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Canadian Intellectual Property Office

CA 3093885 A1 2020/01/30

(21) 3 093 885

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2019/07/25
(87) Date publication PCT/PCT Publication Date: 2020/01/30
(85) Entrée phase nationale/National Entry: 2020/09/11
(86) N° demande PCT/PCT Application No.: KR 2019/009215
(87) N° publication PCT/PCT Publication No.: 2020/022791
(30) Priorités/Priorities: 2018/07/25 (KR10-2018-0086308); 2019/03/15 (KR10-2019-0029758)

(51) Cl.Int./Int.Cl. *C12N 9/26* (2006.01), *A61K 38/16* (2006.01), *A61K 45/06* (2006.01), *A61P 35/00* (2006.01)

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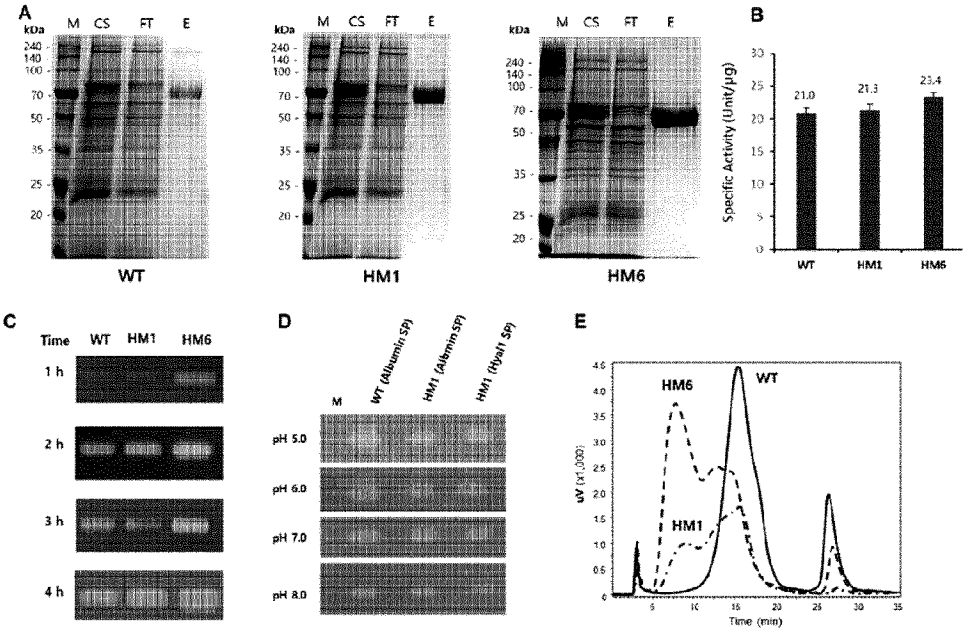
(54) Title: NOVEL HYALURONIDASE VARIANTS AND PHARMACEUTICAL COMPOSITION COMPRISING THE SAME



(57) Abrégé/Abstract:
The present invention relates to a technical field of protein engineering for increasing the enzyme activity and thermal stability of human hyaluronidase, which is a hyaluronic acid-hydrolyzing enzyme, and relates to a hyaluronidase PH20 mutant or a fragment thereof, comprising: the substitution of at least one amino acid residue, among the amino acid sequence of the wild-type PH20 having SEQ ID NO: 1, at an alpha helix site and/or a site corresponding to a connection site thereof; and selectively, the additional deletion of an N-terminal amino acid residue and/or a C-terminal amino acid residue. Specifically, the present invention relates to a PH20 mutant and a fragment thereof, the wild-type PH20, having a sequence of SEQ ID NO: 1, comprising: the substitution of at least one residue selected from the group consisting of T341A, T341C, T341G, S343E, M345T, K349E, L353A, L354I, N356E and I361T; additionally, the substitution of an amino acid positioned at alpha helix site 8 and/or the connection site of alpha helix site 7 and alpha helix site 8; and the deletion of some amino acids of an N-terminal site and a C-terminal site.

ABSTRACT

The present invention is related to the field of protein engineering technology which increases the enzymatic activity and thermal stability of human hyaluronidase which is an enzyme that hydrolyzes hyaluronic acid; and more particularly to hyaluronidase PH20 variants or fragments thereof, which comprise one or more amino acid residue substitutions in the region corresponding to the alpha-helix region and its linker region in the amino acid sequence of wild-type PH20 of SEQ ID NO: 1 and in which one or more amino acid residues at the N-terminus and/or the C-terminus are selectively cleaved additionally.

Specifically, the present invention relates to PH20 variants or fragments thereof, which comprise one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, T341G, S343E, M345T, K349E, L353A, L354I, N356E and I361T in wild-type PH20 having the amino acid sequence of SEQ ID NO: 1, and additionally comprise the substitution of amino acids located in the alpha-helix 8 region and/or a linker region between alpha-helix 7 and alpha-helix 8 in the amino acid sequence of wild-type PH20, and in which one or more amino acids located at the N-terminal and C-terminal regions are deleted.

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NOVEL HYALURONIDASE VARIANTS AND PHARMACEUTICAL COMPOSITION COMPRISING THE SAME

TECHNICAL FIELD

[1] The present invention relates to novel human hyaluronidase variants having increased enzymatic activity and thermal stability compared to human hyaluronidase which is an enzyme that hydrolyzes hyaluronic acid, and more particularly to hyaluronidase PH20 variants or fragments thereof, which comprise one or more amino acid residue substitutions in the region corresponding to the alpha-helix region and/or its linker region in wild-type PH20 having the amino acid sequence of SEQ ID NO: 1, preferably mature wild-type PH20 consisting of amino acid residues L36 to S490, and in which one or more of the N-terminal or C-terminal amino acid residues are selectively deleted, a method for producing the same, and a pharmaceutical composition comprising the same.

BACKGROUND ART

[3] The human skin is composed of epidermis, dermis and a subcutaneous fat layer, and there are six types of glycosaminoglycans in the skin. These glycosaminoglycans include hyaluronic acid, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, and keratin sulfate.

[4] These glycosaminoglycans are composed of repeating disaccharide sugar units. The number of the disaccharide sugar units is different among the glycosaminoglycans, but ranges

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from several hundreds to thousands. Among the glycosaminoglycans, hyaluronic acid is present in the skin more than half of the amount in the body. Hyaluronic acid is synthesized by hyaluronan synthase present in the cell membrane, is present alone without binding to proteoglycans, and is the only glycosaminoglycan having no sulfate group. Other glycosaminoglycans bind to proteoglycans and have a sulfate group. Hyaluronic acid consists of glucuronic acid and N-acetylglucosamine linked via alternating β-1,4 and β-1,3 bonds, and is composed of about 5,000 repeating units of these disaccharides. It is known that about one-third (5 g) of hyaluronic acid in the human body is turned over every day.

[5] Hyaluronidases are enzymes that degrade hyaluronic acid present in the extracellular matrix. It is known that there are six types of hyaluronidases in humans:they are Hyal1, Hyal2, Hyal3, Hyal4, HyalPS1, and PH20/SPAM1. Human Hyal1 and Hyal2 are expressed in most of the tissues. PH20/SPAM1 (hereinafter referred to as PH20) is expressed in the sperm plasma membrane and the acrosomal membrane. However, HyalPS1 is not expressed because it is a pseudogene. Hyaluronidases are divided into, according to a method of cleaving hyaluronic acid, three types: enzymes (EC 3.2.1.35) that cleave β-1,4 bonds between N-acetylglucosamine and glucuronic acid by the use of H_2O; enzymes (EC 3.2.1.36) that cleave β-1,3 bonds between N-acetylglucosamine and glucuronic acid by the use of H_2O; and bacterial hyaluronidases (EC 4.2.99.1) that cleave β-1,4 bonds without using H_2O.

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[6] The catalytic amino acids of Hyal1 are D129 and E131, which hydrolyze hyaluronic acid by substrate-assisted catalysis. Hyal1 exhibits the optimum activity at an acidic pH of 3 to 4, and has no enzymatic activity at pH 4.5 or higher. In contrast to Hyal1, PH20 exhibits enzymatic activity at a wide pH range of 3 to 8.

[7] Arming et al. identified that the catalytic amino acids of PH20 are D111 and E113 (Arming et al., 1997). Arming et al. labelled Leu as the first amino acid of the mature protein, and thus the catalytic amino acids of the full-length PH20 with the signal peptide correspond to D146 and E148, respectively.

[8] Hyaluronidase hydrolyzes hyaluronic acid, thereby reducing the viscosity of hyaluronic acid in the extracellular matrix and increasing the permeability thereof into tissue (skin). The subcutaneous area of the skin has a neutral pH of about 7.0 to 7.5. Thus, among the various types of hyaluronidases, PH20 is widely used in clinical practice (Bookbinder et al., 2006). In examples in which PH20 is used in clinical practice, PH20 is used as an eye relaxant and an anesthetic additive in ophthalmic surgery, and is also co-administered with an antibody therapeutic agent which is injected subcutaneously (Bookbinder et al., 2006). In addition, based on the property of hyaluronic acid that is overexpressed in tumor cells, PH20 is used to hydrolyze hyaluronic acid in the extracellular matrix of tumor cells, thereby increasing the access of an anticancer therapeutic agent to the tumor

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cells. In addition, it is also used to promote resorption of body fluids and blood, which are excessively present in tissue.

[9] PH20 was first identified in guinea pig sperm by Lathrop et al., and is also known to be expressed in sperms of different species. Human PH20 gene was cloned by Lin et al. and Gmachl et al. Human PH20 has the amino acid sequence of SEQ ID NO: 1 which consists of 509 amino acid residues, and exhibits 60% amino acid identity with guinea pig PH20 gene. Human PH20 enzyme is encoded from SPAM1 (sperm adhesion molecule-1) gene, and Ser490 of PH20 is present as binding to the glycosylphosphatidylinositol (GPI) on the surface of the sperm plasma membrane and in the acrosomal membrane. Sperm hydrolyzes hyaluronic acid using PH20 when it penetrates oocytes through the hyaluronan-rich cumulus layer of the oocytes. PH20 is present in the amount corresponding to 1% or less of the amount of proteins in sperm, and has six N-glycosylation sites (N82, N166, N235, N254, N368, and N393).

[10] Currently commercially available PH20 is obtained by extraction from the testes of cattle or sheep. Examples thereof include Amphadase® (bovine hyaluronidase) and Vitrase® (sheep hyaluronidase).

[11] Bovine testicular hyaluronidase (BTH) is obtained by removing a signal peptide and 56 amino acids on the C-terminal from bovine wild-type PH20 during post-translational modification. BTH is also a glycoprotein, and has a mannose content of 5% and a glucosamine content of 2.2%, based on the total components including amino acids. When animal-derived

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hyaluronidase is repeatedly administered to the human body at a high dose, a neutralizing antibody can be produced. Since animal-derived hyaluronidase contains other biomaterials in addition to PH20, it may cause an allergic reaction when administered to the human body (Bookbinder et al., 2006). In particular, the production and the use of PH20 extracted from cattle can be limited due to concerns of mad cow disease. In order to overcome this problem, studies on the recombinant protein of human PH20 have been conducted.

[12] Recombinant protein of human PH20 has been reported to be expressed in yeast (*P. pastoris*), DS-2 insect cells, and animal cells. The recombinant PH20 proteins produced in insect cells and yeast differ from human PH20 in terms of the pattern of N-glycosylation during post-translational modification.

[13] Among hyaluronidases, only three dimensional structures of Hyal1 (PDB ID: 2PE4)(Chao et al., 2007) and bee venom hyaluronidase (PDB ID: 1FCQ, 1FCU, 1FCV) are determined. Hyal1 is composed of two domains, a catalytic domain and an EGF-like domain. The catalytic domain is in the form of $(\beta/\alpha)_8$ in which an alpha-helix and a beta-strand, which characterize the secondary structure of the protein, are each repeated eight times (Chao et al., 2007). The EGF-like domain is completely conserved in variants in which the C-terminus of Hyal1 is spliced differently. The amino acid sequences of Hyal1 and PH20 are 35.1% identical, and the protein structure of PH20 has not yet been found.

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[14] A recombinant protein of human PH20 was developed by Halozyme Therapeutic, Inc. and has been sold under the trade name Hylenex® (Bookbinder et al., 2006; Frost, 2007).

[15] When D146 and E148, which are the catalytic amino acids of PH20, were mutated to asparagine (D146N) and glutamine (E148Q), respectively, there was no enzymatic activity (Arming et al., 1997). In addition, when R246 of PH20 was substituted with glycine, the enzymatic activity was reduced by 90%, and when E319 was substituted with glutamine and R322 was substituted with threonine, the enzymatic activity disappeared. A variant in which 36 amino acids at the C-terminus of PH20 were removed (474-509 amino-acid truncation) showed a 75% reduction in enzymatic activity compared to wild-type PH20. This variant was not secreted extracellularly and remained in HeLa cells. When C-terminal 134 amino acids were removed from PH20, PH20 had no enzymatic activity and was not secreted extracellularly. According to Frost et al., the C-terminal 477-483 region of PH20 is essential for soluble expression (Frost, 2007). The activity of full-length PH20 (1 to 509) or a PH20 variant having a C-terminus truncated at position 467 was merely 10% of a PH20 variant having a C-terminus truncated at one of positions 477 to 483 (Frost, 2007).

[16] Meanwhile, recombinant PH20 still has insufficient thermal stability or expression levels in the recombinant cells. Therefore, there is a great demand in industry for a recombinant hyaluronidase having further improved biological and physico-chemical characteristics.

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DISCLOSURE OF INVENTION

TECHNICAL PROBLEM

[19] It is an object of the present invention to provide a hyaluronidase PH20 variant or fragment thereof which is improved in thermal stability, enzyme activity and expression level, compared to wild-type PH20, preferably mature wild-type PH20.

[20] Another object of the present invention is to provide a composition for treating cancer, comprising the above-described hyaluronidase PH20 variant or fragment thereof, and a method of treating cancer using the same.

TECHNICAL SOLUTION

[21] To achieve the above objects, the present invention provides a hyaluronidase PH20 variant or fragment thereof, which comprises one or more amino acid residue substitutions in the region corresponding to an alpha-helix region and/or its linker region in the amino acid sequence of wild-type PH20, preferably mature wild-type PH20, and in which one or more of the N-terminal or C-terminal amino acid residues are selectively deleted.

[22] The present invention also provides a composition for treating cancer, comprising the above-described hyaluronidase PH20 variant or fragment thereof, and a method of treating cancer using the same.

