obtained from *A. oryzae*, and the proteolytic enzyme mixture has exoprotease, endoprotease and peptidase activity.
2. The proteolytic enzyme mixture of claim 1, wherein the ratio of the 42 kDa protease : 34 kDa protease : 35 kDa fungal neutral protease is:
 - a) 10:1:40; or
 - b) 5-15:0.5-1.5:30-50;as measured in Hemoglobin Unit Tyrosine base (HUT) units.
3. The proteolytic enzyme mixture of claims 1 or 2, wherein the mixture displays peak proteolytic activity at approximately pH 6.
4. The proteolytic enzyme mixture of any one of claims 1-3, wherein the mixture maintains proteolytic activity across a pH range of approximately 3.0 to 9.0.
5. The proteolytic enzyme mixture of any one of claims 1-4, wherein the mixture is stable over a temperature range of approximately 20 to 80 °C.
6. The proteolytic enzyme mixture of any one of claims 1-5, wherein the mixture has a relative activity level of at least 40% over a temperature range of approximately 20 to 80 °C.

7. A proteolytic enzyme mixture comprising a plurality of fungal proteases from the genus *Aspergillus*, wherein the mixture comprises at least two of SEQ ID NOs: 1, 2, and 3.
8. A proteolytic enzyme mixture comprising a plurality of fungal proteases from the genus *Aspergillus*, wherein the mixture includes at least one variant of SEQ ID NOs: 1, 2, or 3, comprising:
- a) an enzyme which shares at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% full-length sequence identity with any one of SEQ ID NOs: 1-3;
 - b) an enzyme which shares at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% full-length sequence identity with the region spanning position 78-404 of SEQ ID NO: 1;
 - c) an enzyme which shares at least 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% full-length sequence identity with the region spanning position 246-634 of SEQ ID NO: 3; or
 - d) any combination of a), b) and/or c);
- wherein the variant retains the enzymatic activity of any one of SEQ ID NOs: 1-3.
9. The proteolytic enzyme mixture of any one of claims 1-8, in a dehydrated, powdered, granular or freeze-dried form.
10. A dietary supplement comprising the proteolytic enzyme mixture of any one of claims 1-9.
11. A dietary supplement comprising the proteolytic enzyme mixture of any one of claims 1-9 and at least one additional enzyme comprising:
- a) a carbohydrase;

- b) an alpha-amylase; and/or
 - c) an alpha-galactosidase.
12. The dietary supplement of claim 10, wherein the dietary supplement is:
- a) a protein supplement or a nutritional supplement; and/or
 - b) formulated as a tablet, capsule, or powder.
13. The dietary supplement of any one of claims 10-12, wherein the dietary supplement contains at least 0.04% by weight solids of a protease preparation from *Aspergillus oryzae*.
14. A method for increasing dietary protein digestion and/or the absorption of amino acids from dietary protein, comprising:
- administering the proteolytic enzyme mixture of any one of claims 1-9 or the dietary supplement of any one of claims 10-13 to a subject at least once per day.
15. The method of claim 14, wherein the dietary supplement is administered at least twice per day, at least three times per day, or at least four times per day.
16. The method of claims 14 or 15, wherein the dietary supplement is administered in an amount effective to increase postprandial plasma concentrations of total amino acids, essential amino acids, branched chain amino acids, and/or leucine in the subject.
17. The method of any one of claims 14-16, wherein the dietary supplement is administered to the subject before, after, or concurrently with a food or beverage comprising at least 10 wt.% of protein.

18. A method for improving muscle health, increasing muscle protein synthesis, increasing muscle size, and/or improving muscle strength, comprising:

administering the proteolytic enzyme mixture of any one of claims 1-9 or the dietary supplement of any one of claims 10-13 to a subject at least once per day.

19. A method of increasing exercise performance, decreasing muscle breakdown during exercise, and/or improving recovery from exercise comprising:

administering the proteolytic enzyme mixture of any one of claims 1-9, a food product comprising the proteolytic enzyme mixture of any one of claims 1-9, or the dietary supplement of any one of claims 10-13, to a subject in an amount sufficient to increase exercise performance and/or decrease muscle breakdown during exercise.

20. The method of claim 19, wherein the food product is a protein shake.

21. The method of claim 20, wherein the protein shake comprises a protein source from dairy, plant, or a mushroom.

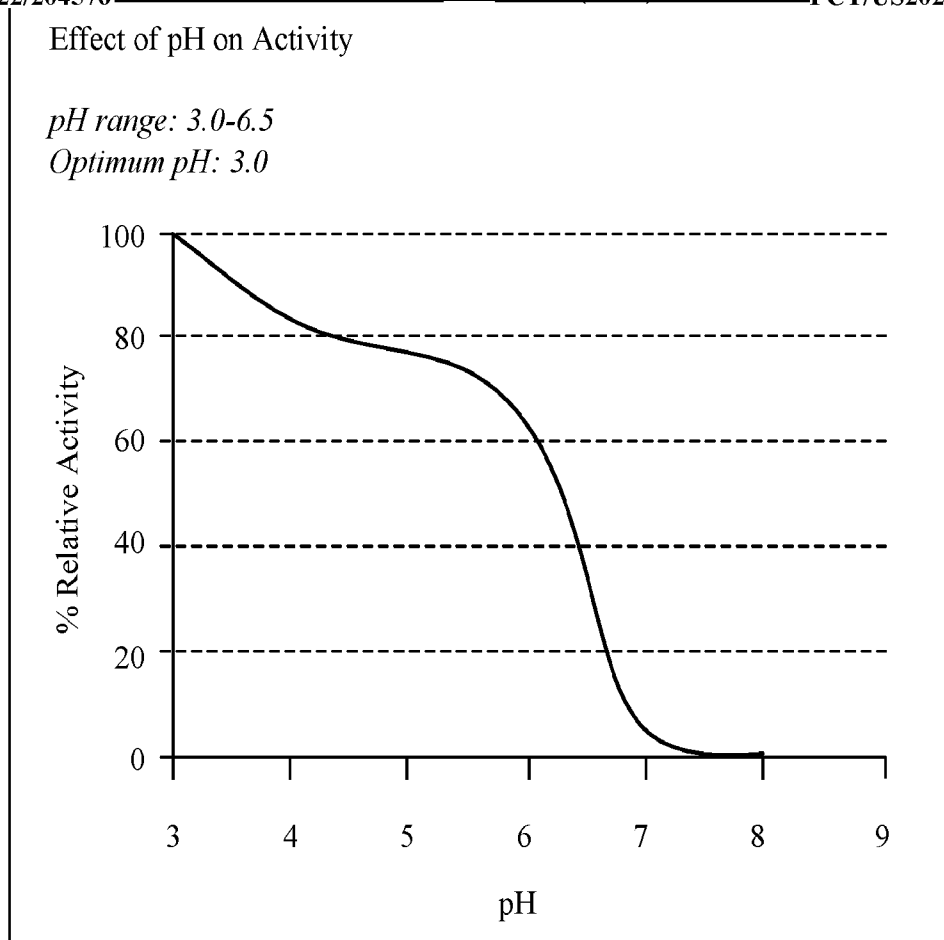


FIG. 1A

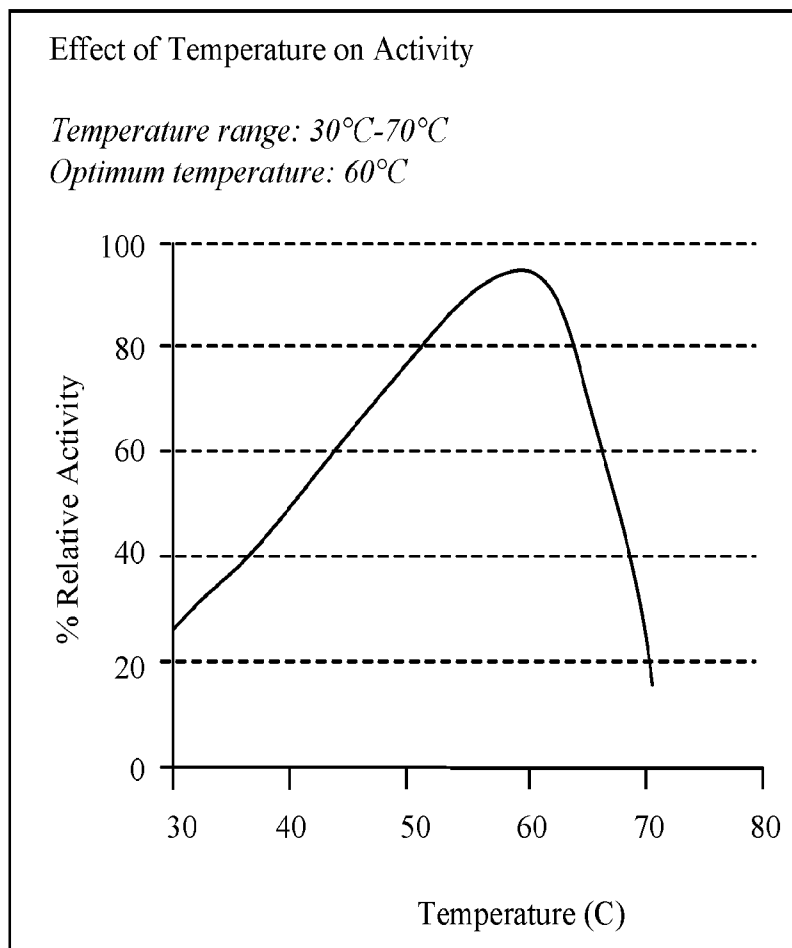


FIG. 1B

Effect of pH on Activity

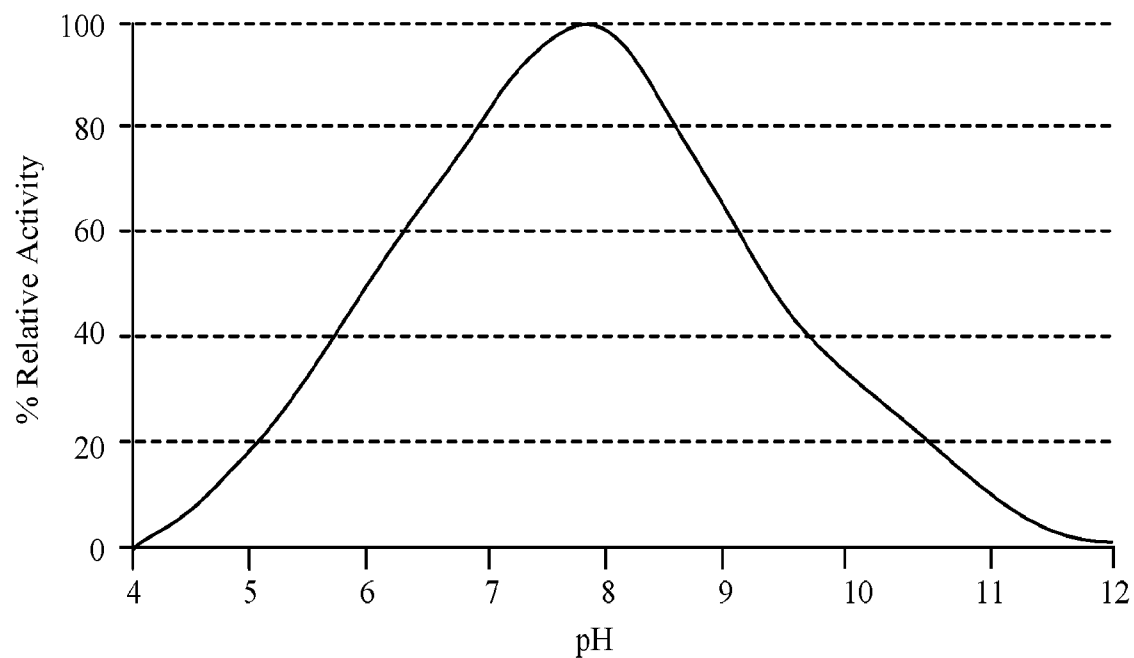
*pH range: 5.5-10.0**Optimum pH: 7.6*

FIG. 2A

Effect of Temperature on Activity

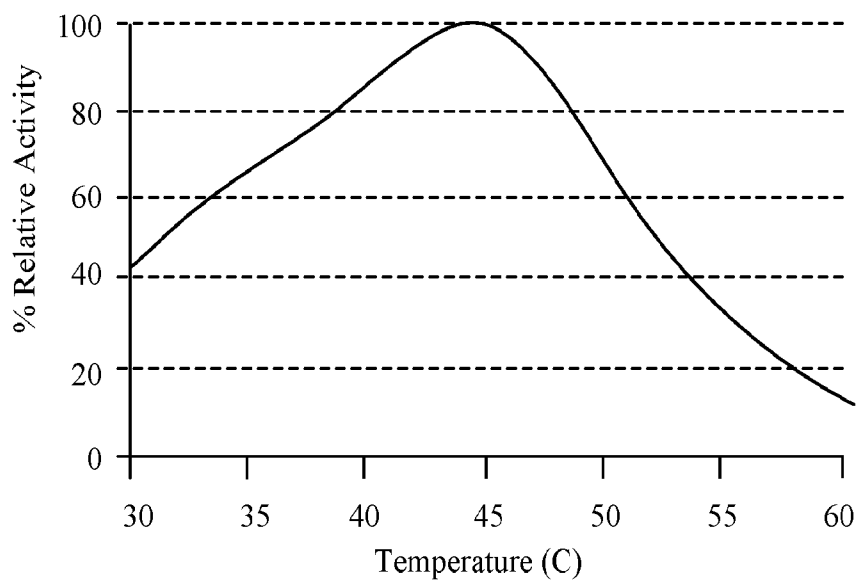
*Temperature range: 30°C-55°C**Optimum temperature: 45°C*