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BRIEF DESCRIPTION OF THE DRAWINGS

[24] FIG. 1 shows the protein tertiary structure model of PH20. The protein tertiary structure of PH20 was modeled using, as a template , Hyal1 (PDB ID: 2PE4)(Chao et.al., 2007) whose protein crystal structure was found, in Swiss-Model server (https://swissmodel.expasy.org/).

[25] FIG. 1A shows the protein tertiary structure model of PH20, and indicates D146 and E148 which are catalytic amino acids. The protein tertiary structure model of PH20 is composed of eight repeats of a beta-strand and an alpha-helix.

[26] FIG. 1B shows an eta (η) 8 loop in which alpha-helix 8 of PH20 and G340 to I344 which form a linker region in the N-terminal region of alpha-helix 8 are located. G340, T341, L342, S343 and I344 residues are each shown.

[27] FIG. 1C shows amino acid residues (C351, Y357, and N363) which interact with the adjacent secondary structure, among the amino acids located in alpha-helix 8 of PH20. C351 forms a disulfide bond with C60 located in alpha-helix 1, Y357 hydrophobically interacts with F315 located between beta-strand 7 and alpha-helix 7, and N363 forms a hydrogen bond with a D69 residue located in alpha-helix 1.

[29] FIG. 2 compares the protein expression levels of WT (wild type) and variants constructed in the present invention. WT and variants were expressed by transient transfection in ExpiCHO cells. WT had an expression level of 16.1 mg/L. The protein expression levels of variants based on the variants HM1 and HM6 were higher than that of WT, and the protein

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expression levels of HM4 and HM7 were the highest. The protein expression level of HM11 obtained by introducing additional amino acid substitutions (Y365F and I367L) into the variant HM6 decreased to 6.4 mg/mL.

[31] FIG. 3 shows experimental results for the variants HM1 and HM6.

[32] FIG. 3A shows the results of SDS-PAGE after purification of WT and the variants HM1 and HM6. Purification was performed using a HisTrap column and a Q Sepharose column. WT and the variants HM1 and HM6 had a molecular weight of ~70 kDa (figure legend: M, molecular weight marker; CS, supernatant; FT, flow-through; and Elution, elution fractions).

[33] FIG. 3B shows the enzymatic activity values of WT and the variants HM1 and HM6, measured by turbidimetric assay at pH 7.0. In the present invention, the enzymatic activity value measured by the turbidimetric assay was expressed as specific activity.

[34] FIG. 3C shows the enzymatic activities of WT and the variants HM1 and HM6, measured by substrate-gel assay. After SDS was removed with 2.5% Triton X-100 (w/v) at 4°C, an enzymatic reaction was performed at 37°C for 1 to 4 h. The variant HM6 renatured faster than WT and the variant HM1 and thus more rapidly hydrolyzed hyaluronic acid on polyacrylamide gel. The white band shows hyaluronic acid degraded by WT and the variant protein.

[35] FIG. 3D shows the enzymatic activities of WT and the variant HM1, measured by substrate-gel assay at pH of 5 to 8.

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WT and the variant HM1 exhibit activity in the pH range of 5 to 8, and show the highest enzymatic activity at pH 5.0. The variant HM1 has the signal peptide of human serum albumin or human Hyal1. The white band shows hyaluronic acid degraded by WT and the variant protein.

[36] FIG. 3E shows the results of separating WT and the variants HM1 and HM6 by a phenyl column. The variants were eluted faster than WT from the phenyl column.

[38] FIG. 4 shows the results of analyzing the final product of hyaluronic acid, degraded by WT and the variant HM6, after 10 min and 1 h by means of an Amide-80 column.

[40] FIG. 5 shows experimental results for G340 to I344 amino acid mutations of PH20.

[41] FIG. 5A shows SDS-PAGE results after HisTrap column purification for variants HM7, HM8, HM9, HM10 and HM21.

[42] FIG. 5B shows the results of measuring the enzymatic activities of WT and variants HM6, HM8, HM9, HM10, HM21 and HM7 by turbidimetric assay at pH 7.0.

[43] FIG. 5C shows the results of measuring the enzymatic activities of WT and variants HM6, HM8, HM9, HM10, HM21 and HM7 by substrate-gel assay. The bar graph at the bottom of FIG. 5C shows the degree of enzymatic activity obtained by quantifying the band after staining the gel with Alcian blue. The white band shows hyaluronic acid degraded by WT and the variant protein.

[44] FIG. 5D shows the results of analyzing WT and variants HM8, HM9, HM10, HM21 and HM7 by phenyl column chromatography.

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[45] FIG. 5E shows the results of separating WT and variants HM6, HM8, HM9, HM10, HM21 and HM7 depending on their isoelectric points at the pH of 3 to 7 by means of IEF gel.

[47] FIG. 6 shows experimental results for the variant HM11.

[48] FIG. 6A shows the results of purifying protein by HisTrap column chromatography for the variant HM11.

[49] FIG. 6B shows the results of measuring the enzymatic activities of WT and the variant HM11 at pH 7.0 by turbidimetric assay.

[51] FIG. 7 shows experimental results for N-terminally truncated PH20 variants HM40, HM13, HM41, HM24, HM42, and HM25.

[52] FIG. 7A shows the results of purifying protein by HisTrap column chromatography for the PH20 variants HM40, HM13, HM41, HM24, HM42 and HM25.

[53] FIG. 7B shows the expression levels of the PH20 variants HM40, HM13, HM41, HM24, HM42, HM25, HP61 and HP62 in ExpiCHO cells.

[54] FIG. 7C shows the enzymatic activities of the PH20 variants HM40, HM13, HM41, HM24, HM42 HM25, HP61 and HP62 measured at pH 7.0 by turbidimetric assay and expressed as specific activities.

[55] FIG. 7D shows the enzymatic activities of the PH20 variants HM40, HM13, HM41, HM24, and HM42, measured by substrate-gel assay. The white band shows hyaluronic acid degraded by WT and the variant protein.

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[56] FIG. 7E shows the results of analyzing WT and the variants HM40, HM13, HM41, HM24 and HM42 by phenyl column chromatography.

[57] FIG. 7F shows the change in particle size with increasing temperature for the PH20 variants HM40, HM13, HM41 and HM42.

[59] FIG. 8 shows experimental results for C-terminally truncated variants HM14, HM15 and HM16 constructed using HM6 as a template.

[60] FIG. 8A shows the results of SDS-PAGE after HisTrap purification for the variants HM14, HM15 and HM16. As controls, WT and the variant HM6 were included.

[61] FIG. 8B shows the results of measuring the enzymatic activities of WT and the variants HM6, HM14, HM15, and HM16 at pH 7.0 by turbidimetric assay.

[62] FIG. 8C shows the results of measuring the enzymatic activities of WT and the variants HM6, HM14, HM15, and HM16 for 1, 2 and 4 h by substrate-gel assay. The right graph of FIG. 8C is a bar graph showing enzymatic activities measured after staining with Alcian-blue after 1 h of enzymatic reaction. The white band shows hyaluronic acid degraded by WT and the variant protein.

[63] FIG. 8D shows the results of analyzing WT and the variants HM6, HM14, HM15, and HM16 by phenyl column chromatography.

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[64] FIG. 8E shows the results of separating the variants HM14, HM15, and HM16 depending on their isoelectric points at the pH of 3 to 7 by IEF gel.

[66] FIG. 9 shows experimental results for PH20 variants HM19 and HM20 constructed using HM10 as a template.

[67] FIG. 9A shows the results of purified protein by HisTrap column chromatography for the PH20 variants HM19 and HM20.

[68] FIG. 9B shows the results of comparing the enzymatic activities of the PH20 variant HM19 and HM20 at pH 7.0 by turbidimetric assay.

[69] FIG. 9C shows the results of staining SDS gel with Alcian blue dye after 1 h of enzymatic reaction at 37°C by substrate-gel assay for WT and the variants HM10, HM19, and HM20. The white band shows hyaluronic acid degraded by WT and the variant protein.

[71] FIG. 10 shows the results of measuring the aggregation temperatures of WT and the PH20 variants by dynamic light scattering (hereinafter referred to as DLS) system. The measurements were performed in triplicate and expressed as mean ± S.E. values.

[73] FIG. 11 shows a Stern-Volmer plot obtained after measuring the change in fluorescence of tryptophan residues of WT and PH20 variants by addition of acrylamide (0 to 0.5 M). Among amino acids, tryptophan is excited at 295 nm and emits maximum fluorescence at 340 nm. Acrylamide is a small molecule that can penetrate a protein structure and quench the fluorescence emission of tryptophan. As the protein structure

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is more flexible, the quenching of fluorescence by acrylamide is greater. F0 is the fluorescence value in the absence of acrylamide, and F is the fluorescence value in the presence of acrylamide (0 to 0.5 M). The change in the fluorescence value measured was expressed as the ratio F0/F.

[74] FIG. 11A is a Stern-Volmer plot for WT and variants HM1, HM4, HM6 and HM7.

[75] FIG. 11B is a Stern-Volmer plots for WT and variants HM14, HM15 and HM16.

[77] FIG. 12 shows the expression levels of HM10-based PH20 variants in ExpiCHO cells.

[78] FIG. 12A graphically shows the expression levels of respective variants.

[79] FIG. 12B shows the expression levels of respective variants in the table. WT and the PH20 variants had a 6xHis-tag at the C-terminus, and the protein expression levels after HisTrap column purification were expressed in mg/L. HM30 to HM33 variants were not expressed in ExpiCHO cells.

[81] FIG. 13 shows Western blot results for cell cultures of variants HM29, HM30, HM31, HM32 and HM33. The C-termini of HM10-based variants HM29, HM30, HM31, HM32 and HM33 were cleaved after A467, C464, D461, C358, or C455, respectively. C-terminally cleaved HM29 was expressed in ExpiCHO cells, but variants having a C-terminus cleaved at C464 or shorter in length was not expressed in ExpiCHO cells. Primary antibody was rabbit anti-PH20 polyclonal antibody (Abcam) diluted at

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1:500. Secondary antibody was Goat anti-rabbit IgG HRP diluted at 1:2,000.

[83] FIG. 14 shows experimental results for C-terminally truncated variants constructed using HM10 as a template.

[84] FIG. 14A shows the results of measuring enzymatic activities at pH 7.0 by turbidimetric assay for C-terminally cleaved variants constructed using HM10 as a template.

[85] FIG. 14B compares enzymatic activities depending on the C-terminal cleavage sites of 17 PH20 variants (HM43, HM44, HM45, HM20, HM19, HM35, HM36, HM37, HM38, HM39, HM47, HM48, HM49, HM50, HM51, HM52 and HM10) constructed using HM10 as a template.

[86] FIG. 14C shows the results of staining SDS gel with Alcian blue dye after 1 h of enzymatic reaction at 37°C by substrate-gel assay for some (HM29, HM35, HM36, HM37, HM38, HM39, HM43, HM44 and HM45) of PH20 variants constructed using HM10 as a template. The white band shows hyaluronic acid degraded by WT and the variant protein.

[88] FIG. 15 shows SDS gel after passage through a final column during protein purification for the HP34 (FIG. 15A) and HP46 (FIG. 15B) expressed in ExpiCHO cells. HP34 was subjected to a four-step chromatography purification procedure consisting of Q Sepharose, Butyl HP, Heparin and Blue Sepharose columns, and SDS gel is a result obtained after Blue Sepharose column chromatography. HP46 was subjected to a three-step chromatography purification procedure consisting of

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Q Sepharose, Butyl HP and Heparin columns, and SDS gel is a result obtained after Heparin column chromatography.

[90] FIG. 16 shows the enzymatic activities of 6xHis-tag-free PH20 variants HP34 and HP46 constructed using HM21 as a template.

[91] FIG. 16A shows the results of measuring the enzymatic activities of WT and the variants HM21, HP34 and HP46 at pH 7.0 by turbidimetric assay.

[92] FIG. 16B shows the results of measuring the enzymatic activities of WT (HW2) and the variants HM21, HP34 and HP46 at pH 5.3 by Morgan-Elson assay (K_m: Michaelis-Menten constant, k_{cat}: turnover number, and k_{cat} / K_m: catalytic efficiency).

[94] FIG. 17 shows the results of characterization of HM21-Based PH20 Variants.

[95] FIG. 17A shows the results of measuring aggregation temperatures by DLS for 6xHis-tag-free PH20 variants HP34 and HP46 constructed using HM21 as a template. As controls, the aggregation temperatures of HW2 and HM21 are shown.

[96] FIG. 17B shows the results of measuring enzymatic activities for 1 h by substrate-gel assay for HW2 and the variants PH20 (HP20, HP34 and HP46).

[97] FIG. 17C shows the results of performing a substrate-gel assay after allowing variant (HW2 and HP46) samples to stand at pH 3.0 and pH 7.0 for 14 h. After SDS-PAGE, SDS was removed with 2.5% Triton X-100 (w/v), and an enzymatic reaction was performed at 37°C for 1 h.

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[98] FIG. 17D shows the expression levels of PH20 variants HM21, HM53, HM54, HM55, HM56, HP59 and HP60 in ExpiCHO cells.

[99] FIG. 17E shows the expression of the enzymatic activity value of PH20 variants HM21, HM53, HM54, HM55, HM56, HP59 and HP60, measured by the turbidimetric assay, as specific activity at pH 5.3.

[101] FIG. 18 shows the results of measuring the stimulating index of CD4+ T cells in treatment with PH20 and PH20 variant at concentrations of 1.5 ng/mL and 15 ng/mL, respectively.

[103] FIG. 19 shows the results of measuring the stimulating index of CD8+ T cells in treatment with PH20 and PH20 variant at concentrations of 1.5 ng/mL and 15 ng/mL, respectively.

BEST MODE FOR CARRYING OUT THE INVENTION

[106] Unless defined otherwise, all the technical and scientific terms used herein have the same meaning as those generally understood by one of ordinary skill in the art to which the invention pertains. Generally, the nomenclature used herein and the experiment methods, which will be described below, are those well-known and commonly employed in the art.

[108] The present invention provides a hyaluronidase PH20 variant or fragment thereof, which comprises one or more amino acid residue substitutions in the region corresponding to an alpha-helix region and/or its linker region, preferably an alpha-helix 8 region (S347 to C381) and/or a linker region (A333 to R346) between alpha-helix 7 and alpha-helix 8, in the amino acid sequence of wild-type PH20, preferably mature wild-

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type PH20, and in which one or more of the N-terminal or C-terminal amino acid residues are selectively cleaved and deleted.

[110] In the present invention, the positions of amino acid residues in each variant correspond to the amino acid positions of wild-type PH20 having the sequence of SEQ ID NO: 1.

[111] In addition, in the present invention, "mature wild-type PH20" means a protein consisting of amino acid residues L36 to S490 of SEQ ID NO: 1, which lack M1 to T35, which form a signal peptide, and A491 to L509, which are not related to the substantial enzymatic function of PH20, in the amino acid sequence of wild-type PH20 having the sequence of SEQ ID NO: 1.