FIG. 2B

WO 2022/204576

3/33

PCT/US2022/022053

pH and Activity

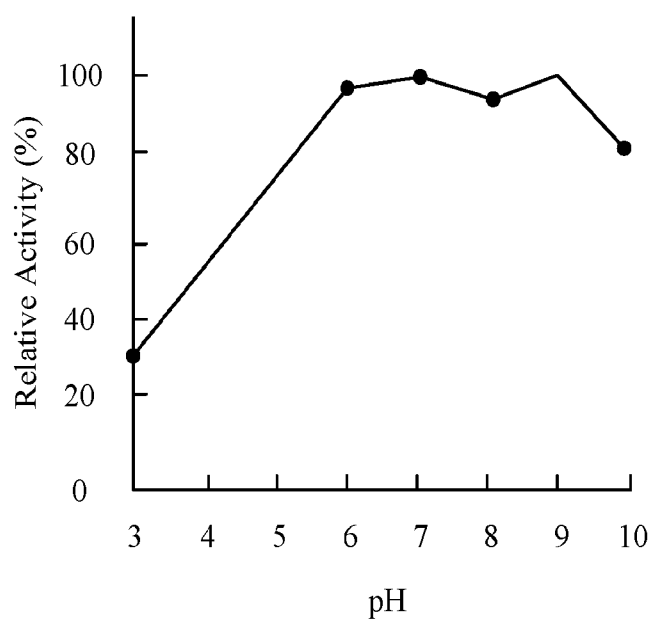


FIG. 3A

Temperature and Activity

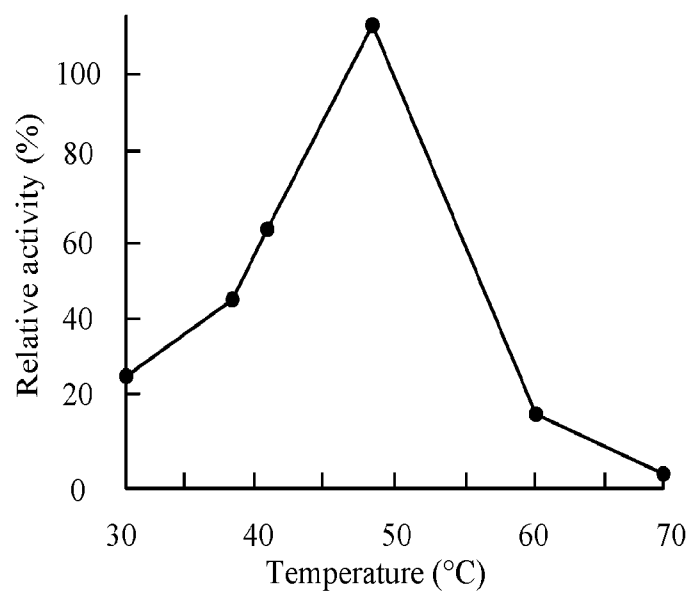
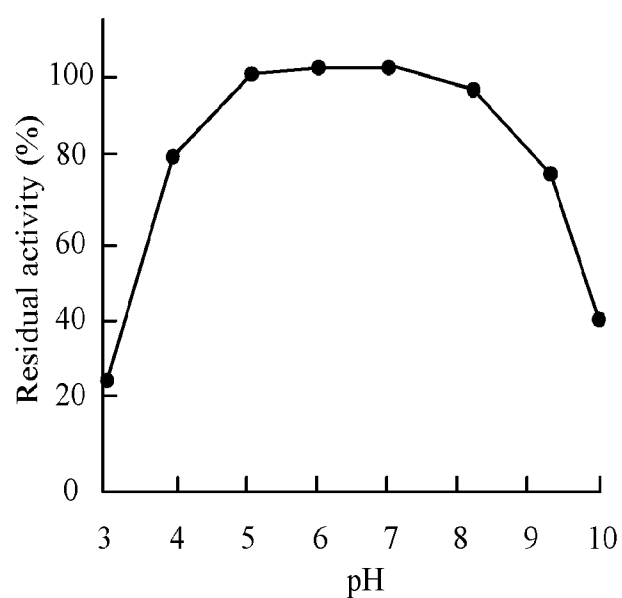


FIG. 3B

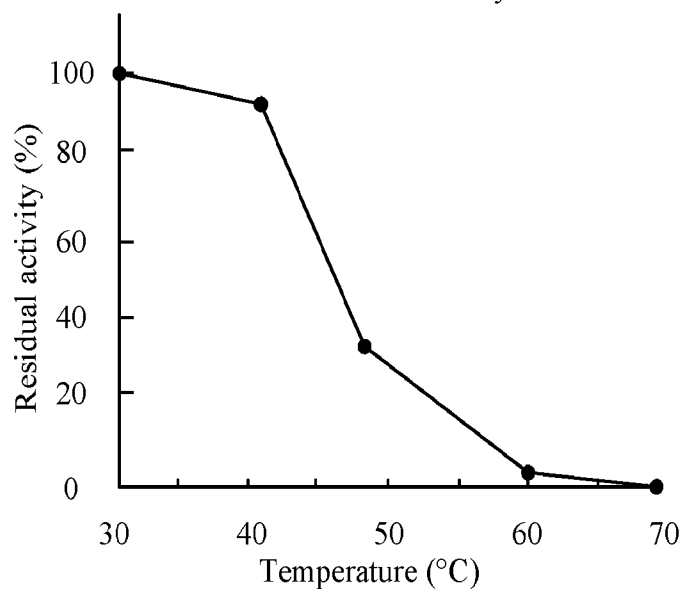
pH Stability



1% Enzyme Solution, 37°C, 60 min

FIG. 3C

Thermostability



1% Enzyme solution, pH 7.0, 60 min

FIG. 3D

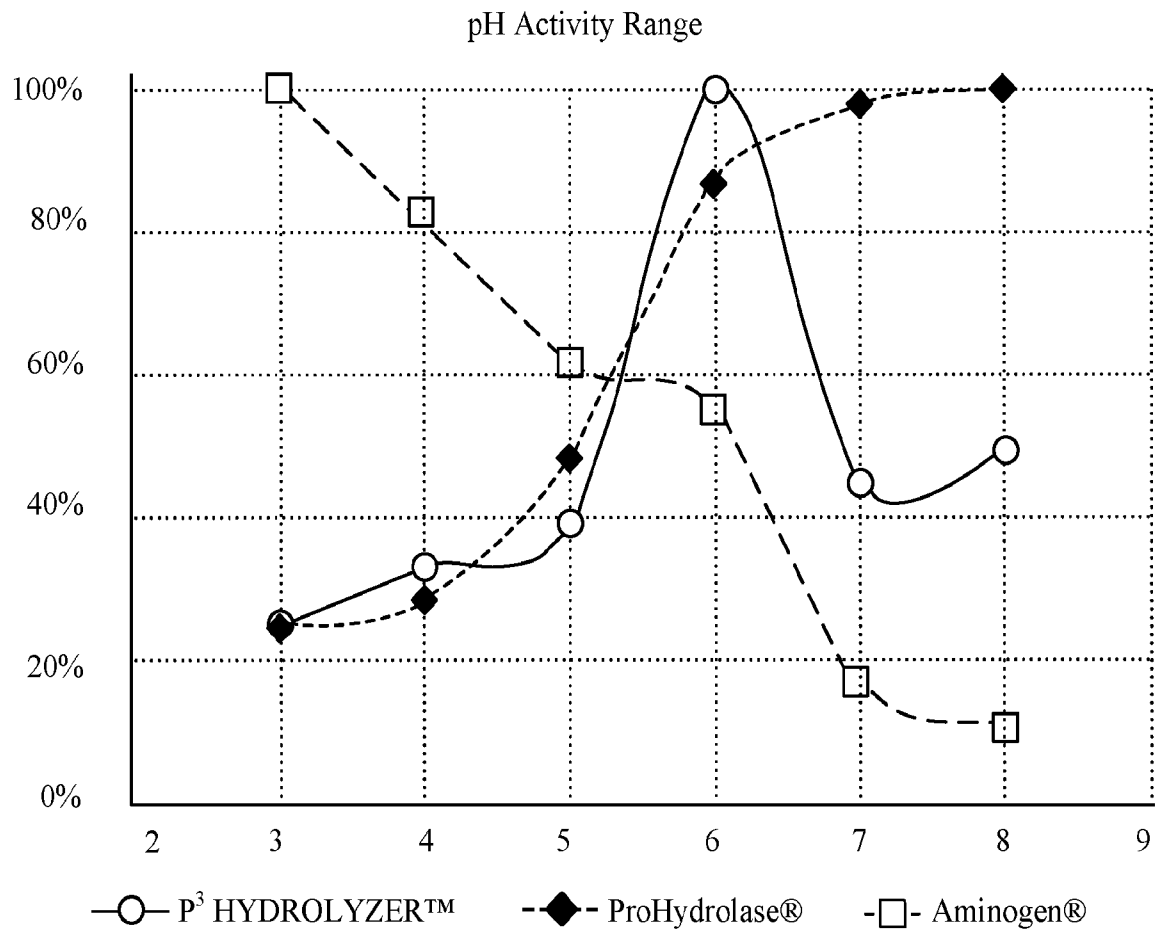


FIG. 4

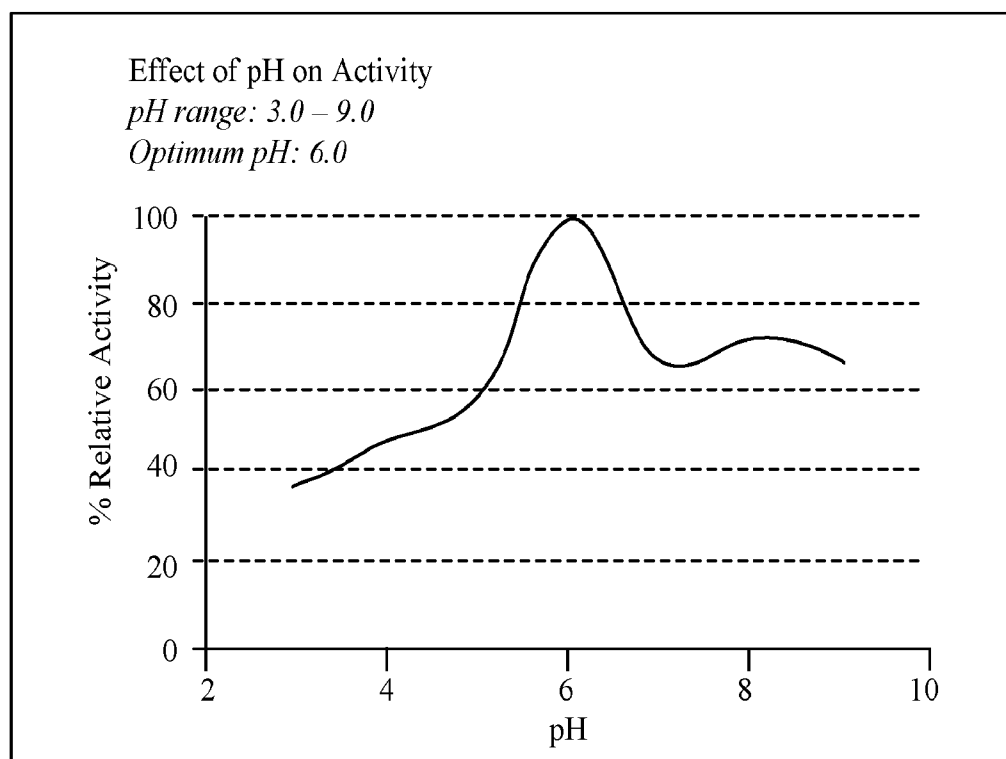


FIG. 5A

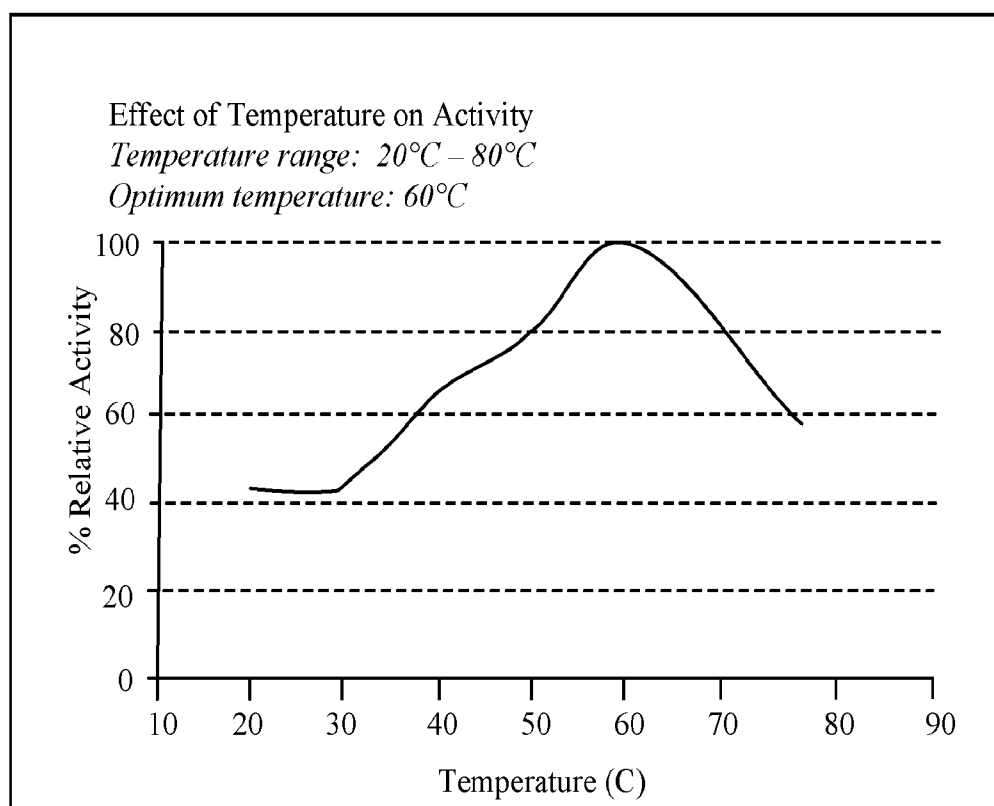


FIG. 5B

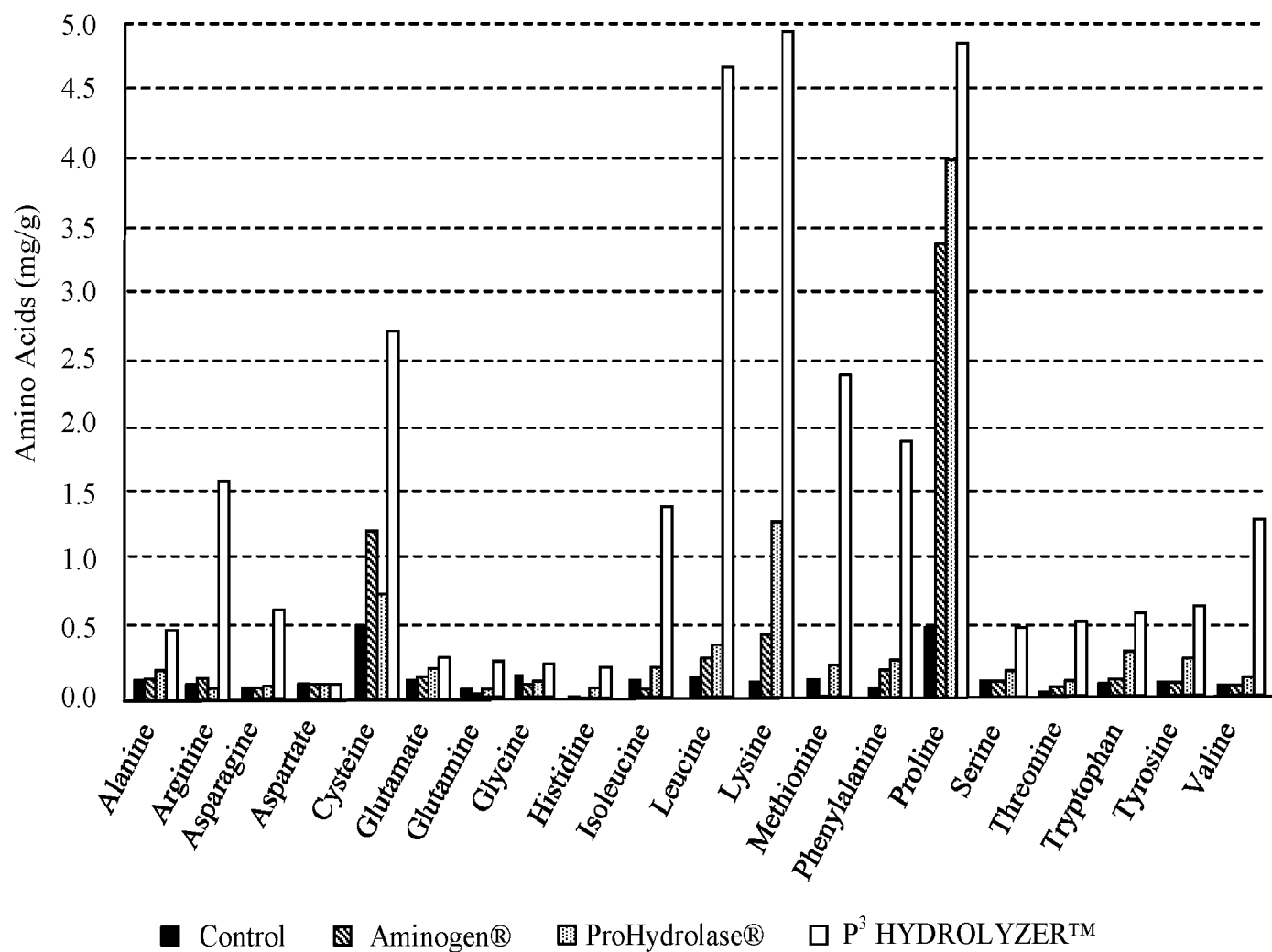


FIG. 6