[113] **Table 1. Amino acid sequence of wild-type PH20 (SEQ ID NO: 1)**

MGVLKFKHIFFRSFVKSSGVSQIVFTFLLIPCCLTLNFRAPPVIPNVPFLWAWNAPSEFCLG
KFDEPLDMSLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITGVTVNGGIPQKISLQDHL
DKAKKDITFYMPVDNLGMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQLSLTEATE
KAKQEFEKAGKDFLVETIKLGKLLRPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDD
LSWLWNESTALYPSIYLNTQQSPVAATLYVRNRVREAIRVSKIPDAKSPLPVFAYTRIVFTD
QVLKFLSQDELVYTFGETVALGASGIVIWGTLSIMRSMKSCLLLDNYMETILNPYIINVTLA
AKMCSQVLCQEQGVCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTLEDLEQFSEKFY
CSCYSTLSCKEKADVKDTDAVDVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLSATMFIV
SILFLIISSVASL

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[114] Specifically, the PH20 variant or fragment thereof according to the present invention may comprise one or more mutations, preferably amino acid residue substitutions selected from the group consisting of T341A, T341C, T341G, S343E, M345T, K349E, L353A, L354I, N356E and I361T, more preferably selected from the group consisting of T341A, T341C, L354I and N356E in wild-type PH20 having the amino acid sequence of SEQ ID NO: 1.

[115] In the present invention, the term "PH20 variant" is intended to include mutation of one or more amino acid residues, preferably substitution of one or more amino acid residues in the amino acid sequence of wild-type PH20, as well as occurrence of deletion of one or more amino acid residues at N-terminus or C-terminus together with substitution of the amino acid residues, and is used as substantially the same meaning as the expression "PH20 variant or fragment thereof".

[117] In the present invention, the protein tertiary structure of PH20 located outside the active site was studied through the protein structure modeling of human PH20 on the basis of Hyal1 (SEQ ID NO: 2) which is a human hyaluronidase whose protein tertiary structure is known. As a result, amino acids located in the alpha-helix 8 region of PH20 were selected and substituted with the amino acid sequence of alpha-helix 8 of Hyal1, thereby attempting to enhance the thermal stability of the protein structure without affecting the catalytic activity of the enzyme. In particular, because alpha-helix 8 is located in the outer portion of the protein tertiary structure of PH20,

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there is less interaction with the adjacent alpha-helix or beta-strand than the other alpha-helices of PH20. According to the present invention, it has been found that when the amino acid sequence of the alpha-helix 8 region of human PH20 and a linker region between alpha-helix 7 and alpha-helix 8 is partially substituted with the amino acid sequence of the alpha-helix 8 region of highly hydrophilic Hyal1 and a linker region between alpha-helix 7 and alpha-helix 8 of Hyal1, the enzymatic activity at neutral pH and the protein aggregation temperature ($T_{agg.}$) increase. Based on these experimental results, it has been found that a novel PH20 variant or fragment thereof, which has increased enzymatic activity and thermal stability compared to wild-type PH20, can be provided.

[119] Thus, the PH20 variant according to the present invention comprises one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, T341G, S343E, M345T, K349E, L353A, L354I, N356E and I361T, preferably selected from the group consisting of T341A, T341C, L354I and N356E in the amino acid sequence of wild-type PH20 (having the amino acid sequence of SEQ ID NO: 1), preferably mature wild-type PH20 (having a sequence consisting of amino acid residues L36 to S490 in the amino acid sequence of SEQ ID NO: 1).

[120] The PH20 variant according to the present invention also comprises one or more amino acid residue substitutions in the region corresponding to an alpha-helix region and/or its linker region, preferably an alpha-helix 8 region (S347 to

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C381) and/or the linker region (A333 to R346) between alpha-helix 7 and alpha-helix 8, more preferably T341 to N363, T341 to I361, L342 to I361, S343 to I361, I344 to I361, M345 to I361, or M345 to N363 in wild-type PH20 having the amino acid sequence of SEQ ID NO: 1.

[122] In particular, in the PH20 variant according to the present invention, the alpha-helix 8 region (S347 to C381) and/or the linker region (A333 to R346) between alpha-helix 7 and alpha-helix 8 of the wild-type PH20, preferably the mature wild-type PH20 may be substituted with one or more amino acid residues of the corresponding region (see Tables 2 and 3) of Hyal1 having the sequence of SEQ ID NO: 2, but is not limited thereto.

[124] **Table 2. Amino acid sequence of wild-type Hyal1 (SEQ ID NO: 2)**

MAAHLLPICALFLTLLDMAQGFRGPLLPNRPFTTVWNANTQWCLERHGVDVDVSVFDVVANP
GQTFRGPDMTIFYSSQLGTYPYYTPTGEPVFGGLPQNASLIAHLARTFQDILAAIPAPDFSG
LAVIDWEAWRPRWAFNWDTKDIYRQRSRALVQAQHPDWPAPQVEAVAQDQFQGAARAWMAGT
LQLGRALRPRGLWGFYGFPDCYNYDFLSPNYTGQCPSGIRAQNDQLGWLWGQSRALYPSIYM
PAVLEGTGKSQMYVQHRVAEAFRVAVAAGDPNLPVLPYVQIFYDTTNHFLPLDELEHSLGES
AAQGAAGVVLWVSWENTRTKESCQAIKEYMDTTLGPFILNVTSGALLCSQALCSGHGRCVRR
TSHPKALLLLNPASFSIQLTPGGGPLSLRGALSLEDQAQMAVEFKCRCYPGWQAPWCERKSM
W

[125] **Table 3. Comparison of alpha-helices and amino acid sequence between PH20 and Hyal1**

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alpha-helix	amino acid sequences of PH20	amino acid sequences of Hyal1
alpha-helix 1	P56~D65	N39~G48
alpha-helix 3	S119~M135	S101~I117
alpha-helix 4'	K161~N176	K144~H159
alpha-helix 4	S180-R211	P163~R194
alpha-helix 5	F239~S256	P222~S239
alpha-helix 6	A274~D293	K257~G277
alpha-helix 7	S317~G332	P299~G314
alpha-helix 8	S347~C381	T329~C363

[126] More specifically, the novel PH20 variant or fragment thereof according to the present invention preferably comprises an amino acid residue substitution of L354I and/or N356E in the amino acid sequence of wild-type PH20, preferably mature wild-type PH20,

[127] and further comprises at least the amino acid residue substitution at one or more positions selected from among T341 to N363, particularly one or more positions selected from the group consisting of T341, L342, S343, I344, M345, S347, M348, K349, L352, L353, D355, E359, I361 and N363, but is not limited thereto.

[128] More preferably, the amino acid residue substitution at one or more positions selected from the group consisting of T341, L342, S343, I344, M345, S347, M348, K349, L352, L353,

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D355, E359, I361 and N363 may be one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, T341D, T341G, T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, D355K, E359D, I361T and N363G , but is not limited thereto.

[130] Preferably, the novel PH20 variant or fragment thereof according to the present invention may comprise one or more amino acid residue substitutions selected from the group consisting of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T,

[131] and may further comprise one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, T341D, T341G, T341S, L342W, S343E, I344N and N363G, but is not limited thereto.

[133] More preferably, the novel PH20 variant or fragment thereof according to the present invention may comprise any one amino acid residue substitution selected from the following amino acid residue substitution groups, but is not limited thereto:

[135]~[145]

(a) T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(b) L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(c) M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D, I361T and N363G;

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(d) T341G, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(e) T341A, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(f) T341C, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(g) T341D, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(h) I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T; and

(i) S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T.

[147] In the present invention, an expression described by one-letter amino acid residue code together with numbers, such as "S347", means the amino acid residue at each position in the amino acid sequence of SEQ ID NO: 1.

[148] For example, "S347" means that the amino acid residue at position 347 in the amino acid sequence of SEQ ID NO: 1 is serine.

[149] In addition, "S347T" means that serine at position 347 of SEQ ID NO: 1 is substituted with threonine.

[151] The PH20 variant according to the present invention is interpreted as including variants or fragments thereof in which the amino acid residue at the specific amino acid residue position is conservatively substituted.

[152] As used herein, the term "conservative substitution" refers to modifications of a PH20 variant that involves the

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substitution of one or more amino acids for amino acids having similar biochemical properties that do not result in loss of the biological or biochemical function of the PH20 variant.

[153] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined and are well known in the art to which the present invention pertains. These families include amino acids with basic side chains (e.g., lysine, arginine and histidine), amino acids with acidic side chains (e.g., aspartic acid and glutamic acid), amino acids with uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, and cysteine), amino acids with nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan), amino acids with beta-branched side chains (e.g., threonine, valine, and isoleucine), and amino acids with aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, and histidine).

[154] It is envisioned that the PH20 variant or fragments thereof of the present invention may still retain the activity although it has conservative amino acid substitutions.

[156] In addition, the PH20 variant or fragment thereof according to the present invention is interpreted to include PH20 variants or fragments thereof having substantially the same function and/or effect with those/that of the PH20 variant or fragment thereof according to the present

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invention, and having an amino acid sequence homology of at least 80% or 85%, preferably at least 90%, more preferably at least 95%, most preferably at least 99% to the PH20 variant or fragment thereof according to the present invention.

[158] The PH20 variants according to the present invention have increased expression levels and protein refolding rate, thereby increasing high thermal stability, compared to mature wild-type PH20. Furthermore, the enzymatic activity of the PH20 variants was more increased than or similar to that of mature wild-type PH20 despite an increase in the thermal stability.

[160] Meanwhile, even if the mature wild type PH20 variants with the C-terminal deletion showed the decrease in enzymatic activities, based on the present invention, the C-terminal deleted PH20 variants showed the similar or increased enzymatic activities due to the more rapid protein refolding and thermal stabilities. In addition, PH20 variants in this present invention maintained the enzymatic activities when the N-terminal amino acids were deleted up to five amino acid residues. This indicated that for the protein expression and enzyme activities P41 of the N-terminus was important.

[162] Accordingly, the PH20 variant or fragment thereof according to the present invention is characterized in that it comprises one or more amino acid residue substitutions in the alpha-helix 8 region (S347 to C381) and/or the linker region (A333 to R346) between alpha-helix 7 and alpha-helix 8 in the amino acid sequence of wild-type PH20, and one or more of the

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N-terminal and/or C-terminal amino acid residues are additionally deleted, but is not limited thereto.

[164] In one aspect, the PH20 variant or fragment thereof according to the present invention may be one in which cleavage occurs before an amino acid residue selected from the group consisting of M1 to P42 of the amino acid sequence of SEQ ID NO: 1, preferably before an amino acid residue L36, N37, F38, R39, A40, P41, or P42 at the N-terminus, so that one or more amino acid residues at the N-terminus are deleted, and/or cleavage occurs after an amino acid residue selected from the group consisting of V455 to L509, preferably after an amino acid residue selected from the group consisting of V455 to S490, most preferably after an amino acid residue V455, C458, D461, C464, I465, D466, A467, F468, K470, P471, P472, M473, E474, T475, E476, P478, I480, Y482, A484, P486, T488, or S490 at the C-terminus, so that one or more amino acid residues at the C-terminus are deleted.

[166] The expression "cleavage occurs before an amino acid residue selected from the group consisting of M1 to P42 at the N-terminus" means that an amino acid residue immediately before an amino acid residue selected from among M1 to P42 at the N-terminus is cleaved and deleted.

[167] For example, the expression "cleavage occurs before an amino acid residue L36, N37, F38, R39, A40, P41, or P42" respectively means that all amino acid residues from M1 to T35 immediately before L36, all amino acid residues from M1 to L36 immediately before N37, all amino acid residues from M1 to N37

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immediately before F38, all amino acid residues from M1 to F38 immediately before R39, all amino acid residues from M1 to R39 immediately before A40, all amino acid residues from M1 to A40 immediately before P41, or all amino acid residues from M1 to P41 immediately before P42 in the amino acid sequence of SEQ ID NO: 1 is cleaved and removed, respectively.

[169] In addition, the expression "cleavage occurs after an amino acid residue selected from the group consisting of V455 to L509 at the C-terminus" means that an amino acid residue immediately after an amino acid residue selected from among V455 to L509 at the C-terminus is cleaved and deleted.

[171] For example, the expression "cleavage occurs after an amino acid residue V455, C458, D461, C464, I465, D466, A467, F468, K470, P471, P472, M473, E474, T475, E476, P478, I480, Y482, A484, P486, T488 or S490 at the C-terminus" means that an amino acid residue after the amino acid residue V455, C458, D461, C464, I465, D466, A467, F468, K470, P471, P472, M473, E474, T475, E476, P478, I480, Y482, A484, P486, T488 or S490 in the amino acid sequence of SEQ ID NO: 1 is cleaved and removed.

[173] Preferably, the novel PH20 variant or fragment thereof according to the present invention may be selected from the group consisting of amino acid sequences of SEQ ID NOs: 60 to 115, but is not limited thereto.

[174] Most preferably, the novel PH20 variant or fragment thereof according to the present invention may have the amino acid sequence of SEQ ID NO: 99. The novel PH20 variant or

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fragment thereof having an amino acid sequence of SEQ ID NO: 99 may comprise 15 amino acid substitutions of T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, and deletion before F38 at the N-terminus, and truncation after F468 at the C-terminus.

[176] The sequences of the substituted or cleaved amino acids in the PH20 variants constructed in the specific embodiment according to the present invention are as shown in Table 11.

[178] A study focused on increasing the enzymatic activity and thermal stability of PH20 by amino acid substitution of an alpha-helix and its linker region, which are secondary structures forming the tertiary structure of the protein, as disclosed in the present invention, has not been previously reported. Previous studies reported that the enzymatic activity of wild-type PH20 changes depending on the cleavage positions of amino acid residues located at the C-terminal region. However, in the present invention, a specific alpha-helix forming the secondary structure of PH20 was substituted with the alpha-helix of other human hyaluronidase, thereby constructing PH20 variants having higher stability than wild-type PH20. These variants may be variants in which the interaction of the substituted alpha-helix domain with portions forming other secondary structures of PH20 shows a pattern different from that of wild-type PH20, indicating that the variants have consistent enzymatic activity regardless of the C-terminal cleavage position.

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[180] In specific embodiment, the novel PH20 variant or fragment thereof according to the present invention, which has increased enzymatic activity and thermal stability compared to mature wild-type PH20, may be one which comprises one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, T341G, S343E, M345T, K349E, L353A, L354I, N356E and I361T, and in which one or more amino acids located in an alpha-helix 8 region (S347 to C381) and/or the linker region (A333 to R346) between alpha-helix 7 and alpha-helix 8 in the amino acid sequence of wild-type PH20, preferably mature wild-type PH20, are substituted with other amino acids.