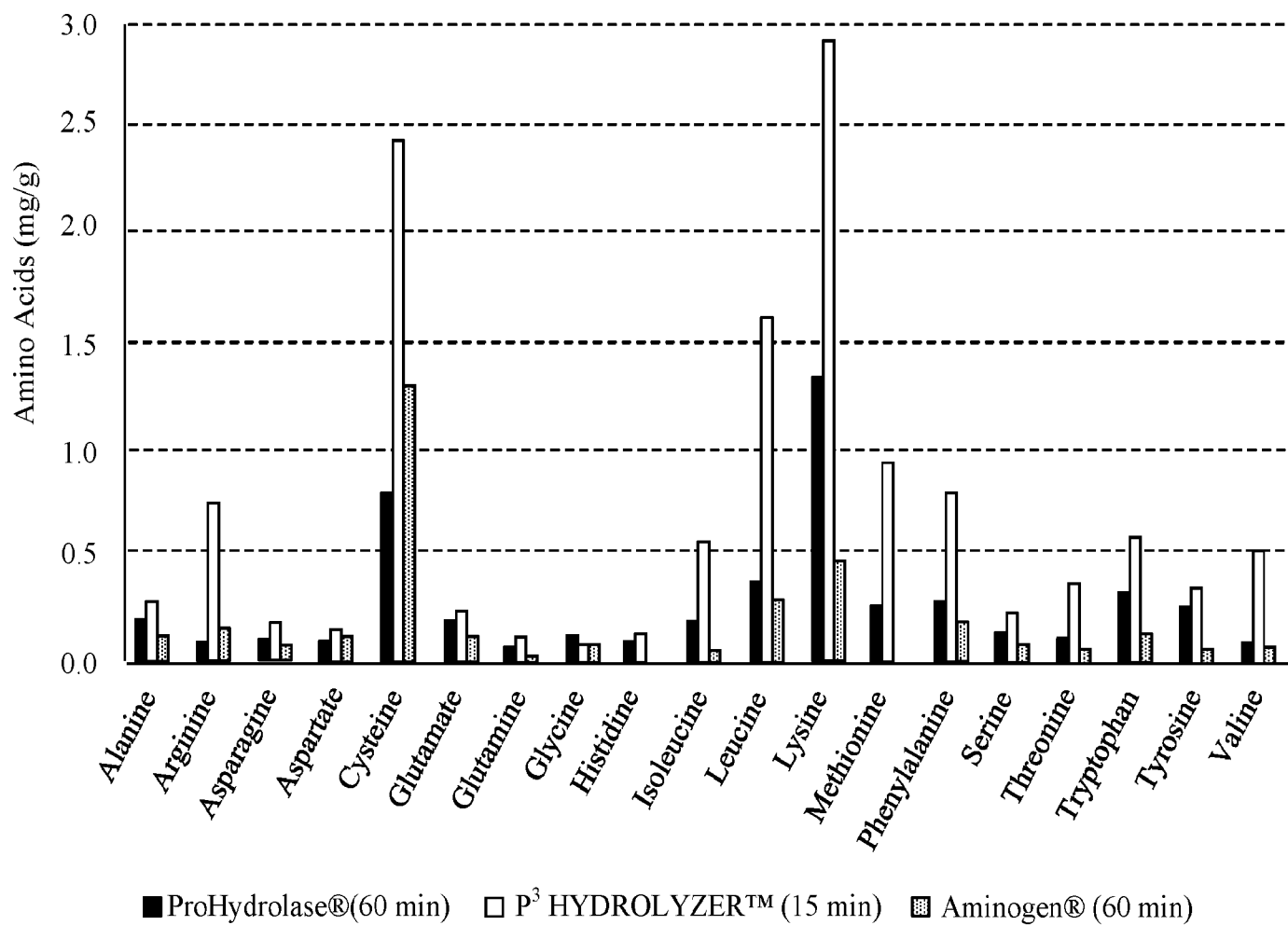


FIG. 7

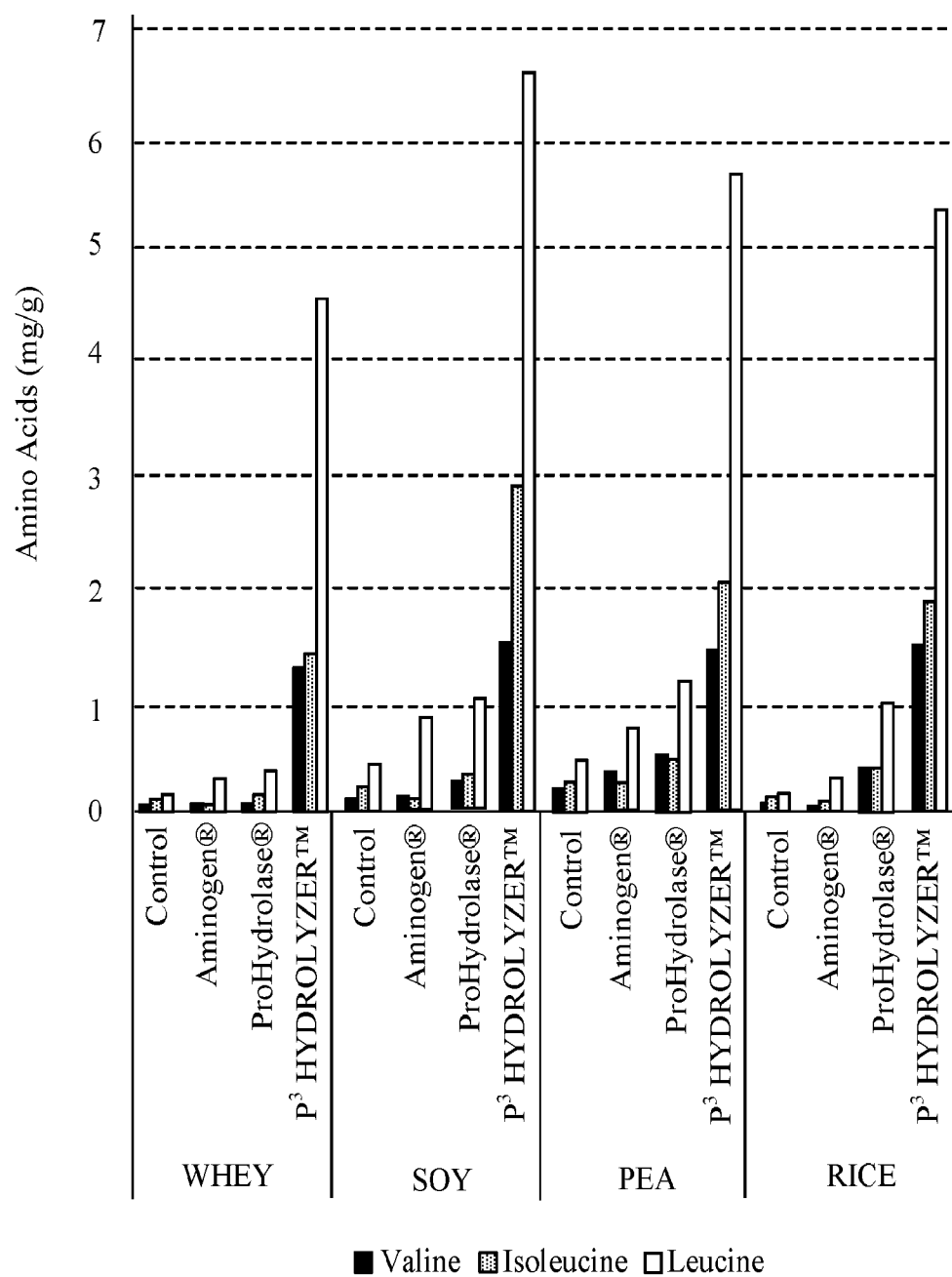


FIG. 8

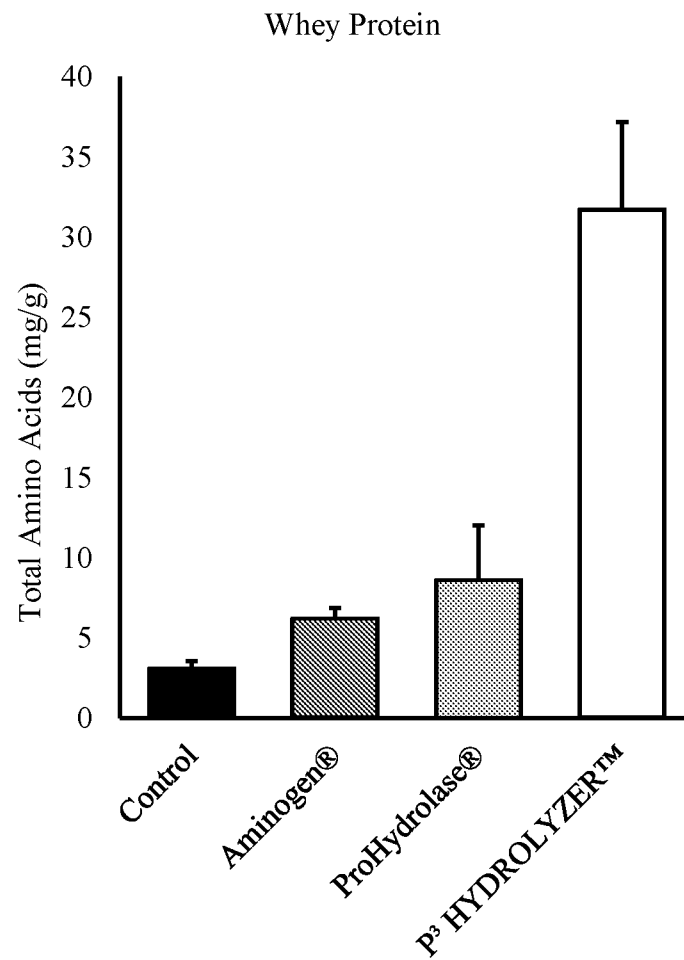


FIG. 9A

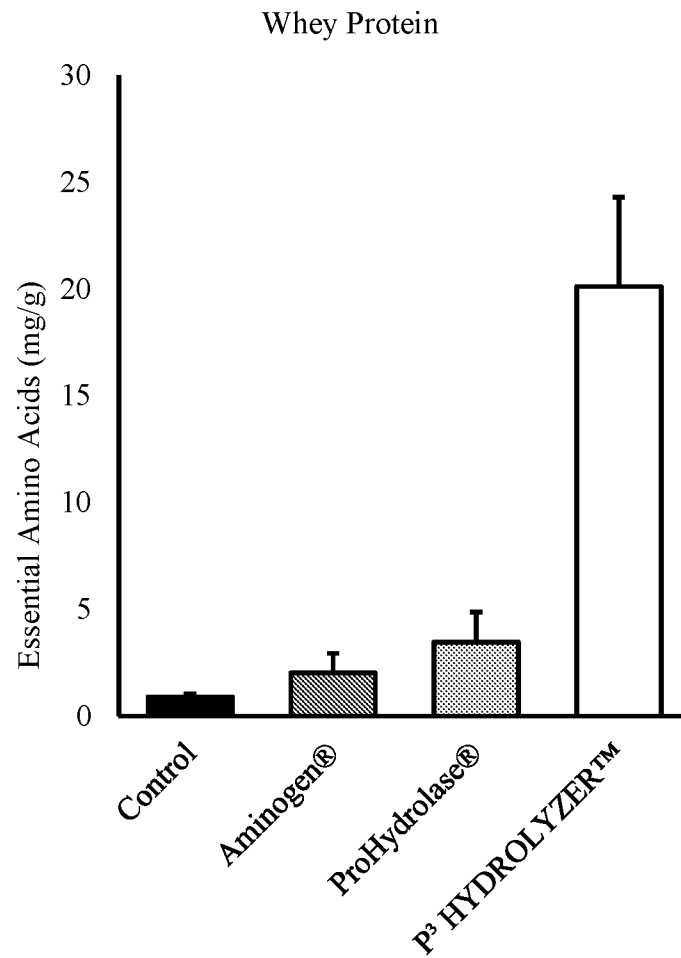


FIG. 9B

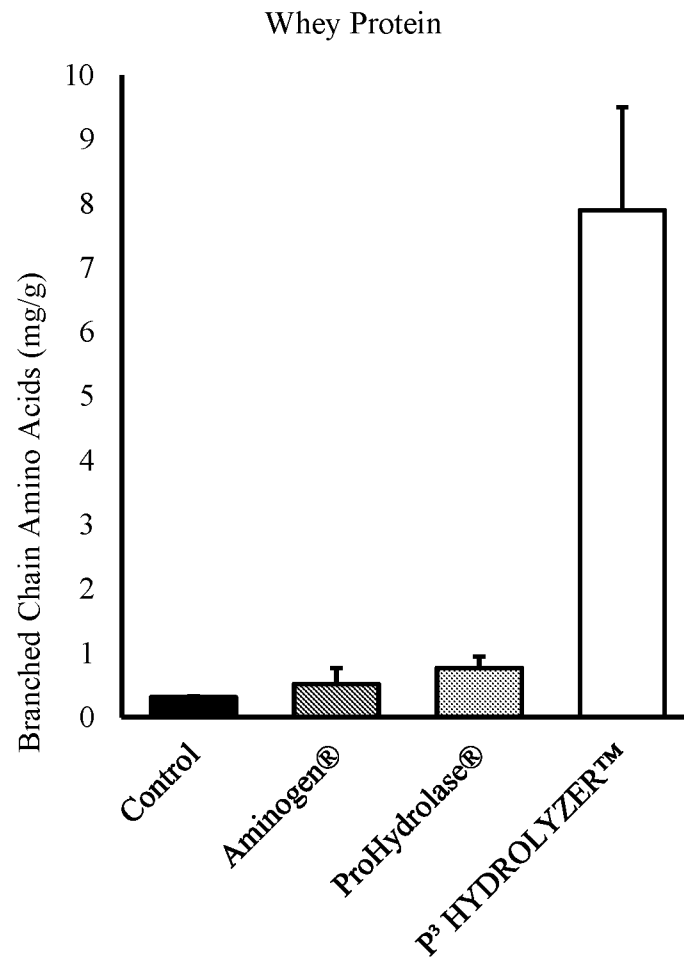


FIG. 9C

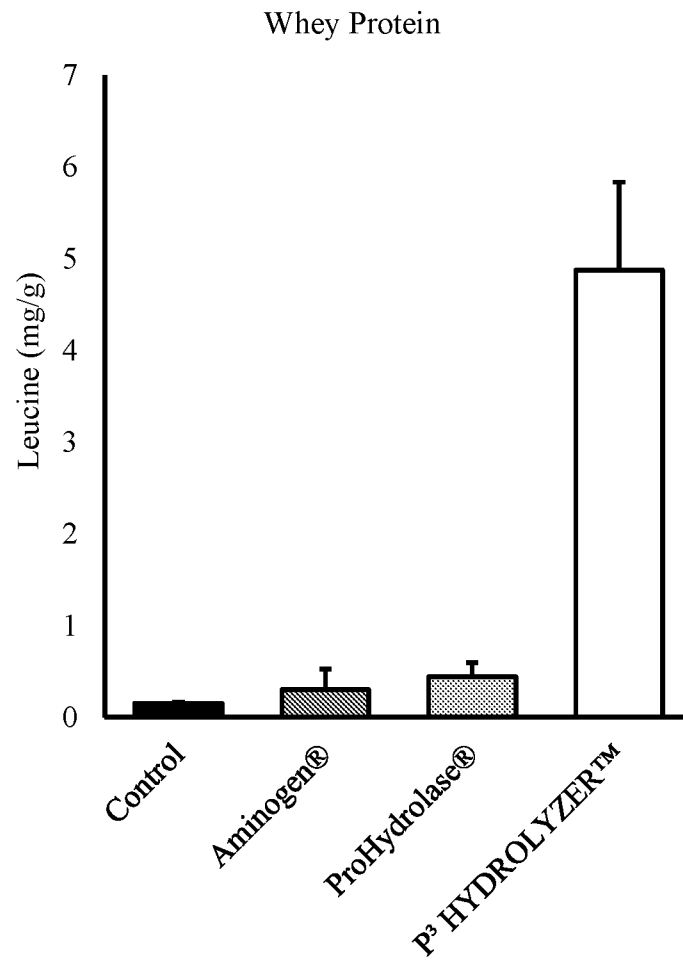


FIG. 9D

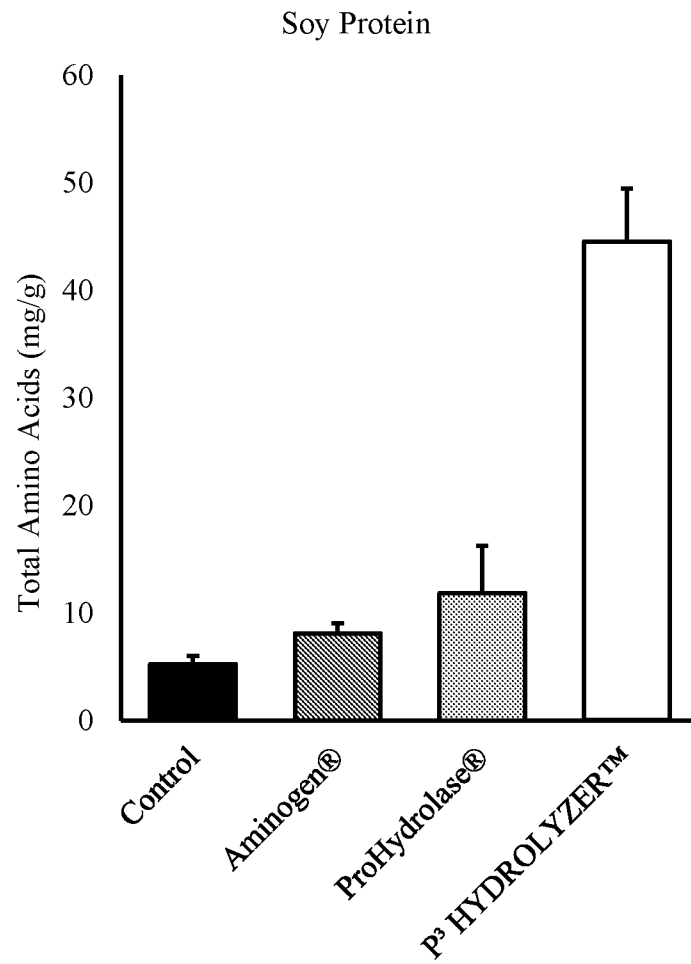


FIG. 10A

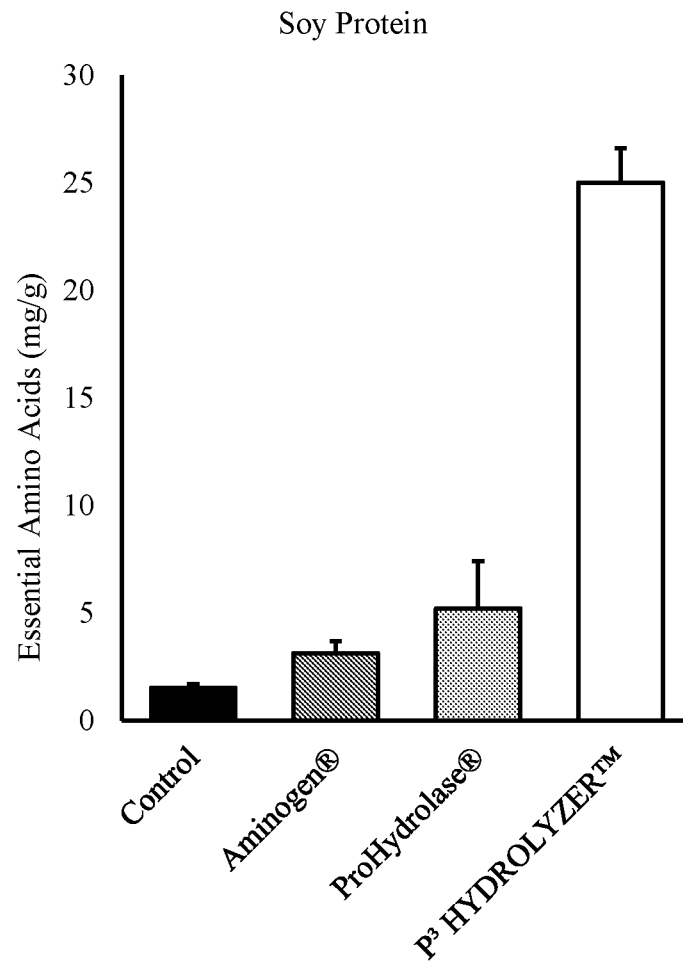


FIG. 10B

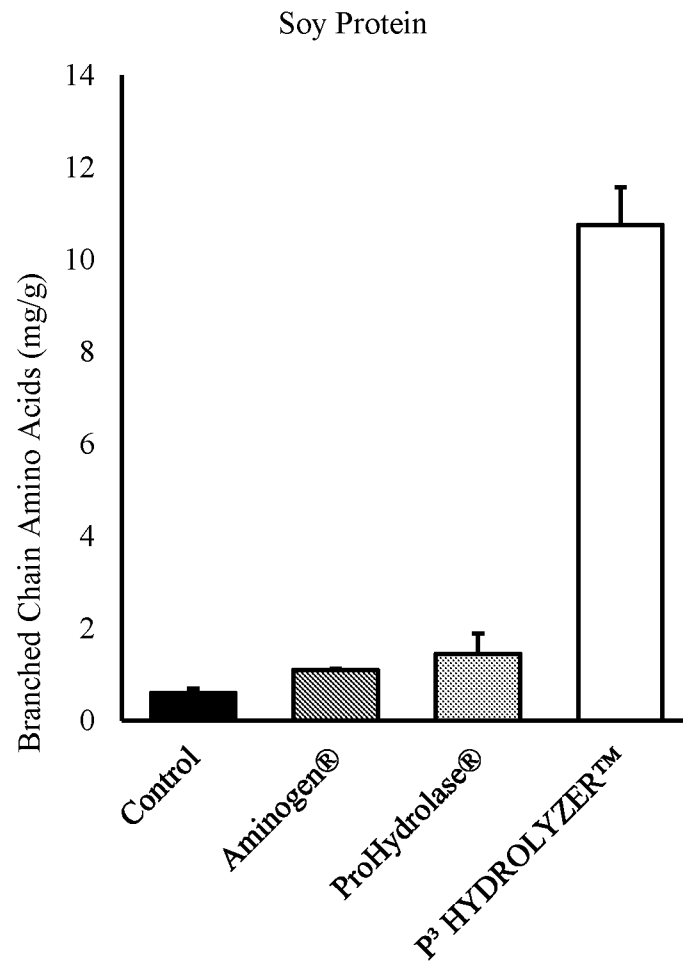


FIG. 10C

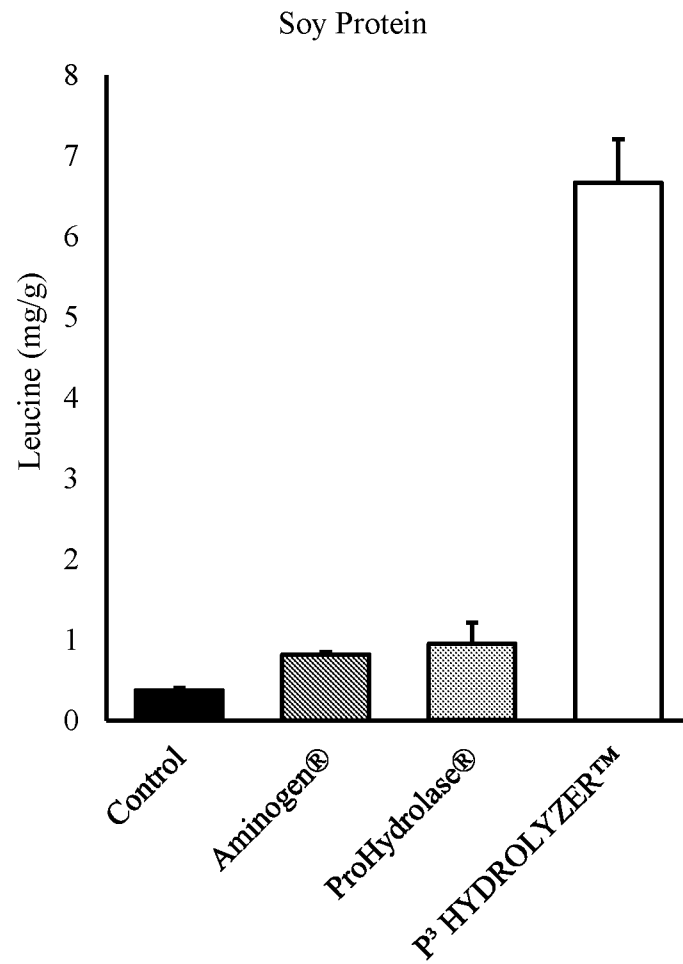


FIG. 10D

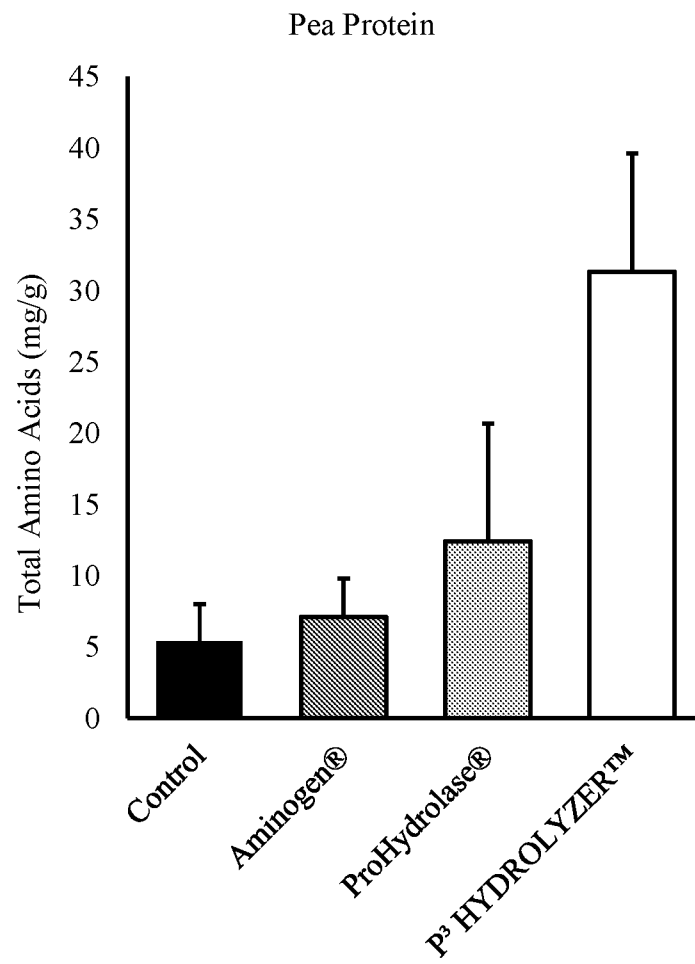


FIG. 11A

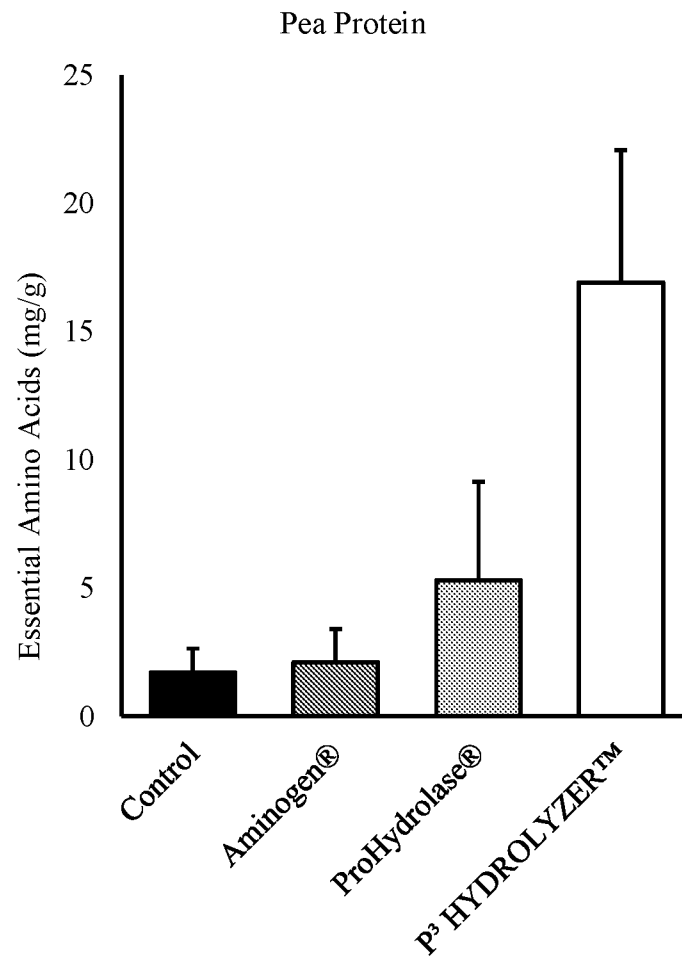


FIG. 11B

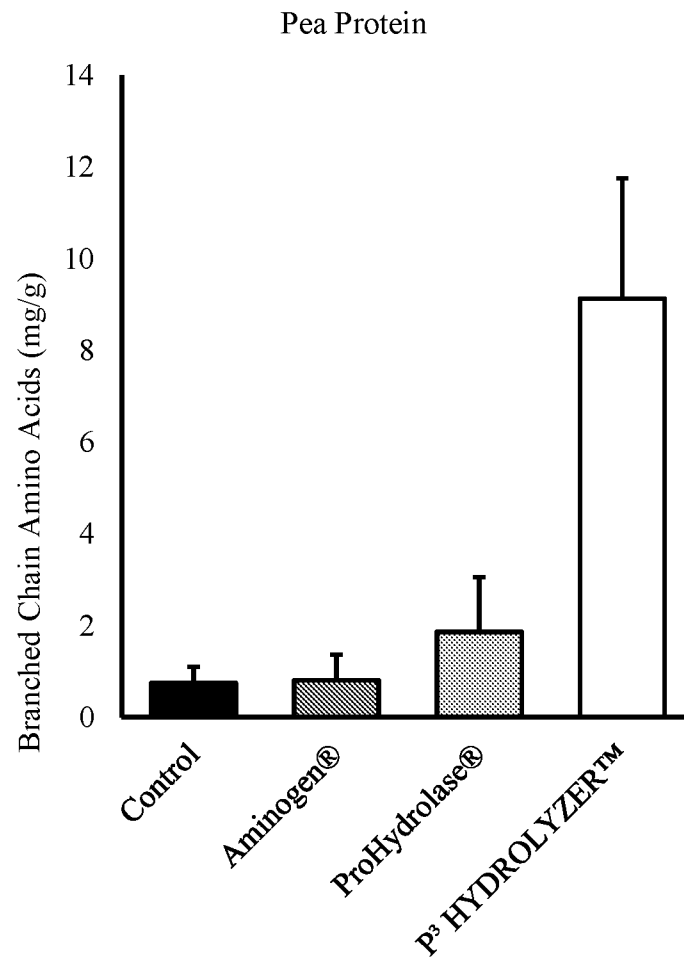


FIG. 11C

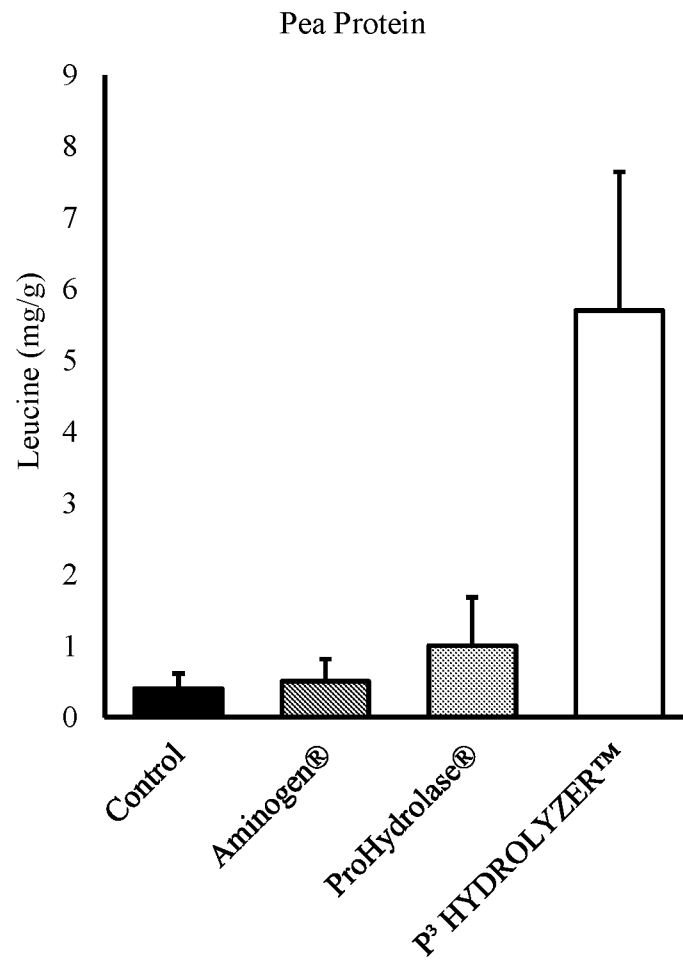


FIG. 11D

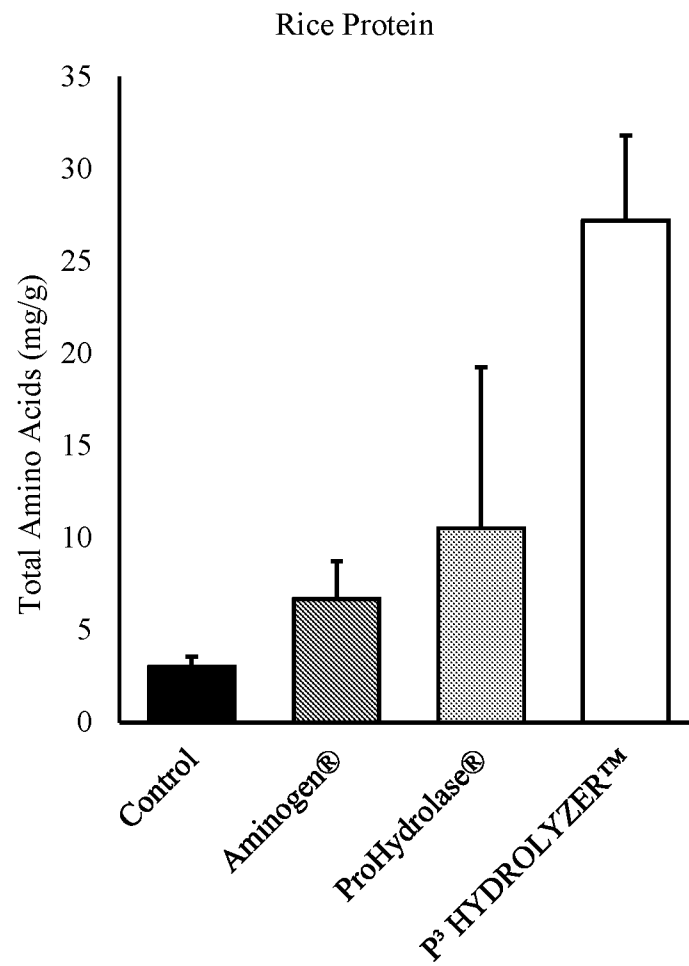


FIG. 12A

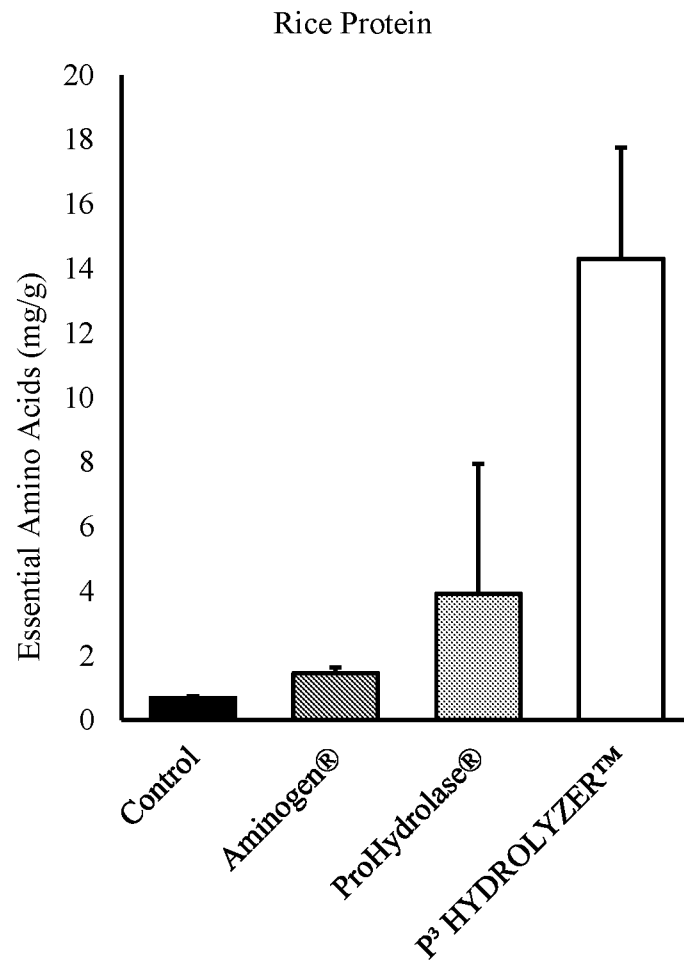