[181] Specifically, the amino acid substitution in the linker region between alpha-helix 7 and alpha-helix 8 comprises the substitution of one or more amino acid residues in the region consisting of amino acid residues T341 to N363, T341 to I361, L342 to I361, L342 to I361, S343 to I361, I344 to I361, M345 to I361, or M345 to N363.

[182] In order to examine the effect of C-terminal truncation in PH20 variants in which alpha-helix 8 and a linker region between alpha-helix 7 and alpha-helix 8 are substituted, three PH20 variants (HM6, HM10 and HM21) were selected as templates.

[184] HM6 is a variant in which amino acids in M345 to N363 region are substituted with the amino acid sequence of Hyal1 (M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T in SEQ ID NO: 1 are substituted). In addition, HM6 is a variant in which the substitution of alpha-helix 8

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and a linker region between alpha-helix 7 and alpha-helix 8 is the least substituted variant among the PH20 variants according to the present invention, which do not comprise additional C-terminal cleavage (that is, a form in which the C-terminal amino acid residue is S490, like mature wild-type PH20).

[185] HM10 is a variant in which amino acids in L342 to I361 region are substituted with the amino acid sequence of Hyal1 (L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T in SEQ ID NO: 1 are substituted), and which has the highest thermal stability while having an enzymatic activity similar to that of mature wild-type PH20 among the PH20 variants according to the present invention, which do not comprise additional C-terminal truncation.

[186] HM21 is a variant in which amino acids in T341 to I361 region are substituted with the amino acid sequence of Hyal1 (T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T in SEQ ID NO: 1 are substituted), and which has an enzymatic activity which is about two times higher than that of wild-type PH20 at pH 7.0 among the PH20 variants according to the present invention, which do not comprise additional C-terminal truncation.

[187] The HM6-based PH20 variants constructed in the present invention have the N-terminus starting at L36 and the C-terminus terminating at I465, F468, or P471 as shown in Table 4 below.

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[189] **Table 4. C-terminal amino acid truncated PH20 variants constructed using HM6 as a template**

Variant	Total length	Substituted region	Number of substituted amino acids	Number of truncated amino acids
HM6	36~490	M345~I361	11	0
HM14	36~465		11	25
HM15	36~468		11	22
HM16	36~471		11	19

[190] The HM10-based PH20 variants commonly have the N-terminus cleaved before the F38 residue and the C-terminus cleaved after the V455, C458, D461, C464, I465, D466, A467, F468, K470, P472, M473, E474, T475, E476, P478, I480, Y482, A484, P486, or T488 residue as shown in Table 5 below.

[192]~[193]

Table 5. C-terminal amino acid truncated PH20 variants constructed using HM10 as a template

Variant	Total length	Substituted region	Number of substituted amino acids	Number of truncated amino acids
HM10	36~490	L342~I361	14	0
HM52	38~488		14	4
HM51	38~486		14	6
HM50	38~484		14	8
HM49	38~482		14	10

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HM48	38~480	14	12
HM47	38~478	14	14
HM39	38~476	14	16
HM38	38~475	14	17
HM37	38~474	14	18
HM36	38~473	14	19
HM35	38~472	14	20
HM19	38~470	14	22
HM20	38~468	14	24
HM45	38~467	14	25
HM29	36~467	14	23
HM44	38~466	14	26
HM43	38~465	14	27
HM30	36~464	14	26
HM31	36~461	14	29
HM32	36~458	14	32
HM33	36~455	14	35
HP19	38~470	14	22
HP20	38~468	14	24

[194] As shown in the examples of variants which comprise amino acid substitutions in the L342 to I361 region corresponding to the alpha-helix 8 region and the linker region between alpha-helix 7 and alpha-helix 8 of HM10 as a template and in which the N-terminus was cleaved before the F38 residue and the C-terminus was cleaved at I465, D466, A467, F468, K470, P472, M473, E474, T475, E476, P478, I480, Y482, A484, P486, or T488, the PH20 variants according to the present invention exhibited an enzymatic activity similar to

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that of mature wild-type PH20 regardless of the C-terminal cleavage position.

[196] As shown in Table 6 below, two HM21-based PH20 variants commonly have the N-terminus cleaved before the F38 residue and the C-terminus cleaved after the F468 or K470 residue.

[198] **Table 6. C-terminal amino acid truncated PH20 variants constructed using HM21 as a template**

Variant	Total length	Substituted region	Number of substituted amino acids	Number of truncated amino acids
HM21	38~490	T341~I361	15	2
HP34	38~470		15	22
HP46	38~468		15	24

[199] Variants were constructed using, as a template, HM21 having an enzymatic activity which is about two times higher than that of mature wild-type PH20. These variants are those which comprise amino acid substitutions in the T341 to I361 region corresponding to the alpha-helix 8 region and the linker region between alpha helix 7 and alpha-helix 8 and in which the N-terminus was cleaved before the F38 residue and the C-terminus was cleaved after F468 or K470. Surprisingly, these variants maintained the high enzymatic activity of HM21 regardless of the C-terminal cleavage position.

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[200] In the study conducted by Frost et al., when the length of PH20 is shorter due to the cleavage before the amino acid position of 477, the enzymatic activity decreased to about 10% of a variant having a C-terminus cleaved after the position 477. However, in the present invention, when one or more amino acids in alpha-helix 8 of PH20 and its linker region were substituted, the enzymatic activity was maintained due to the increase in the stability of the protein regardless of the C-terminal cleavage position. This result is very significant in that it solves the problem that the enzymatic activity of wild-type PH20 is reduced due to the C-terminal truncation of wild-type PH20.

[202] In addition, in the present invention, the effect of the N-terminal amino acids of PH20, which has not been known previously, was studied.

[203] In order to examine the effects of N-terminal cleavage sites in HM6 variants in which one or more amino acid residues in the region (M345 to I361) corresponding to the alpha-helix 8 region of wild-type PH20 and the linker region between alpha-helix 7 and alpha-helix 8 (substituted with M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D or I361T in SEQ ID NO: 1 and comprising no additional C-terminal cleavage) were substituted with amino acid residues of the corresponding alpha-helix 8 region of Hyal1 and the linker region between alpha-helix 7 and alpha-helix 8, variants, in which amino acid residues L36 to V47 in SEQ ID NO: 1 were substituted with FRGPLLPNR or amino acid residues L36 to A52

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in SEQ ID NO: 1 were substituted with FRGPLLPNRPFTTV, were constructed using HM6 as a template. In addition, using HM6 as a template, the variants HM40, HM13, HM41, HM24, HM42 and HM25 were constructed in which the N-terminus in the amino acid sequence of SEQ ID NO: 1 was cleaved before the N37, F38, R39, A40, P41 or P42 residue (see Table 7).

[205] **Table 7. N-terminal amino acid cleaved variants based on HM6**

Variant	Total length	Substituted region	Number of substituted amino acids	Number of truncated amino acids
HM6	36~490	M345~I361	11	0
HM40	37~490		11	1
HM13	38~490		11	2
HM41	39~490		11	3
HM24	40~490		11	4
HM42	41~490		11	5
HM25	42~490		11	6
HM17		L36~V47, M345~I361	23	
HM18		L36~A52, M345~I361	28	

[206] As a result, it was shown that when the N-terminus of HM6 was cleaved before the N37, F38, R39, A40 or P41 residue, the enzymatic activity was not greatly influenced; however, when the N-terminus was cleaved before the P42 residue, the

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enzymatic activity significantly decreased, indicating that the N-terminal region of PH20, located after P41, is important for protein expression and enzymatic activity. In addition, when one or more amino acids in the N-terminal L36 to V47 or L36 to A52 region of HM6 were substituted with the amino acids of Hyal1, the variant protein was not expressed in ExpiCHO cells, indicating that the N-terminal region is important for protein expression.

[208] In addition, in the present invention, it was attempted to increase the expression of a recombinant PH20 protein in animal cells by using the signal peptide of other proteins, instead of using the original signal peptide of PH20.

[209] Therefore, in another aspect, the novel PH20 variant according to the present invention may be one in which the N-terminus further comprises a human growth hormone signal peptide having an amino acid sequence MATGSRTSLLLAFGLLCLPWLQEGSA of SEQ ID NO: 3, a human serum albumin signal peptide having an amino acid sequence MKWVTFISLLFLFSSAYS of SEQ ID NO: 4, or a human Hyal1 signal peptide having an amino acid sequence MAAHLLPICALFLTLLDMAQG of SEQ ID NO: 5 as shown in Table 8 below, instead of the signal peptide of wild-type PH20, which consists of M1 to T35, but is not limited thereto.

[211] The expression "instead of the signal peptide of wild-type PH20, which consists of M1 to T35" means a case in which the signal peptide of wild-type PH20 is partially or completely deleted; thus it does not perform its function. In

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addition, the expression is meant to include a case in which a portion of the N-terminus is further deleted, for example, a case in which cleavage occurs before the N37, F38, R39, A40, P41 or P42 residue occurs so that an additional deletion of the N-terminus together with the deletion of the signal peptide of wild-type PH20 occurs.

[213] **Table 8. Amino acid sequences of signal peptide of human growth hormone, human serum albumin or human Hyal1**

	Amino acid sequences	SEQ NO.
human growth hormone	MATGSRTSLLLAFGLLCLPWLQEGSA	3
human serum albumin	MKWVTFISLLFLFSSAYS	4
human Hyal1	MAAHLLPICALFLTLLDMAQG	5

[214] In another aspect, the present invention provides a composition for treating cancer comprising the novel PH20 variant according to the present invention, and a method for treating cancer using the same.

[216] Cancers or carcinomas that can be treated by the novel PH20 variant according to the present invention are not limited particularly, but includes both solid cancers and blood cancers. The cancer may be selected from the group consisting of liver cancer, hepatocellular carcinoma, gastric cancer, breast cancer, lung cancer, ovarian cancer, bronchial

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cancer, nasopharyngeal cancer, laryngeal cancer, pancreatic cancer, bladder cancer, colorectal cancer, colon cancer, uterine cervical cancer, brain cancer, prostate cancer, bone cancer, thyroid cancer, parathyroid cancer, renal cancer, esophageal cancer, biliary tract cancer, testis cancer, rectal cancer, head and neck cancer, ureteral cancer, osteosarcoma, neurocytoma, fibrosarcoma, rhabdomyosarcoma, astrocytoma, neuroblastoma and neuroglioma, but is not limited thereto. Preferably, cancers that can be treated by the composition according to the present invention may be selected from the group consisting of colorectal cancer, breast cancer, lung cancer and renal cancer, but is not limited thereto.

[218] The composition of the present invention may be a pharmaceutical composition. The pharmaceutical composition may further comprise a pharmaceutically acceptable component. The component that is typically used in the formulation of drugs may be one or more selected from the group consisting of, but not limited to, lactose, dextrose, sucrose, sorbitol, mannitol, starch, gum acacia, calcium phosphate, alginate, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrups, methyl cellulose, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate, and mineral oil. In addition, the pharmaceutical composition may further comprise one or more selected from the group consisting of diluents, excipients, lubricants, wetting agents, sweeteners, aromatics, emulsifiers, suspensions, and preservatives.

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[219] The pharmaceutical composition of the present invention may be administered orally or parenterally. The parenteral administration is carried out by intravenous injection, subcutaneous injection, intramuscular injection, intraperitoneal injection, endothelial administration, topical administration, intranasal administration, intrapulmonary administration, rectal administration, and the like. For the oral administration, the active ingredient in the composition needs to be formulated into a coated dosage form or into a dosage form which can protect the active ingredient from being disintegrated in stomach considering that peptides and proteins are digested in stomach. Alternatively, the present composition may be administered via any device by which the active ingredient can move to the target cell of interest.

[220] The pharmaceutical composition may be formulated in the form of solutions, suspensions, syrups or emulsions in oils or aqueous media, or in the form of extracts, grains, suppositories, powders, granules, tablets or capsules, and may additionally include dispersing or stabilizing agents for the purpose of formulation.

[221] In particular, the composition for treating cancer according to the present invention may be used for combined treatment with other anticancer drugs.

[222] An anticancer drug that can be used for combined treatment with the novel PH20 variant according to the present invention is preferably a chemical anticancer drug, an

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antibody-based anticancer drug, a biological anticancer drug, an RNAi, or a cell therapeutic agent, but is not limited thereto.

[223] Preferably, the anticancer drug that can be used for combined treatment with the novel PH20 variant according to the present invention is preferably an immuno-oncologic agent, more preferably an immune checkpoint inhibitor, but is not limited thereto.

[225] In another aspect, the present invention is directed to a nucleic acid encoding the PH20 variant or fragment thereof.

[226] The nucleic acids, as used herein, may be present in cells, in the cell lysate, or in the partially purified or substantially pure form. "Isolated" or "substantially pure", when referring to nucleic acids, refer to those that have been purified away from other cellular components or other contaminants, e.g., other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art. The nucleic acids of the present invention may be DNA or RNA.

[227] In still another aspect, the present invention is directed to recombinant expression vector comprising the nucleic acid. For expression of the PH20 variant or fragment thereof according to the present invention, a DNA encoding the PH20 variant can be obtained by standard molecular biology techniques (e.g., PCR amplification or cDNA cloning using a

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hybridoma that expresses the PH20 variant), and the DNA can be inserted into an expression vector such that it is "operatively linked" to transcriptional and translational control sequences.

[228] As used herein, the term "operatively linked" is intended to mean that a gene encoding the PH20 variant or fragment thereof is ligated into a vector such that transcriptional and translational control sequences serve their intended function of regulating the transcription and translation of the gene encoding the PH20 variant or fragment thereof. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The genes encoding the PH20 are inserted into the expression vector by standard methods (e.g., ligation of complementary restriction enzyme sites on a fragment of the gene encoding the PH20 variant or fragment thereof and vector, or blunt end ligation if no restriction enzyme sites are present).

[229] In addition, the recombinant expression vectors carry regulatory sequences that control the expression of a gene encoding the PH20 variant in the host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals) that control the transcription or translation of the genes encoding the PH20 variant or fragment thereof. It will be appreciated by those skilled in the art that the design of the expression vector, including the

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selection of regulatory sequences, may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc.

[231] In yet another aspect, the present invention is directed to a host cell comprising the nucleic acid or the vector. The host cell according to the present invention is preferably selected from the group consisting of animal cells, plant cells, yeasts, *E. coli.*, and insect cells, but is not limited thereto.