FIG. 12B

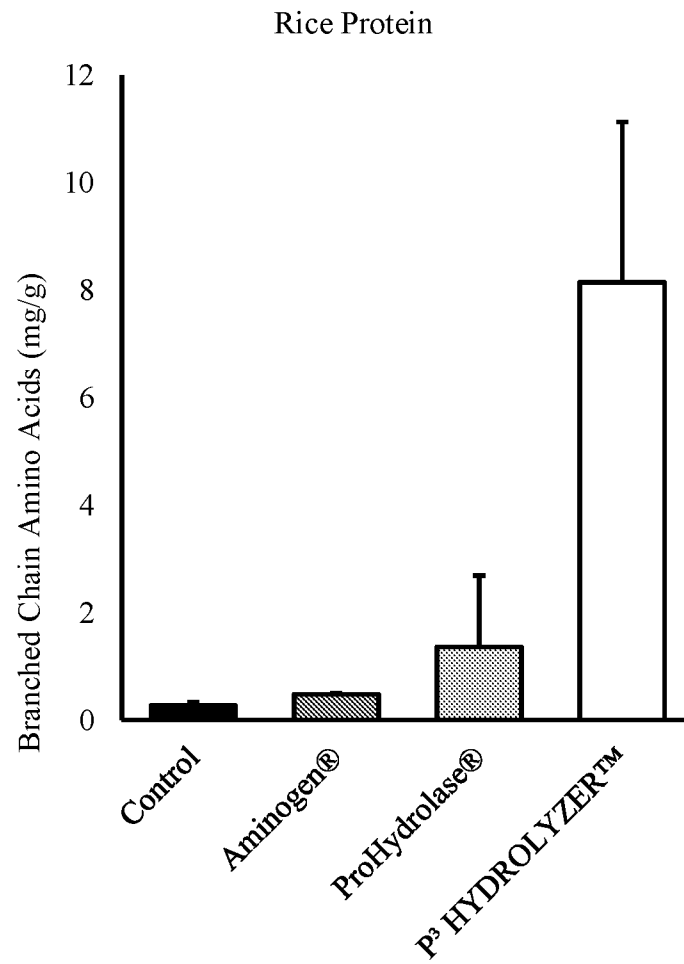


FIG. 12C

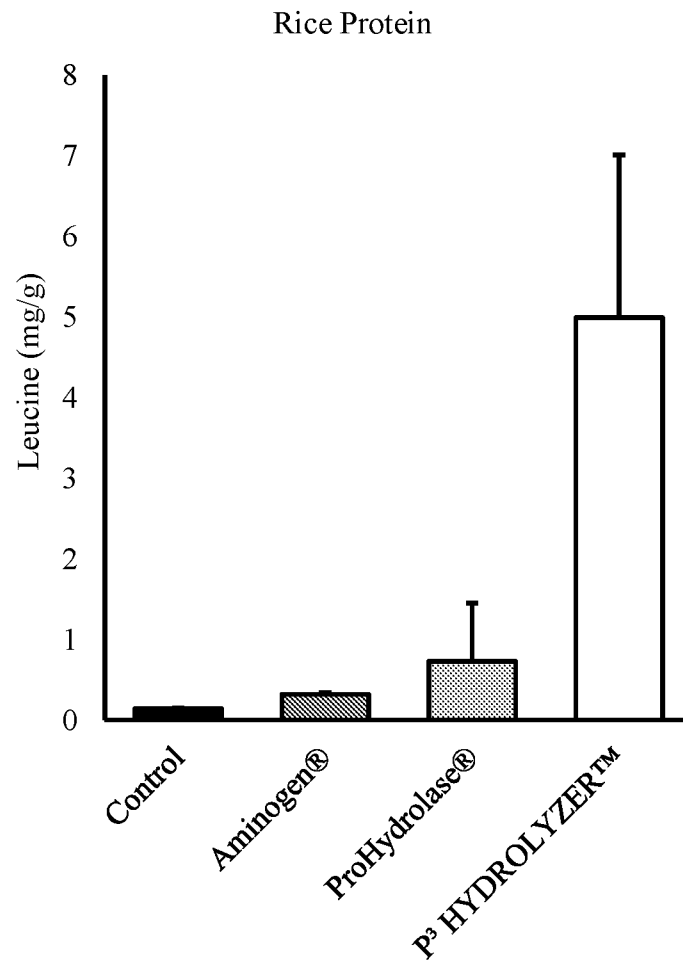


FIG. 12D

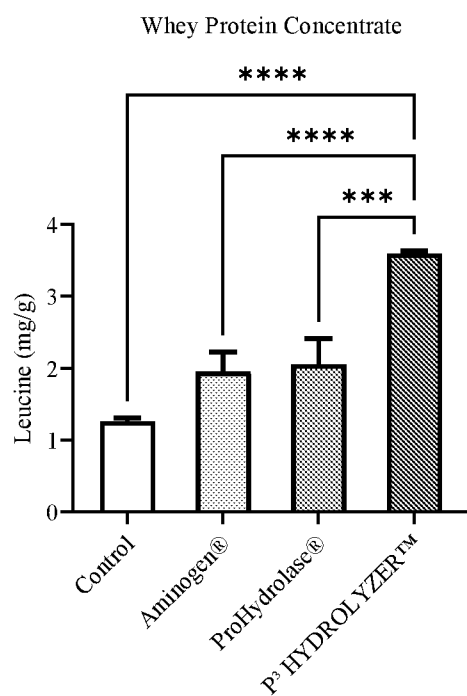


FIG. 13A

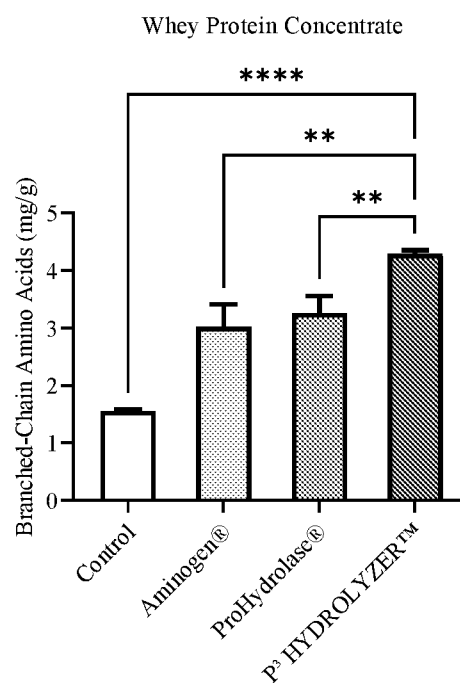


FIG. 13B

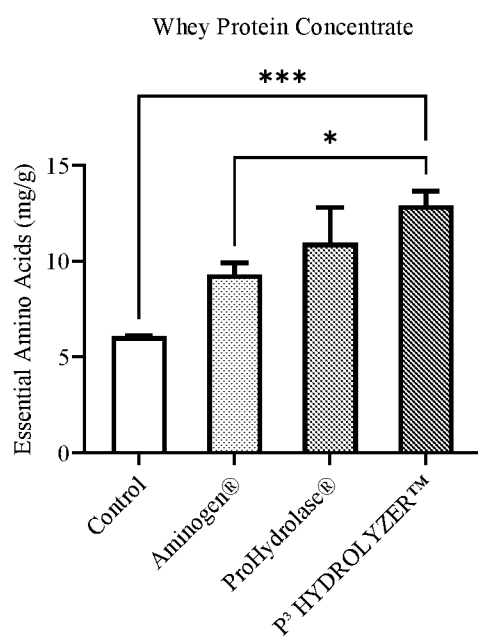


FIG. 13C

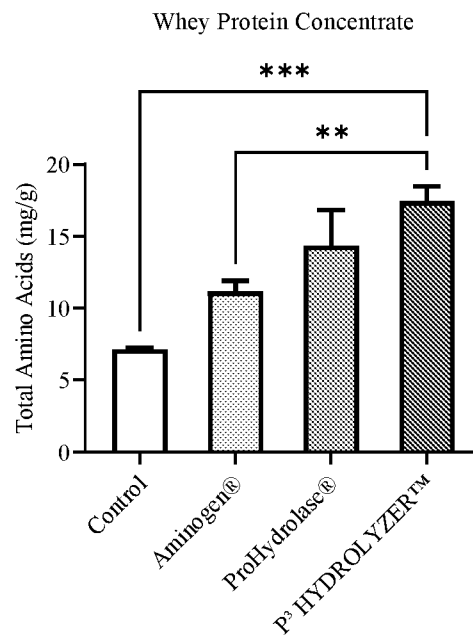


FIG. 13D

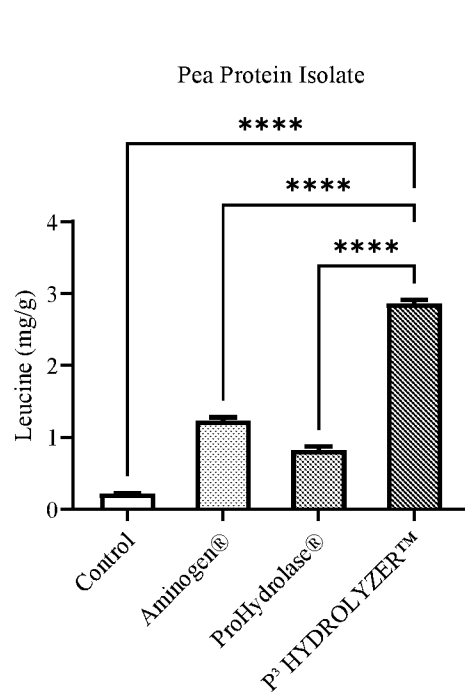


FIG. 14A