[232] Specifically, the host cell according to the present invention include prokaryotic cells such as *E. coli*, *Bacillus subtilis*, *Streptomyces* sp., *Pseudomonas* sp., *Proteus mirabilis* or *Staphylococcus* sp., fungi such as *Aspergillus* sp., yeasts such as *Pichia pastoris*, *Saccharomyces cerevisiae*, *Schizosaccharomyces* sp. and *Neurospora crassa*, and eukaryotic cells such as lower eukaryotic cells, and higher other eukaryotic cells such as insect cells.

[233] In addition, the host cells that can be used in the present invention may be derived from plants or mammals. Preferably, examples of the host cells include, but are not limited to, monkey kidney cells (COS7), NSO cells, SP2/0, Chinese hamster ovary (CHO) cells, W138, baby hamster kidney (BHK) cells, MDCK, myeloma cells, HuT 78 cells and HEK293 cells. More preferably, CHO cells may be used.

[235] The nucleic acid or the vector is transfected into a host cell. Transfection can be performed using various techniques that are generally used to introduce foreign

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nucleic acid (DNA or RNA) into prokaryotic or eukaryotic cells, for example, electrophoresis, calcium phosphate precipitation, DEAE-dextran transfection or lipofection. In order to express the PH20 variant or fragment thereof of the present invention, various combinations of recombinant expression vectors and host cells can be employed. The preferred expression vector for eukaryotic cells comprises gene expression regulatory sequences derived from, but not limited to, SV40, bovine papillomavirus, adenovirus, adeno-associated virus, cytomegalovirus and retrovirus. The expression vector, which can be used for bacterial hosts, comprises bacterial plasmids, such as, pET, pRSET, pBluescript, pGEX2T, pUC vector, col E1, pCR1, pBR322, pMB9 and the derivatives thereof, obtained from *E. coli*; a plasmid having broad host range, such as, RP4; phage DNAs exemplified by various phage lambda derivatives, such as, λgt10, λgt11 and NM989; and other DNA phages, such as, M13 and filamentous single-stranded DNA phage. The expression vector available for yeast cells may be 2-μm plasmid and its derivatives. The expression vector for insect cells includes pVL941.

[237] In a further aspect, the present invention is directed to a method for producing an PH20 variant or fragment thereof, the method comprising a step of culturing the host cell and expressing the PH20 variant or fragment thereof according to the present invention.

[238] When a recombinant expression vector capable of expressing the PH20 variant or fragment thereof is introduced

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into mammalian host cells, the PH20 variant or fragment thereof can be produced by culturing the host cells for a period of time such that the PH20 variant or fragment thereof is expressed in the host cells, preferably a period of time such that the PH20 variant is secreted into the medium during culture of the host cells.

[239] In some cases, the expressed PH20 variant can be isolated and purified from the host cells. Isolation or purification of the PH20 variant can be performed by conventional isolation/purification methods (e.g., chromatography) that are used for proteins. The chromatography may include one or more combinations selected from affinity chromatography, ion exchange chromatography, and hydrophobic chromatography, but is not limited thereto. In addition to the chromatography, a combination of filtration, ultrafiltration, salting out, dialysis, and the like may be used to isolate and purify the antibody.

:

EXAMPLES

[244] Hereinafter, the present invention will be described in further detail with reference to examples. It will be obvious to a person having ordinary skill in the art that these examples are illustrative purposes only and are not to be construed to limit or change the scope of the present invention.

Example 1. Construction of PH20 Variants

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[247] For construction of PH20 variants, the cDNA (clone ID: hMU002604) of wild-type PH20 was purchased from the Korean Human Gene Bank. Wild-type PH20 encodes amino acids from L36 to S490. The PH20 gene was amplified by polymerase chain reaction (hereinafter referred to as PCR) and inserted into the *XhoI* and *NotI* restriction enzyme sites of a pcDNA3.4-TOPO vector. For expression in ExpiCHO cells, the signal peptide of human growth hormone, human serum hormone or human Hyal1 was used as a signal peptide instead of the original signal peptide of PH20. For protein purification using a HisTrap column, the DNA sequence of a 6xHis-tag was located at the 3'-end of the PH20 cDNA. The amino acid substitution of PH20 variants was performed using PCR method, and the amino acid substitution was confirmed by DNA sequencing.

[248] The list of primers used in cloning of the PH20 variants are summarized in Table 9 below, and the specific sequences of the primers are summarized in Table 10 below.

[250]~[252]

Table 9. List of primers used in cloning of PH20 variants

Clone	Primer		
	1	2	3
cB4202	ALB-SP-Xho	ALB-PH20-MR	SPAM1-6H-Not
cB4203-HM1	ALB-SP-Xho	PH20_M345-364-F	SPAM1-6H-not
cB4203-HM2	ALB-SP-Xho	PH20_Y365-L380-F	SPAM1-7H-not
cB4203-HM3	ALB-SP-Xho	PH20_M345-L380-F	SPAM1-8H-not
cB4203-HM4	ALB-SP-Xho	B4203-HM4-F	SPAM1-9H-not
cB4203-HM5	ALB-SP-Xho	B4203-HM5-F	SPAM1-10H-not
cB4203-HM6	ALB-SP-Xho	PH20-G363N	SPAM1-6H-not
cB4203-HM7	ALB-SP-Xho	PH20-G363N	SPAM1-12H-not

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cB4203-HM8	ALB-SP-Xho	cB4203-HM8-M	SPAM1-13H-not
cB4203-HM9	ALB-SP-Xho	cB4203-HM9-M	SPAM1-14H-not
cB4203-HM10	ALB-SP-Xho	cB4203-HM10-M	SPAM1-6H-Not
cB4203-HM11	ALB-SP-Xho	4203-HM11	SPAM1-16H-not
cB4203-HM12	ALB-SP-Xho	4203-HM12	SPAM1-17H-not
cB4203-HM13	ALB-SP-Xho	SASP-LN-del-R	SPAM1-18H-not
cB4203-HM14	ALB-SP-Xho	-	I465-6H-not
cB4203-HM15	ALB-SP-Xho	-	F468-6H-not
cB4203-HM16	ALB-SP-Xho	-	P471-6H-not
cB4203-HM17	ALB-SP-Xho	PH20-HM17	SPAM1-22H-not
cB4203-HM18	ALB-SP-Xho	PH20-HM18	SPAM1-23H-not
cB4203-HM19	ALB-SP-Xho	SASP-LN-del-R	K470-6H-not
cB4003-HP19	ALB-SP-Xho	K470-not	
cB4203-HM20	ALB-SP-Xho	SASP-LN-del-R	F468-6H-not
cB4003-HP20	ALB-SP-Xho	F468-not	
cB4203-HM21	ALB-SP-Xho	M21-mega-F	SPAM1-6H-Not
cB4203-HM24	ALB-SP-Xho	M24-R	SPAM1-6H-not
cB4203-HM25	ALB-SP-Xho	M25-R	SPAM1-6H-not
cB4203-HM26	ALB-SP-Xho	B4-HM26	SPAM1-6H-not
cB4203-HM27	ALB-SP-Xho	B4-HM27	SPAM1-6H-not
cB4203-HM28	ALB-SP-Xho	B4-HM28	SPAM1-6H-not
cB4203-HM29	ALB-SP-Xho	B4-HM29	
cB4203-HM30	ALB-SP-Xho	B4-HM30	
cB4203-HM31	ALB-SP-Xho	B4-HM31	
cB4203-HM32	ALB-SP-Xho	B4-HM32	
cB4203-HM33	ALB-SP-Xho	B4-m33	
cB4003-HP34	ALB-SP-Xho	M21-mega-F	K470-not
cB4203-HM35	ALB-SP-Xho	SASP-LN-del-R	P472-6H-not
cB4003-HP35	ALB-SP-Xho	P472-not	
cB4203-HM36	ALB-SP-Xho	SASP-LN-del-R	M473-6H-not
cB4003-HP36	ALB-SP-Xho	M473-not	
cB4203-HM37	ALB-SP-Xho	SASP-LN-del-R	E474-6H-not
cB4003-HP37	ALB-SP-Xho	E474-not	
cB4203-HM38	ALB-SP-Xho	SASP-LN-del-R	T475-6H-not
cB4003-HP38	ALB-SP-Xho	T475-not	
cB4203-HM39	ALB-SP-Xho	SASP-LN-del-R	E476-6H-not
cB4003-HP39	ALB-SP-Xho	SASP-LN-del-R	E476-not
cB4203-HM40	ALB-SP-Xho	M40-mega	SPAM1-6H-Not
cB4203-HM41	ALB-SP-Xho	M41-mega	SPAM1-6H-Not

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cB4203-HM42	ALB-SP-Xho	m42-mega	SPAM1-6H-not
cB4203-HM43	ALB-SP-Xho	I465-6H-not	
cB4203-HM44	ALB-SP-Xho	D466-6H-not	
cB4203-HM45	ALB-SP-Xho	B4-HM29	
cB4003-HP46	ALB-SP-Xho	F468-Not	
cB4203-HM47	ALB-SP-Xho	P478-H-Not	
cB4203-HM48	ALB-SP-Xho	I480-H-Not	
cB4203-HM49	ALB-SP-Xho	Y482-H-Not	
cB4203-HM50	ALB-SP-Xho	A484-H-not	
cB4203-HM51	ALB-SP-Xho	P486-H-not	
cB4203-HM52	ALB-SP-Xho	SASP-LN-del-R	T488-H-not

[253]~[257]

Table 10. Primer sequences used in cloning of PH20 variants

Primer	SEQ NO.	Nucleotide Sequences (5'-> 3')
ALB-SP-Xho	6	GAA TAT CTC GAG GCC ACC ATG AAG TGG GTT ACA
SPAM1-6H-Not	7	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGG AAG AAA CCA ATT CTG C
ALB-PH20-MR	8	TAA CAG GAG GTG CTC TGA AAT TCA GAG AGT AAG CAG AGG AG
PH20_M345-P364-F	9	ATG GGG AAC CCT CAG TAT AAC AAG AAC CAA GGA ATC ATG TCA GGC CAT CAA GGA GTA TAT GGA CAC TAC ACT GGG GCC CTA CAT AAT CAA CGT CAC AC
PH20_Y365-L380-F	10	ATG GAG ACT ATA CTG AAT CCT TTC ATC CTG AAC GTG ACC AGT GGG GCC CTT CTC TGC AGT CAA GCC CTG TGC CAG GAG CAA GGA GTG TG
PH20_M345-L380-F*	11	ATG GAC ACT ACA CTG GGG CCC TTC ATC CTG AAC GTG ACC AGT GGG GCC CTT CTC TGC AGT CAA GCC CTG TGC CAG GAG CAA GGA GTG TG
B4203-HM4-F	12	ACT GTT GCT CTG GGT GCT TCT GGA ATT GTA ATA TGG GTA AGC TGG GAA AAT ACA AGA ACC AAG GAA TCA TGT CA

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B4203-HM5-F	13	AGC AAG GAG TGT GTA TAA GGA AAA CCA GCC ACC CAA AAG ACT ATC TTC ACC TCA ACC CAG A
PH20-G363N	14	AGT ATA TGG ACA CTA CAC TGA ACC CCT ACA TAA TCA ACG TCA C
cB4203-HM8-M	15	ATT GTA ATA TGG GGA ACC CTC AGT AAT ACA AGA ACC AAG GAA TC
cB4203-HM9-M	16	ATT GTA ATA TGG GGA ACC CTC GAA AAT ACA AGA ACC AAG GAA TC
cB4203- HM10-M	17	ATT GTA ATA TGG GGA ACC TGG GAA AAT ACA AGA ACC AAG GAA TC
4203-HM11	18	ACA CTA CAC TGA ACC CCT TCA TAC TCA ACG TCA CCC TAG CAG CCA
4203-HM12	19	ACA CTA CAC TGA ACC CCT TCA TAC TCA ACG TCA CCC TAT CAG GCA AAA TGT GTA GCC AAG TGC
SASP-LN-del-R	20	TAA CAG GAG GTG CTC TGA AAG AGT AAG CAG AGG AG
I465-6H-not	21	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGT ATA CAG ACA CCA TCA GC
F468-6H-not	22	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA AAA GCA TCT ATA CAG ACA CC
P471-6H-not	23	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA GGT TTT AGA AAA GCA TCT ATA C
PH20-HM17	24	TCC AGG CCC AGA GGA AAG GCC GGT TGG GTA GCA AGG GGC CCC TAA AAG AGT AAG CAG AGG AG
PH20-HM18	25	TCA CTT GGG GCA TTC CAG ACG GTG GTG AAG GGC CGG TTG GGT AGC AAG GGG CCC CTA AAA GAG TAA GCA GAG GAG
K470-not	26	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGT TTT AGA AAA GCA TCT ATA C
F468-not	27	CTA ATT GCG GCC GCT CAT TAA AAA GCA TCT ATA CAG ACA CC
M21-mega-F	28	AAT TGT AAT ATG GGG AAG CTG GGA AAA TAC AAG AA
M24-R	29	TGG AAT AAC AGG AGG TGC AGA GTA AGC AGA GGA GA
M25-R	30	TTT GGA ATA ACA GGA GAG TAA GCA GAG GAG A

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B4-HM26	31	AGT TTT GAA ATT CCT TTC TCT GGA TGA GCT GGA GCA CAG CCT GGG GGA GAG TGC GGC CCA GGG TGC TTC TGG AAT TG
B4-HM27	32	ATG AGC TGG AGC ACA GCT TTG GGG AGA GTG CGG CCC AG
B4-HM28	33	ATT CCT TTC TCA AGA TGA ACT TGA GCA CAG CTT TGG CGA AAC TGT TGC
B4-HM29	34	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA GCA TCT ATA CAG ACA CC
B4-HM30	35	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA CAG ACA CCA TCA GCA ATA C
B4-HM31	36	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA TCA GCA ATA CAC ACA TC
B4-HM32	37	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA CAC ACA TCA ACA GCA TC
B4- HM33	38	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA ACA GCA TCA GTG TCT TTT AC
P472-not	39	GTT ATA GCG GCC GCT CAT TAG GGA GGT TTT AGA AAA GCA TC
M473-not	40	GTT ATA GCG GCC GCT CAT TAC ATG GGA GGT TTT AGA AAA GCA TC
E474-not	41	GTT ATA GCG GCC GCT CAT TAC TCC ATG GGA GGT TTT AGA AAA GC
T475-not	42	GTT ATA GCG GCC GCT CAT TAT GTC TCC ATG GGA GGT TTT AG
M40-mega	43	TAA CAG GAG GTG CTC TGA AAT TAG AGT AAG CAG AGG AG
M41-mega	44	TGG AAT AAC AGG AGG TGC TCT AGA GTA AGC AGA GGA G
M42-mega	45	TTT GGA ATA ACA GGA GGA GAG TAA GCA GAG GAG
D466-6H-not	46	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA TCT ATA CAG ACA CCA TCA GC
P478-H-Not	47	GAT AAT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA GGT TCT TCT GTC TCC ATG GG
I480-H-Not	48	GAT AAT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGA ATT TGA GGT TCT TCT GTC TCC
Y482-H-Not	49	GAT AAT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGG TAG AAA ATT TGA GGT TCT TCT G
A484-H-not	50	GAT AAT GCG GCC GCT CAT TAG TGG TGA TGG

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		TGA TGA TGA GCA TTG TAG AAA ATT TGA GGT TC
P486-H-not	51	GAT AAT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGG GGT GAA GCA TTG TAG AAA ATT TGA GG
T488-H-not	52	GAT AAT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGT GTG GAG GGT GAA GCA TTG TAG
K470-6H-not	53	CTA ATT GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGT TTT AGA AAA GCA TCT ATA C
P472-6H-not	54	GTT ATA GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGG GGA GGT TTT AGA AAA GCA TC
M473-6H-not	55	GTT ATA GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGC ATG GGA GGT TTT AGA AAA GCA TC
E474-6H-not	56	GTT ATA GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGC TCC ATG GGA GGT TTT AGA AAA GC
T475-6H-not	57	GTT ATA GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGT GTC TCC ATG GGA GGT TTT AG
E476-6H-not	58	GTT ATA GCG GCC GCT CAT TAG TGG TGA TGG TGA TGA TGT TCT GTC TCC ATG GGA GG
E476-not	59	GTT ATA GCG GCC GCT CAT TAT TCT GTC TCC ATG GGA GG

[258] After finding the PH20 variant with increased enzymatic activity and thermal stability, the 6xHis-tag-free cDNA of the PH20 variant was also constructed.

[259] When the cell density of ExpiCHO cells reached 6×10^6/mL, a plasmid comprising the wild-type or variant PH20 cDNA inserted in the pcDNA3.4-TOPO vector was transfected into the ExpiCHO cells by ExpiFectamine CHO reagent. As a cell culture medium, ExpiCHO expression medium (100 to 500 mL) was used. After transfection, the ExpiCHO cells were shake-cultured at 130 rpm for total 6 days, during which the cells were cultured at 37°C for 1 day and further cultured at lower temperature of

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32°C for 5 days. After completion of the culture, the cell supernatant was collected by centrifugation at 10,000 rpm for 30 min.

[260] The recombinant proteins of the C-terminal 6xHis-attached wild-type PH20 and variant PH20, produced in the ExpiCHO cells, were purified in three steps (performed using a HisTrap column, a Q Sepharose column and a Phenyl column, respectively) by an AKTA prime system.

[261] For protein purification using the HisTrap column, buffer A (20 mM sodium phosphate, pH 7.5, 0.5 M NaCl) and buffer B (20 mM sodium phosphate, pH 7.5, 0.5 M NaCl, 0.5 M imidazole) were prepared. The protein was bound to the HisTrap column, and the column was flushed with 5 column volumes (CV) of buffer A to remove non-specifically bound proteins. It was confirmed that the conductivity was maintained at constant level, the column was flushed with 5 CV of 20% buffer B to elute the protein. The eluted protein was dialyzed with dialysis buffer (20 mM sodium phosphate, pH 7.5, 50 mM NaCl). For protein purification using the Q Sepharose column, buffer A (20 mM sodium phosphate, pH 7.5) and buffer B (20 mM sodium phosphate, pH 7.5, 0.5 M NaCl) were prepared. The protein was bound to the Q Sepharose column, and the column was flushed with 5 CV of buffer A to remove nonspecifically bound proteins, and then 5 CV of buffer B was flushed at concentration gradient of 0 to 100% to elute the protein.

[262] For protein purification using the phenyl column, buffer A (20 mM sodium phosphate, pH 7.0, 1.5 M $(NH_4)_2SO_4$) and buffer

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B (20 mM sodium phosphate, pH 7.0) were prepared. The protein was bound to the phenyl column, and the column was flushed with 5 CV of buffer A to remove nonspecifically bound proteins, and then 5 CV of buffer B was flushed at concentration gradient of 0 to 100% to elute the protein.

[263] The enzymatic activities of wild-type PH20 and variant PH20 were measured by turbidimetric assay, substrate-gel assay, and Morgan-Elson assay.

[264] The turbidimetric assay is a method of measuring the absorbance in the precipitate produced when hyaluronic acid is mixed with albumin (BSA). When hyaluronic acid is hydrolyzed by PH20, the absorbance of the precipitate produced when mixed with albumin decreases. Hyaluronidase PH20 (Sigma) was diluted to 1, 2, 5, 7.5, 10, 15, 20, 30, 50 and 60 units/mL, and prepared in each tube. The purified protein sample was dissolved in enzyme diluent buffer (20 mM Tris·HCl, pH 7.0, 77 mM NaCl, 0.01% (w/v) bovine serum albumin) and diluted to 100X, 300X, 600X, 1200X and 2400X and prepared in each tube. In fresh tubes, the hyaluronic acid solution having a concentration of 3 mg/mL was diluted 10-fold to a concentration of 0.3 mg/mL so that the volume of each tube became 180 μL. 60 μL of enzyme was added to and mixed with the diluted hyaluronic acid solution and allowed to react at 37°C for 45 min. After completion of the reaction, 50 μL of the reacted enzyme and 250 μL of acidic albumin solution were added to each well of a 96-well plate and shaken for 10 min,

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and then the absorbance was measured at 600 nm by spectrophotometer.

[265] In the substrate-gel assay, the protein was electrophoresed on 10% SDS gel (including 0.17 mg/mL hyaluronic acid) for 1 h, and SDS was removed with 2.5% Triton X-100 (w/v) at 4°C for 2 h. Thereafter, an enzymatic reaction was performed in the buffer (50 mM sodium phosphate, pH 7.0, 150 mM NaCl) at 37°C (which is the optimum temperature for PH20) for 1 to 4 h, and the protein was stained with 0.5% Alcian blue reagent. Alcian blue reagent not bound to the hyaluronic acid was removed using de-staining solution. The SDS gel stained with Alcian blue was imaged, and then the band was quantified.

[266] The thermal stability of the protein was measured by a method of measuring the aggregation temperature by dynamic light scattering (DLS), a method of measuring the melting temperature (T_m) in real-time PCR using Sypro-Orange dye, a method of measuring the enzymatic activity after leaving the protein to stand at predetermined temperature for a predetermined time, etc. In the method of measuring the aggregation temperature by DLS, the aggregation of molecules is measured using light scattering, and thus the sensitivity is high and the aggregation temperature is generally lower than the melting temperature of the protein.

[267] The sequences of the substituted or cleaved amino acids in the PH20 variants constructed in the present invention are shown in Table 11 below.

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[268] Among the variants according to the present invention, the variant having the 6xHis-tag attached to the C-terminus of PH20 was named HM; the variant free of the 6xHis-tag was named HP; mature PH20 (L36 to S490) having the 6xHis-tag attached to the C-terminus was named WT; and mature wild-type PH20 (L36 to Y482) having the C-terminus cleaved after Y482 while being free of the 6xHis-tag was named HW2.

[270]~[303]

Table 11. Amino acid sequences of PH20 variants according to the present invention and substitution/cleavage characteristics thereof

Name	SEQ NO.	Substitution	Amino Acid Sequence
HM1	60	12 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D, I361T and N363G.	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**S C**QAIKE**YM**D**T**T**L**G**PYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM2	61	7 amino acids substitution of Y365F, I367L, L371S, A372G, K374L, M375L and V379A	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSITRTKES CQAIKEYMDTTLNP**F**I**L**NVT**SG**A**LL**CSQ**A**LCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV

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			DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM3	62	19 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D, I361T, N363G, Y365F, I367L, L371S, A372G, K374L, M375L and V379A	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**S C**QAIKE**YM**D**T**T**L**G**P**F**I**L**NVT**SG**A**LL**CSQ**A**LCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM4	63	17 amino acids substitution of G340V, T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D, I361T and N363G	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIW**VSWENT**R**TKE**S C**QAIKE**YM**D**T**T**L**G**PYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM6	64	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM7	65	16 amino acids substitution of G340V, T341S L342W, S343E, I344N, M345T, S347T, M348K,	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR

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		K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T	NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIW**VSWENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM8	66	12 amino acids substitution of I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLS**NT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM9	67	13 amino acids substitution of S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTL**ENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM10	68	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK

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			PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM11	69	13 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D, I361T, Y365F and I367L	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**S C**QAIKE**YM**D**T**T**LNP**F**I**L**NVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM12	70	15 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D, I361T, Y365F, I367L, L371S and A372G	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**S C**QAIKE**YM**D**T**T**LNP**F**I**L**NVT**SG**AKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM13	71	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, and truncation before F38 at the N-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM14	72	11 amino acids substitution of M345T, S347T,	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL

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		M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T and truncation after I465 at the C-terminus	GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCI
HM15	73	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, and truncation after F468 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAF
HM16	74	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, and truncation after P471 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKP
HM17	75	Substitution of L36~V47 with FRGPLLPNR, and amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A,	**FRGPLLPNR**PFLWAWNAPSEFCLGKFDEPLDMSLF SFIGSPRINATGQGVTIFYVDRLGYYPYIDSITGV TVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMA VIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQ LSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPN HLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDD LSWLWNESTALYPSIYLNTQQSPVAATLYVRNRVR EAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQ

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		L354I, D355K, N356E, E359D and I361T	DELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**SC**QA** **IKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVCI RKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTL EDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDVC IADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM18	76	Substitution of L36~A52 with FRGPLLPNRPFTTV, and amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T	**FRGPLLPNRPFTTV**WNAPSEFCLGKFDEPLDMSLF SFIGSPRINATGQGVTIFYVDRLGYYPYIDSITGV TVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMA VIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQ LSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPN HLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDD LSWLWNESTALYPSIYLNTQQSPVAATLYVRNRVR EAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQ DELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**SC**QA** **IKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVCI RKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTL EDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDVC IADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM19	77	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after K470 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLK
HM20	78	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT

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		F38 at the N-terminus, and truncation after F468 at the C-terminus	LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAF
HM21	79	15 amino acids substitution of T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWG**SWENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM24	80	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, and truncation before A40 at the N-terminus	APPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFS FIGSPRINATGQGVTIFYVDRLGYYPYIDSITGVT VNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAV IDWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQL SLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNH LWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDL SWLWNESTALYPSIYLNTQQSPVAATLYVRNRVRE AIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQD ELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**SC**QAI KE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVCIR KNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTLE DLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDVCI ADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM25	81	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before P42 at the N-terminus	PVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSFI GSPRINATGQGVTIFYVDRLGYYPYIDSITGVTVN GGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVID WEEWRPTWARNWKPKDVYKNRSIELVQQQNVQLSL TEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHLW GYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLSW LWNESTALYPSIYLNTQQSPVAATLYVRNRVREAI RVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDEL VYTFGETVALGASGIVIWGTLSI**T**R**TKE**SC**QAIKE** YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVCIRKN WNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTLEDL EQFSEKFYCSCYSTLSCKEKADVKDTDAVDVCIAD GVCIDAFLKPPMETEEPQIFYNASPSTLS

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HM29	82	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after A467 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDA
HM30	83	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after C464 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVC
HM31	84	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after D461 at the C-	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIAD

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		terminus	
HM32	85	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after C458 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVC
HM33	86	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after V455 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV
HP34	87	15 amino acids substitution of T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWG**SWENT**R**TKE**SC**Q AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLK

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		truncation after K470 at the C-terminus	
HM35	88	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after P472 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPP
HM36	89	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after M473 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPM
HM37	90	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT

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		terminus, and truncation after E474 at the C-terminus	LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPME
HM38	91	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after T475 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMET
HM39	92	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after E476 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMETE
HM40	93	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, and truncation before	NFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMS LFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSIT GVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLG MAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQN VQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLR PNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRN DDLSWLWNESTALYPSIYLNTQQSPVAATLYVRNR VREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFL SQDELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**SC

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		N37 at the N-terminus	**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGV CIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKP TLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVD VCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM41	94	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, and truncation before R39 at the N-terminus	RAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLF SFIGSPRINATGQGVTIFYVDRLGYYPYIDSITGV TVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGMA VIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQ LSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRPN HLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDD LSWLWNESTALYPSIYLNTQQSPVAATLYVRNRVR EAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQ DELVYTFGETVALGASGIVIWGTLSI**T**R**TKE**SC**QA IKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVCI RKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTL EDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDVC IADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM42	95	11 amino acids substitution of M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, and truncation before P41 at the N-terminus	PPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSLFSF IGSPRINATGQGVTIFYVDRLGYYPYIDSITGVTV NGGIPQKISLQDHLDKAKKDITFYMPVDNLGMAVI DWEEWRPTWARNWKPKDVYKNRSIELVQQQNVQLS LTEATEKAKQEFEKAGKDFLVETIKLGKLLRPNHL WGYYLFPDCYNHHYKKPGYNGSCFNVEIKRNDDLS WLWNESTALYPSIYLNTQQSPVAATLYVRNRVREA IRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLSQDE LVYTFGETVALGASGIVIWGTLSI**T**R**TKE**SC**QAIK E**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVCIRK NWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPTLED LEQFSEKFYCSCYSTLSCKEKADVKDTDAVDVCIA DGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM43	96	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after I465 at the C-	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGS**WENT**R**TKE**SC**Q AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCI

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		terminus	
HM44	97	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after D466 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGS**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCID
HM45	98	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after A467 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGS**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDA
HP46	99	15 amino acids substitution of T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWG**SWENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV

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		terminus, and truncation after F468 at the C-terminus	CIADGVCIDAF
HM47	100	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after P478 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMETEEP
HM48	101	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after I480 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**TT**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMETEEPQI
HM49	102	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T,	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q**

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		truncation before F38 at the N-terminus, and truncation after Y482 at the C-terminus	**AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMETEEPQIFY
HM50	103	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after A484 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMETEEPQIFYNA
HM51	104	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after P486 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMETEEPQIFYNASP
HM52	105	14 amino acids substitution of L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I,	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV

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		D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after T488 at the C-terminus	REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGT**WENT**R**TKE**SC**Q AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAFLKPPMETEEPQIFYNASPST
HM53	106	15 amino acids substitution of T341G, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after S490 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWG**GWENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM54	107	15 amino acids substitution of T341A, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after S490 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWG**AWENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HM55	108	15 amino acids substitution of T341C, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A,	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN