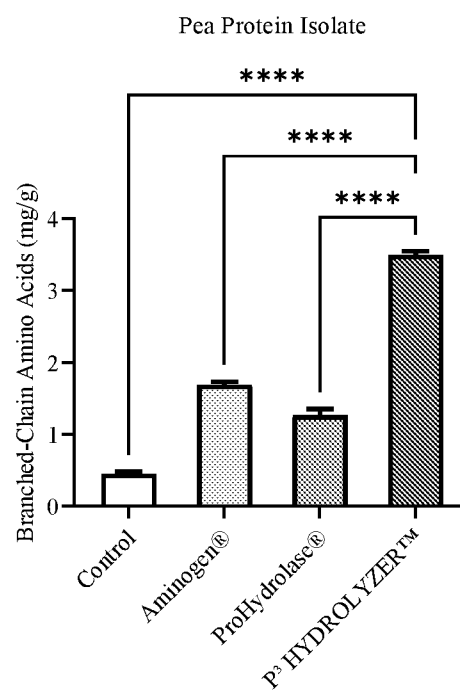


FIG. 14B

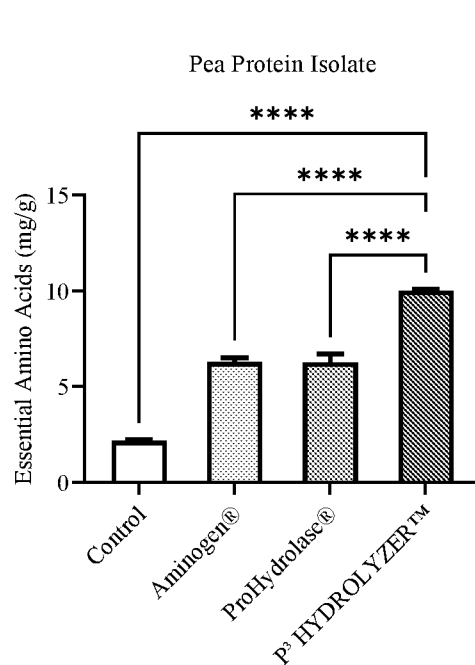


FIG. 14C

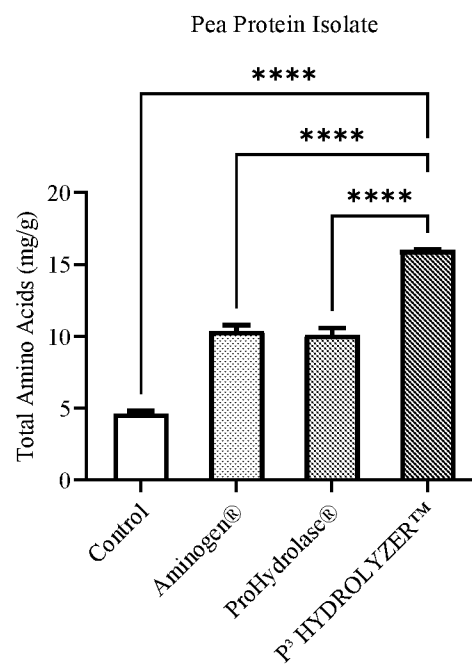


FIG. 14D

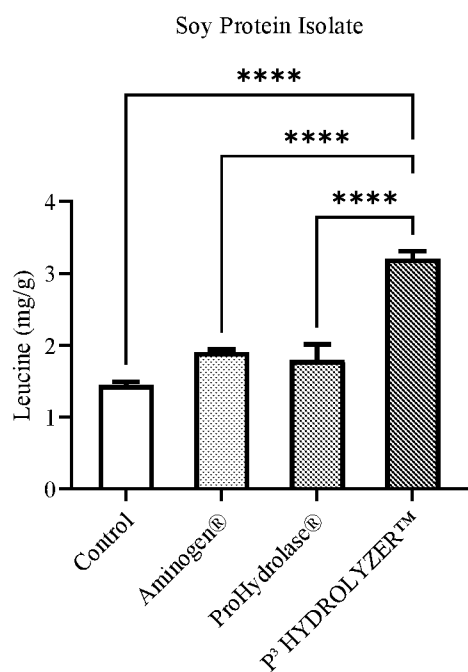


FIG. 15A

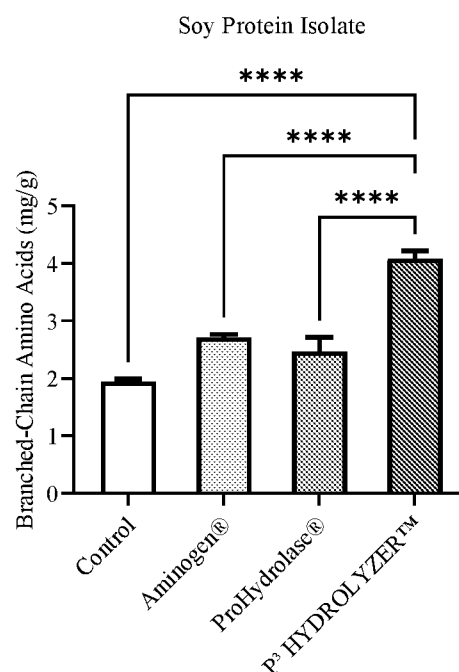


FIG. 15B

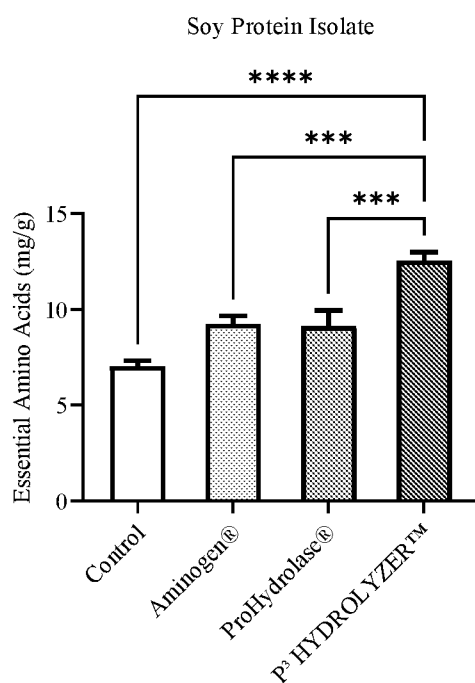


FIG. 15C

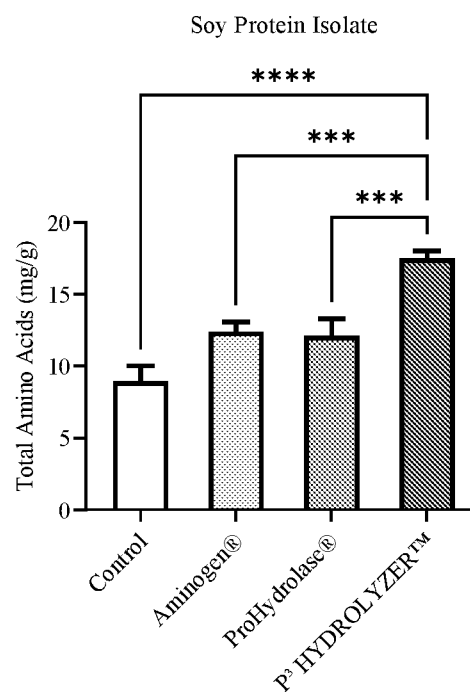


FIG. 15D

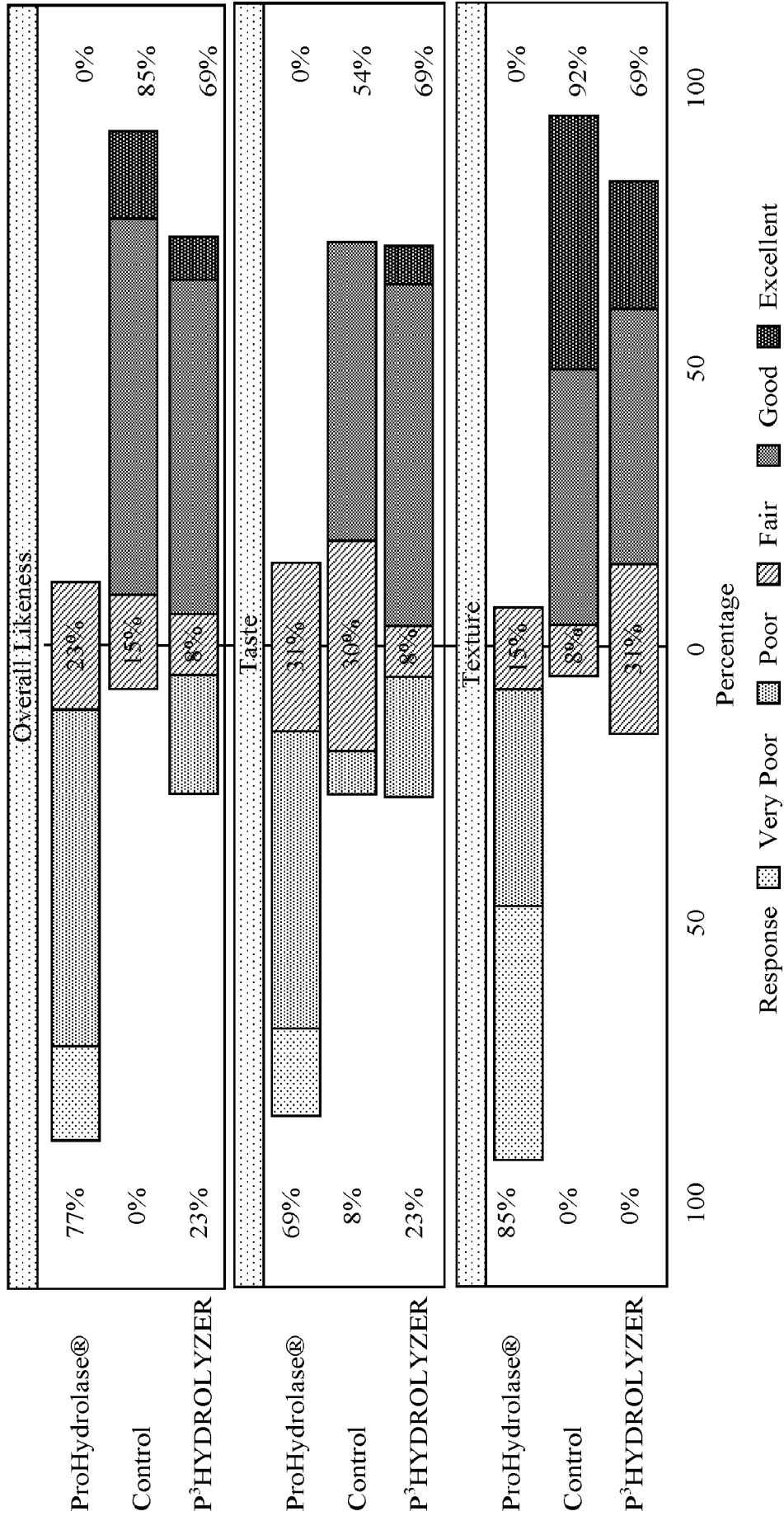