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		L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after S490 at the C-terminus	RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWG**CWENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQ109IFYNASPS TLS
HM56	109	15 amino acids substitution of T341D, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before L36 at the N-terminus, and truncation after S490 at the C-terminus	LNFRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDM SLFSFIGSPRINATGQGVTIFYVDRLGYYPYIDSI TGVTVNGGIPQKISLQDHLDKAKKDITFYMPVDNL GMAVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQ NVQLSLTEATEKAKQEFEKAGKDFLVETIKLGKLL RPNHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKR NDDLSWLWNESTALYPSIYLNTQQSPVAATLYVRN RVREAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKF LSQDELVYTFGETVALGASGIVIWG**DWENT**R**TKE**S C**QAIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQG VCIRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGK PTLEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAV DVCIADGVCIDAFLKPPMETEEPQIFYNASPSTLS
HP57	110	12 amino acids substitution of I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after F468 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGTLS**NT**R**TKE**SC**Q AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAF
HP58	111	13 amino acids substitution of S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWGTL**ENT**R**TKE**SC**Q**

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		before F38 at the N-terminus, and truncation after F468 at the C-terminus	**AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAF
HP59	112	15 amino acids substitution of T341A, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after F468 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWG**AWENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAF
HP60	113	15 amino acids substitution of T341G, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after F468 at the C-terminus	FRAPPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWG**GWENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAF
HP61	114	16 amino acids substitution of A40G, T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before	FR**G**PPVIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWG**SWENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT

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		F38 at the N-terminus, and truncation after F468 at the C-terminus	LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAF
HP62	115	Removal of P42, 15 amino acids substitution of T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T, truncation before F38 at the N-terminus, and truncation after F468 at the C-terminus	FRGP**P**VIPNVPFLWAWNAPSEFCLGKFDEPLDMSL FSFIGSPRINATGQGVTIFYVDRLGYYPYIDSITG VTVNGGIPQKISLQDHLDKAKKDITFYMPVDNLGM AVIDWEEWRPTWARNWKPKDVYKNRSIELVQQQNV QLSLTEATEKAKQEFEKAGKDFLVETIKLGKLLRP NHLWGYYLFPDCYNHHYKKPGYNGSCFNVEIKRND DLSWLWNESTALYPSIYLNTQQSPVAATLYVRNRV REAIRVSKIPDAKSPLPVFAYTRIVFTDQVLKFLS QDELVYTFGETVALGASGIVIWG**SWENT**R**TKE**SC**Q** **AIKE**YM**D**T**T**LNPYIINVTLAAKMCSQVLCQEQGVC IRKNWNSSDYLHLNPDNFAIQLEKGGKFTVRGKPT LEDLEQFSEKFYCSCYSTLSCKEKADVKDTDAVDV CIADGVCIDAF

Example 2. Construction of PH20 Variants HM1 and HM6

[305] As shown in FIG. 1B, amino acid residues M345 to N363 of PH20 correspond to the alpha-helix 8 region and the linker region between alpha-helix 7 and alpha-helix 8 in the protein tertiary structure model. Among the amino acids of alpha-helix 8, C351 forms a disulfide bond with C60 of alpha-helix 1; Y357 forms hydrophobic interaction with F315 of alpha-helix 7; and N363 forms a hydrogen bond with D69 of alpha-helix 1, thereby stabilizing secondary structures adjacent to alpha-helix 8 (FIG. 1C).

[306] In order to construct variants having higher enzymatic activity and thermal stability than WT by substituting amino acids located in the alpha-helix 8 region of PH20 and the

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linker region between alpha-helix 7 and alpha-helix 8, the following variants were constructed: the variant HM1 in which 12 amino acids in the M345 to N363 region were substituted; the variant HM2 in which 7 amino acids in the Y365 to V379 region were substituted; and the variant HM3 in which 19 amino acids in the M345 to V379 region were substituted. Among the amino acids located in alpha-helix 8 of PH20, C351, which is involved in disulfide bonding, and Y357 which is involved in hydrophobic interaction, were not substituted. The substituted amino acid sequences in the variants HM1, HM2 and HM3 are shown in Table 11 above. When ExpiCHO cells were transfected with the pcDNA3.4-TOPO plasmid comprising the gene of the variant HM1, HM2 or HM3, the variant HM1 was expressed in the ExpiCHO cells (FIG. 3A), and the variants HM2 and HM3 were not expressed in the cells. Whether the protein would be expressed was confirmed not only by measurement of the enzymatic activity, but also by Western blot analysis using an antibody (Abcam, ab193009) against human PH20. The epitope of the antibody is Q173 to P222 region. These experimental results suggest that amino acid substitution of Y365 to V379 region of the amino acid sequence of alpha-helix 8, used in construction of the variants HM2 and HM3, causes a serious effect on the protein structure; thus the proteins of the variants HM2 and HM3 were not expressed. The expression level of the variant HM1 was 3.4-fold higher than that of wild-type WT (FIG. 2). The variant HM1 is one in which the hydrogen bond between N363 of alpha-helix 8 and D69 of alpha-helix 1 was removed by

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substituting N363 with glycine (FIG. 1C). To restore the hydrogen bond between N363 and D69, G363 in HM1 was substituted with asparagine, thereby constructing the variant HM6. The substituted amino acids in the variant HM6 are shown in Table 11 above.

[307] The variant HM6 was expressed in ExpiCHO cells, and the expression level thereof was similar to that of the variant HM1 (the expression level was 3.4-fold higher than that of WT) (FIG. 2). When the enzymatic activity was measured by turbidimetric assay, it was shown that the enzymatic activity of the variant HM6 was 1.3-fold higher than that of WT (FIG. 3B). In the substrate-gel assay, generally, SDS is removed with 2.5% Triton X-100 (w/v) after SDS-PAGE, and enzymatic reaction is performed at 37°C for 1 to 4 h, during which the extent to which hyaluronidase hydrolyzes hyaluronic acid is measured using Alcian blue dye. It is known that when SDS is removed from the substrate gel, protein folding immediately occurs, and the substrate does not affect the protein folding. When the enzymatic activities of WT and the variants HM1 and HM6 were measured by the substrate-gel assay at 37°C for 1 to 4 h, HM6 exhibited higher enzymatic activity than those of WT and the variant HM1 (FIG. 3C). This result suggests that protein folding of the variants HM1 and HM6 and the resulting renaturation were faster than those of WT and that the constructed variants exhibit higher enzymatic activity than WT. When the signal peptide of PH20 itself was used, the expression level of the protein in ExpiCHO cells was low, and

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to solve this problem, the signal peptide sequence of human serum albumin or human Hyal1 was used. As shown in FIG. 3C, when each of the signal peptides of human serum albumin and human Hyal1 was used as the signal peptide of the variant HM1, the expression of the protein increased, and there was no significant difference between the two signal peptides. When the enzymatic activity was measured by the substrate-gel assay, WT having the signal peptide of human serum albumin and the variant HM1 having the signal peptide of human serum albumin or Hyal1 exhibited enzymatic activity at the pH ranging from 5 to 8 (FIG. 3D). In the present invention, the signal peptide of human serum albumin was used as the signal peptide of the variant constructed after HM1.

[308] When the thermal stabilities of WT and the variants HM1 and HM6 were compared based on the aggregation temperatures, the aggregation temperatures were 46.5°C, 53.0°C and 50.5°C, respectively, indicating that the aggregation temperatures of the variants HM1 and HM6 were 6.5°C and 4.0°C higher, than that of WT, respectively (FIG. 10). The results of measuring the aggregation temperatures were consistent with the protein refolding results shown in the substrate-gel assay. These results suggest for the first time that the hydrogen bond formed by the N363 residue of alpha-helix 8 plays an important role in the thermal stability and enzymatic activity of PH20.

[309] In addition, when the hydrophilic/hydrophobic natures of WT and the variants HM1 and HM6 were compared using a phenyl column chromatography, HM1 and HM6 were all eluted earlier

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than WT. This suggests that the variants HM1 and HM6 have a more hydrophilic nature than WT due to the substitution of the amino acids. However, the variants HM1 and HM6 eluted from the phenyl column showed two peaks, unlike WT, and exhibited the same molecular weight when treated with PNGase F. This appears to be a difference caused by N-glycosylation (Fig. 3E).

Example 3. Construction of PH20 Variants HM4, HM7, HM8, HM9, HM10, HM11 and HM12

[312] It is believed that the amino acid substitution of the M345 to N363 and M345 to I361 regions by construction of the variants HM1 and HM6 in Example 2 resulted in an increase in both the enzymatic activity and thermal stability of PH20, has made great advances in terms of protein engineering. Thus, based on the variants HM1 and HM6, other amino acids in the N-terminal and C-terminal directions were additionally substituted.

[313] First, the variants HM4 and HM7 were constructed, which comprise the substituted amino acids in the variants HM1 and HM6 and in which the amino acids between G340 and I344 were additionally substituted with G340V, T341S, L342W, S343E and I344N. The sequences of the substituted amino acid sequences in the variants HM4 and HM7 are shown in Table 11 above. The variants HM4 and HM7 were expressed in ExpiCHO cells. The results of protein purification for HM7 are shown in FIG. 5A. HM4 and HM7 showed an increase in protein expression of 6.3-fold compared to WT, and showed increases in aggregation

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temperature of 10°C and 11.5°C, respectively, compared to WT. However, the enzymatic activities of HM4 and HM7, measured by turbidimetric assay, were about 15% of that of WT (FIG. 5B). Enzymatic activity and thermal stability generally have a trade-off relationship, but in the present invention, it appeared that the substitution introduced in the variants HM1 and HM6 increased the thermal stability of the variants while maintaining the enzymatic activity, and the enzymatic activities of the variants HM4 and HM7 decreased due to an excessive increase in the thermal stability. An increase in aggregation temperature of 11.5°C, which appeared in the variants HM4 and HM7 is a very significant result in terms of protein engineering. When the structural flexibilities of the proteins were analyzed by a Stern-Volmer plot, it was shown that the structural flexibilities of HM1, HM6, HM4 and HM7, obtained by substituting alpha-helix 8 and its linker region, were all higher than that of WT (FIG. 11A). This result suggests that the increase in local thermal stability resulted in an increase in the flexibility of the entire protein structure.

[314] The difference between the variants HM6 and HM7 is amino acids between G340 and I344. In order to identify amino acids which are involved in the increased thermal stability of the variant HM7, the following variants were constructed based on the variant HM6: HM8 in which I344N was substituted; HM9 in which S343 and I344N were substituted; HM10 in which L342W, S343E and I344N were substituted; and HM21 in which T341S,

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L342W, S343E and I344N were substituted. The sequences of the substituted amino acids in the variants HM8, HM9, HM10 and HM21 are shown in Table 11 above. The variants HM8, HM9, HM10 and HM21 were expressed in ExpiCHO cells (FIG. 5A). As I344N, S343E and L342W were introduced in the N-terminal direction loop of alpha-helix 8 based on the variant HM6, the aggregation temperatures of the variants HM8, HM9 and HM10 increased to 52.5°C, 53°C and 55.5°C, respectively (FIG. 10). However, the variants HM8, HM9 and HM10 maintained enzymatic activities similar to that of WT (FIG. 5B). This result suggests that the amino acid substitution introduced in the variants HM8, HM9 and HM10 had a local effect on the thermal stabilities of the enzymes, but had no significant effect on the enzymatic activities. However, HM21 showed reduced thermal stability compared to HM10, but showed approximately 2-fold higher enzymatic activity compared to WT at pH 7.0. When WT and each variant were allowed to react with the substrate for 1 h in the substrate-gel assay, the enzymatic activity was reduced in the order of HM21> HM10> HM9> HM8> HM6> WT (FIG. 5C).

[315] When the physical properties of the variants HM7, HM8, HM9, HM10 and HM21 were examined using a phenyl column chromatography, these PH20 variants were eluted earlier than WT, suggesting that these variants all have a hydrophilic nature. However, the pattern of the main peak at the amino acid substitution position appeared differently (FIG. 5D).

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[316] The variant HM7 also showed two peaks in the phenyl column chromatography, like the variants HM1 and HM6, suggesting that two different types were present.

[317] In order to examine the patterns of migration of WT and the variants depending on their isoelectric points, isoelectric focusing (hereinafter referred to as IEF) analysis was performed (FIG. 5E). On IEF gel, WT and the variant HM6 and HM8 showed similar migration patterns, and the variants HM9, HM10, HM21 and HM7 comprising the S343E mutation migrated to a more acidic region. This result suggests that a change in the isoelectric point of the protein occurs due to the introduction of glutamic acid by S343E substitution of the amino acid between G340 and I344.

[318] In addition, based on the variant HM6, other amino acids in the C-terminal regions of alpha-helix 8 were substituted, thereby constructing the variants HM11 and HM12. The sequences of the substituted amino acids in the variants HM11 and HM12 are shown in Table 11 above. The variant HM11 was expressed in ExpiCHO cells, but the expression level thereof was lower than that of WT (FIG. 2), and the variant HM12 was not expressed in ExpiCHO cells. The variant HM11 exhibited an activity corresponding to 32% of that of WT (FIG. 6B).

Example 4. Construction of N-terminal amino acid-truncated PH20 variants based on HM6

[321] The C-terminal region of PH20 is already well known to play an important role in the expression and enzymatic

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activity of PH20, but the role of the N-terminal region of PH20 is not well known. In order to examine the effect of cleavage of the amino acid at the N-terminal region of PH20 on the enzymatic activity, variants HM40, HM13, HM41, HM24, HM42 and HM25 having the N-terminus cleaved at N37, F38, R39, A40, P41 or P42 were constructed based on the variant HM6 (Table 11). Furthermore, HP61 and HP62 with modifications at the N-terminal amino acids were additionally constructed.

[322] The variants HM40, HM13, HM41, HM24, HM42, HP61 and HP62 were expressed in ExpiCHO cells, but HM25 was not expressed (FIGS. 7A and 7B). The N-terminal truncated PH20 variants showed a difference in enzyme activity depending on the position where the N-terminus started. The variants HM40, HM13 and HM41, in which one to three amino acids were cleaved, showed enzymatic activity similar to that of the template HM6, but HM24 and HM42, in which four to five amino acids were cleaved, showed slightly lower activity than HM6 (FIG. 7C). However, HM25 in which six amino acids were cleaved were little expressed in ExpiCHO cells, and the enzymatic activity was also significantly low (3.5 U/μg). It appears that changes in the enzymatic activities of HP61 and HP62 with modifications at N-terminal amino acids are not significant.

[323] Regarding the enzymatic activities of the N-terminal cleaved PH20 variants, measured by the substrate-gel assay (1 h of reaction), the enzymatic activities of HM40, HM13 and HM41 were similar to that of the HM6 variant, but the enzymatic activities of HM24 and HM42 were lower than that of

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HM6 (FIG. 7D). HM25 in which six amino acids were cleaved could not be analyzed, because the amount of the protein produced was small.