FIG. 16

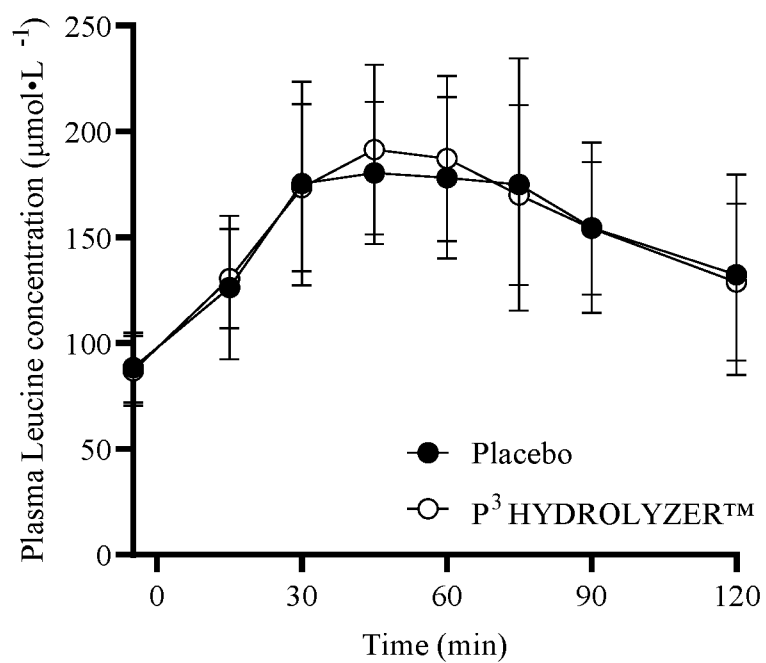


FIG. 17A

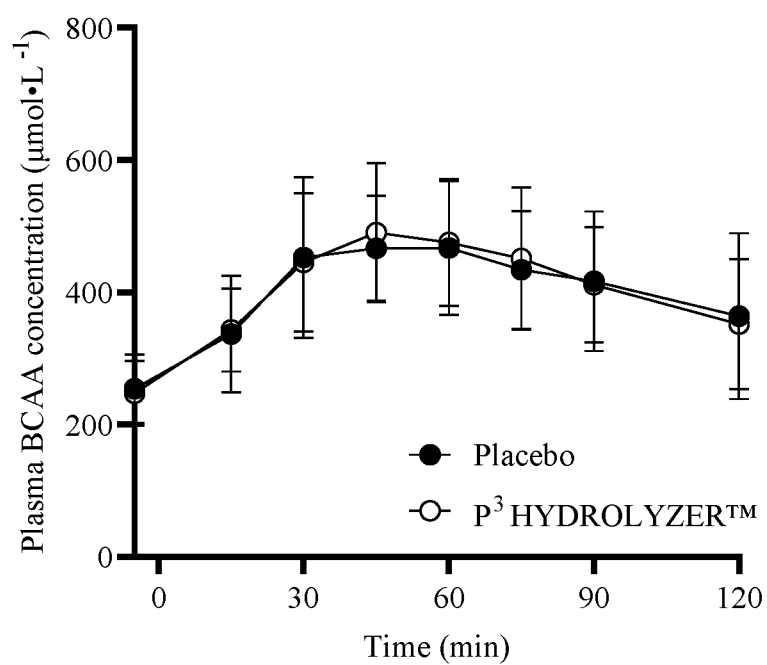


FIG. 17B

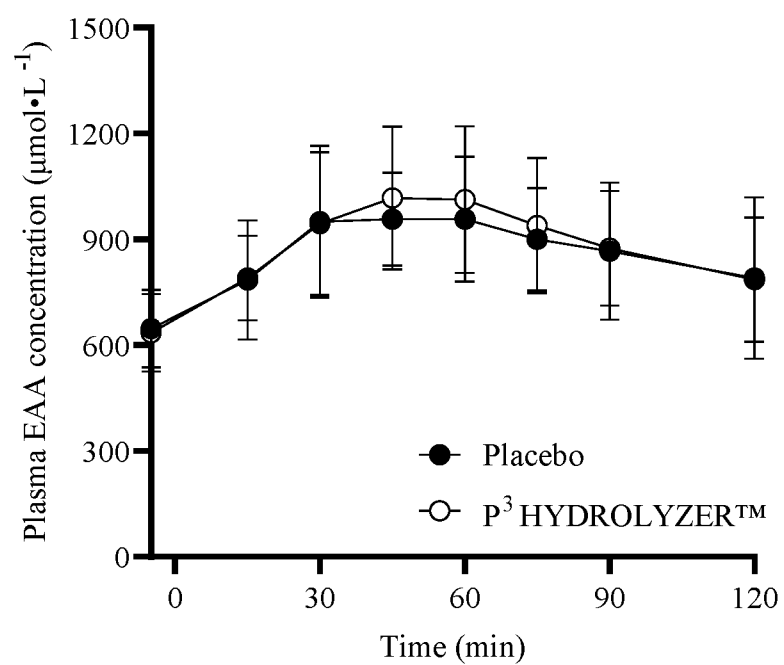


FIG. 17C

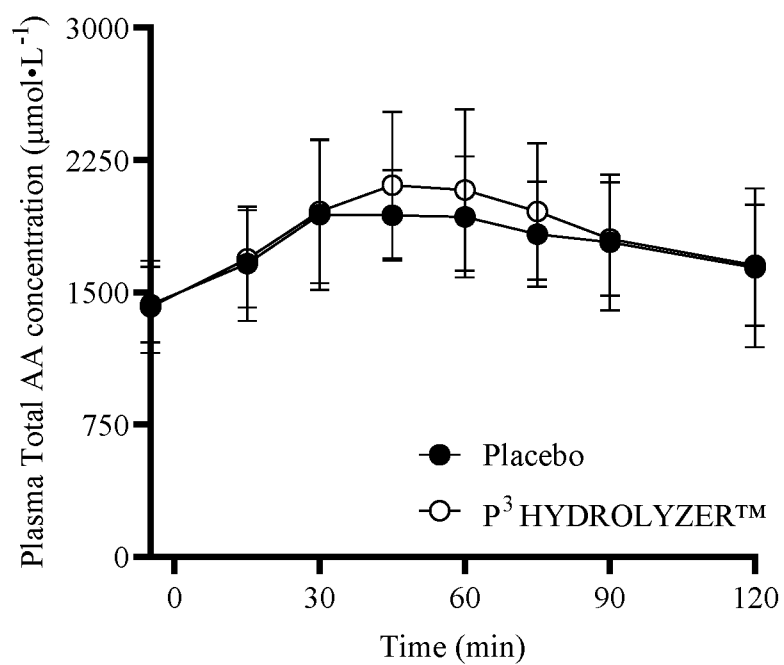


FIG. 17D

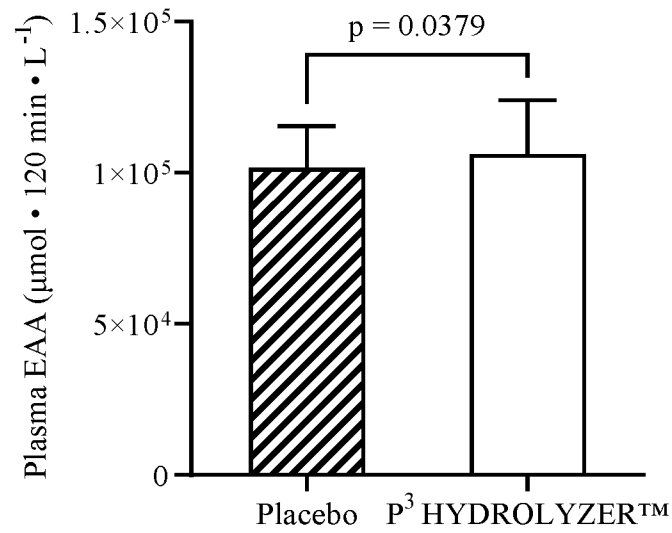


FIG. 18A

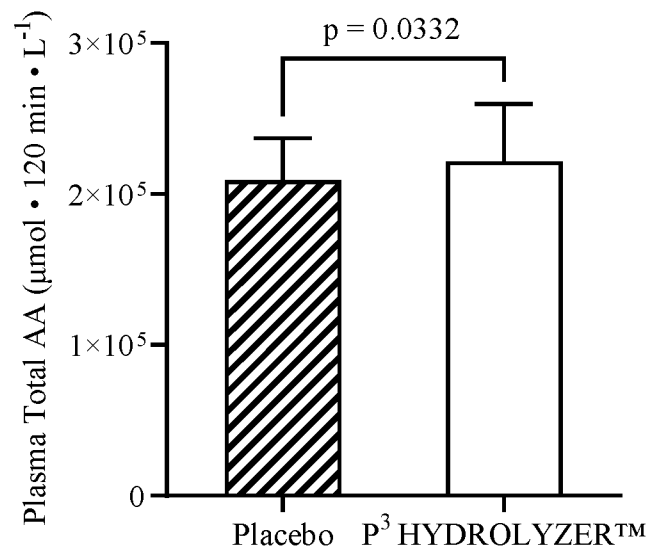


FIG. 18B

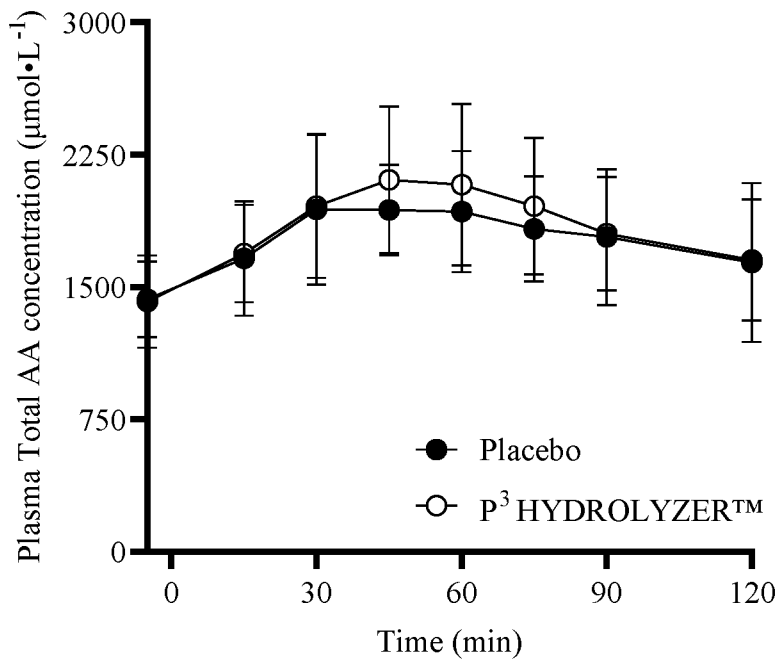


FIG. 17D