[324] When the physical properties of the variants HM40, HM13, HM41, HM24 and HM42 were analyzed using a phenyl column chromatography, these variants were eluted from the column earlier than WT, suggesting that these variants have a hydrophilic nature (FIG. 7E). This result suggests that the variants HM40, HM13, HM41, HM24 and HM42 constructed based on the variant HM6 maintained the hydrophilic nature of HM6 when considering the characteristics of L36 to A40 residues.

[325] The aggregation temperatures of the N-terminal cleaved PH20 variants, measured by DLS, were different among the variants depending on the position where the amino acid started (FIG. 7F). Although the N-terminal amino acid residues of the variants HM40, HM13, HM41 and HM42 were cleaved, they showed an aggregation temperature of 50°C or higher, indicating that the characteristics of the template HM6 were remained intact. Among these variants, the variants HM40 and HM42 exhibited an aggregation temperature which was 3 to 4°C higher than that of HM6, indicating that the thermal stability of these variants increased. In addition, in order to examine the effect of substitution of the N-terminal amino acids of PH20 on protein expression and enzymatic activity, the following variants were constructed: the variant HM17 in which N-terminal amino acid residues 36 to 47 (LNFRAPPVIPNV) of PH20 were substituted with FRGPLLPNR; and the variant HM18 in which

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N-terminal amino acid residues 36 to 52 (LNFRAPPVIPNVPFLWA) of PH20 were substituted with FRGPLLPNRPFTTV. The sequences of the substituted amino acids in the variants HM17 and HM18 are shown in Table 11 above. The variants HM17 and HM18 were not expressed in ExpiCHO cells. This suggests that, even when up to five amino acids located at the N-terminus were cleaved, the variants showed protein expression and enzymatic activity; however, substitution of more amino acid residues, such as 36 to 47 residues or 36 to 52 residues, had an effect on protein folding.

Example 5. Construction of the C-terminal amino acid-truncated HM6-based variants HM14, HM15 and HM16 of PH20

[328] The C-terminal region of PH20 is known to play an important role in protein expression and enzymatic activity. In the present invention, the variants HM14, HM15 and HM16, in which the C-terminal amino acids were cleaved at I465, F468 and K471, respectively, were constructed based on the variant HM6. The sequences of the substituted amino acids in these variants HM14, HM15 and HM16 are shown in Table 11 above. These variants HM14, HM15 and HM16 were expressed in ExpiCHO cells (FIG. 8A), and the protein expression levels thereof were in the order of HM16 > HM15 > HM14, indicating that the protein expression levels decreased as the number of C-terminal amino acids cleaved increased (FIG. 8A). However, the enzymatic activities of the variants HM14, HM15 and HM16 were in the order of HM16 > HM14 (≈WT) > HM15 (FIG. 8B). According

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to Frost et al., the C-terminal 477-483 region of PH20 is necessary for soluble expression, and when the C-terminus is cleaved at 467, the enzymatic activity of the variant is only 10% of a PH20 variant whose C-terminus was cleaved at residues 477 to 483, and when the C-terminus is cleaved before residue 467, the variant has no enzymatic activity. However, the C-terminal cleaved variants HM14, HM15 and HM16 constructed based on the variant HM6 in the present invention showed increased protein folding due to amino acid substitution of the M345 to I361 region, and thus the thermal stability thereof increased. For this reason, even when the C-terminus was cleaved after I465, F468 or P471, the variant showed enzymatic activity similar to that of WT, and the enzymatic activity thereof did not significantly decrease.

[329] The structural flexibilities of WT and the variants HM14, HM15 and HM16 were examined by fluorescence quenching using acrylamide (FIG. 11B). The variants HM14, HM15 and HM16 were all structurally more flexible than WT. This result suggests that the C-terminal cleaved variants constructed using the variant HM6 also maintained their structural flexibility.

[330] The enzymatic activities of the variants HM14, HM15 and HM16, measured by turbidimetric assay, were also confirmed in substrate-gel assay (FIG. 8C).

[331] When the physical properties of the C-terminal cleaved variants were analyzed using a phenyl column chromatography, the variants HM14, HM15 and HM16 were all eluted earlier than WT, indicating that they had a hydrophilic nature. In addition,

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the hydrophobicities of these variants were in the order of HM16 > HM14 > HM15 (FIG. 8D).

Example 6. Construction of the HM10-Based Variants HM19 and HM20 Comprising N-Terminal and C-Terminal Amino Acid Cleavage

[334] The PH20 variants constructed in the present invention were based on HM6, and HM8, HM9 and HM10, in which amino acid residues G340 to I344 were additionally substituted, exhibited better performance than WT in terms of protein expression levels, enzymatic activities and thermal stabilities. The variants HM19 and HM20 were constructed which had an N-terminus cleaved at F38 and a C-terminus cleaved at F468 based on HM10 having high enzymatic activity and thermal stability among variants HM8, HM9 and HM10. HM19 and HM20 were all expressed in ExpiCHO cells and purified using a HisTrap column chromatography (FIG. 9A). When the enzymatic activities of these variants were measured by turbidimetric assay, HM19 and HM20 exhibited an enzymatic activity which was 10% higher than that of WT (FIG. 9B). In substrate-gel assay, HM19 and HM20 also exhibited higher enzymatic activity than WT (FIG. 9C).

Example 7. Characterization of HM10-Based PH20 Variants

[337] The expression levels of HM10-based C-terminal truncated variants in ExpiCHO cells showed a tendency to decrease as the length of the C-terminal region became shorter, and these variants were not expressed when the C-terminus was truncated

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at C464 or shorter (FIG. 12). C464 is necessary because it forms a disulfide bond with C437 and is important for maintaining the protein structure.

[338] In order to examine whether the variant is not expressed in ExpiCHO cells when the C-terminus is cleaved at residue 464 or shorter, Western blot analysis was performed. As shown in FIG. 13, the HM30, HM31, HM32 and HM33 variants were not detected in Western blots.

[339] The enzymatic activities of the HM10-based C-terminal truncated variants, measured by turbidimetric assay, are shown in FIGS. 14A and 14B. The C-terminal truncated PH20 variants exhibited an enzymatic activity of ±20% compared to WT. When the C-terminus was cleaved after I480, the enzyme activity increased overall. In addition, the variants HP19 and HP20, obtained by removing the 6xHis-tag from HM19 and HM20, showed decreases in enzymatic activity of 23% and 9.6%, respectively, compared to when the 6xHis-tag was present. This suggests that the 6xHis-tag has an effect on the enzymatic activity.

[340] When the enzymatic activities of the HM10-based C-terminal truncated variants were measured by substrate-gel assay, these variants exhibited higher enzymatic activity than WT, and showed enzymatic activity similar to that of the template HM10, indicating that the difference in enzymatic activity depending on the length of the C-terminal region was not significant (FIG. 14C).

Example 8. Characterization of HM21-Based PH20 Variants

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[343] The variant HP34 was purified by four-step column chromatography (FIG. 15A), and the variant HP46 was purified by three-step column chromatography (FIG. 15B). The amounts of HP34 and HP46 produced were 1.73 mg/L and 25.6 mg/L, respectively. HP34 and HP46 are 6xHis-tag-free variants, and the process of purifying these variants differs from the process of purifying variants having the 6xHis-tag, and thus it is difficult to compare the expression levels of the proteins.

[344] In turbidimetric assay, the activities of HP34 and HP46 were 45.6 U/μg and 47.2 U/μg, respectively, which were about 2-fold higher than that of WT and were about 10% higher than that of the template HM21 (FIG. 16A).

[345] The kinetics of each variant was measured by Morgan-Elson assay, and the results of the measurement are shown in FIG. 16B. The catalytic efficiencies (k_{cat}/K_m) of HP34 and HP46 were 1.7 to 2 times higher than that of wild-type HW2. This result is consistent with the result that the specific activity was higher than that of WT. The Michaelis constant (K_m) was lower in these variants than in HW2, indicating the substrate affinities of these variants increased. From these results, it can be concluded that the HM21, HP34 and HP46 variants bind strongly to the substrate and have the property of converting the substrate into a product with high efficiency. This property is attributable to the effect of substitution of T341 with serine. When threonine located at position 341 is substituted with an amino acid, such as

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alanine, glycine, aspartic acid or the like, the effect of the substitution on the enzymatic activity can be predicted.

[346] The aggregation temperatures of HP34 and HP46, measured by DLS, were 51.5°C and 51.0°C, respectively, which were similar to that of the template HM21 and were about 5°C higher than that of HW2, indicating that these variants were thermally stable (FIG. 17A). The enzymatic activity of HP20, measured by substrate-gel assay, was similar to that of HP20, whereas HP46 exhibited higher enzymatic activity than HP20, indicating that the protein folding of HM21 as a template was better than that of HM10 (FIG. 17B).

[347] Wild-type HW2 and the variant HP46 were left to stand overnight at pH 7.0 and pH 3.0, and then the enzymatic activities thereof were compared by substrate-gel assay. As a result, it was shown that HP46 exhibited high activity not only at pH 7.0 but also at pH 3.0, indicating that it had excellent stability (FIG. 17C).

[348] HM53, HM54, HM55, HM56, HP59 and HP60 are variants having a mutation at the amino acid at position 341, among HM21-based variants. It was confirmed that the mutation of the amino acid at position 341 had various effects on the expression level and activity of the variants (FIGS. 17D and 17E).

Example 9. *In Vitro* Immunogenicity Assay of PH20 Variant

[351] Biopharmaceuticals with higher molecular weights than those of low-molecular-weight synthetic chemicals have a risk

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of triggering unintended immune responses when they enter the human body. An outside contact surface, created by folding or interaction with adjacent domains in the secondary structure or tertiary structure of a large-molecular-weight biomaterial, can promote immune response to the biomaterial by providing an epitope to the immune system in the human body. This immune response can produce an anti-drug antibody (ADA), and this response can inhibit the drug's effectiveness, or cause hypersensitivity to the drug, or promote clearance of the drug in the human body. The immune response to the drug may therefore affect the results in clinical trials, and may cause serious abnormal reactions upon long-term use. This immune response can be influenced by various factors, and triggered by the specific response to the drug itself or disease, or by factors that depend on the method of drug administration or individual patients. Factors caused by the drug itself include the similarity or dissimilarity of the biopharmaceuticals to human peptides, posttranslational modification, impurities, aggregate formation, and the characteristics of the formulation. Factors that varies among individual patients include sex, reactivity with other drugs being taken, and genetic factors depending on the type of human leukocyte antigen (HLA).

[352] This immunogenic response is triggered by CD4 + T cells or CD8 + T cells that recognize epitopes regardless of the cause of the immune response. Because of HLA class II gene diversity in individuals, epitopes of CD4 + T cells are

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different among individuals, and therefore the responsiveness to biopharmaceuticals in CD4+ T cells in each blood provided by the healthy donor can be a very important criterion for evaluating immune responses that may arise in clinical processes. CD4 + T cells are activated by antigen presenting cells (APCs) recognizing the antigens presented through their type II MHC (major histocompatibility complex). Activated CD4 + T cells release cytokines that activate macrophages, cytotoxic T cells, and B cells, resulting in high levels of antibody production. On the contrary, CD8 + T cells have direct cytotoxicity and directly remove cells infected with antigens, damaged cells, or cells that lost their function. CD8 + T cells have T cell receptors that can recognize specific antigen peptides bound to type I MHC molecules located on the surface of every cell. CD8 + T cells can also be activated by recognizing antigens presented by antigen presenting cells, and this activation can be further enhanced by cytokines of CD4 + T cells. Therefore, when the activation level of CD4 + T cells and CD8 + T cells by new biomaterials is measured *in vitro*, it is possible to predict immunogenic responses that can be induced in clinical processes. In this example, in order to predict the immunogenicity of the PH20 variant in comparison with a control, CD4+ T cells and CD8+ T cells were isolated from PBMCs, and then treated with 1.5 ng/mL and 15 ng/mL of the control PH20 and the PH20 variant (HP46), and then the distributions of activated CD4+ T cells and CD8+ T cells were measured. The activation level of each

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type of T cells was measured using Stimulating Index, and Stimulating Index (SI) is defined as follows:

[353] Stimulating Index (SI) = (T-cell activation level after treatment with test sample) / (T-cell activation level after treatment with vehicle)

If the SI value of cells is 2 or more, it can be determined that the cells were activated at the significant level.

[355] Immunogenic responses may vary depending on the HLA type. Therefore, experiments for measuring responses in more various HLA types were performed using T cells isolated from PBMCs provided from 10 healthy donors. The HLA types of the 10 PBMCs used are shown in Table 12 below.

Table 12. HLA types of tested PBMCs

No.	HLA A		HLA B		HLA C1		DRB1		DQB1		DPB1	
1	0206	3303	3501	5801	0302	0801	1405	1501	0303	0602	0201	0301
2	0201	0207	4601	5101	0102	1502	0803	1101	0301	0601	0201	0202
3	0206	3101	1501	3501	0304	0401	0803	0901	0303	0601	0201	0501
4	0101	3101	1501	1517	0303	0701	1302	1501	0602	0604	0401	0501
5	0201	3101	5101	6701	0702	1402	0401	0403	0301	0302	0201	0401
6	0201	1101	1501	3901	0401	0702	0102	0901	0402	0501	0101	1401
7	0201	0206	4002	5502	0102	0304	0802	1454	0302	0502	0402	0501
8	0201	0201	1501	4001	0303	0304	0802	1406	0301	0302	0501	1301
9	1101	2402	4006	5101	0801	1502	0701	0901	0202	0303	0201	1301
10	2602	3101	4002	5101	0304	1502	0405	1501	0401	0602	0201	0501

[358] The results of measuring the activation levels of CD4+ and CD8+ T cells treated with each of PH20 and the PH20

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variant are summarized in Table 13 below. When looking at the results of measuring the activation levels of CD4+ and CD8+ T cells, it appears that PH20 and the PH20 variant all show relatively low activation levels. In the case of PH20, the activation level of CD4+ T cells was measured to be 2 or more in two experiments, and in the case of the PH20 variant, the activation of CD4+ T cells was not detected. In the case of PH20, the activation of CD8+ T cells was detected in one experiment, and in the case of the PH20 variant, the activation of CD8+ T cells was also detected in one experiment. However, in the case of PH20, the SI value was measured to be 2 or more at both 1.5 ng/mL and 15 ng/mL, but in the case of the PH20 variant, an SI value of 2 or less at 1.5 ng/mL and an SI value of 2 or more at 15 ng/mL were measured (see FIGS. 18 and 19).

[359] Therefore, at the lower concentration, the activation level of CD8 + T cells was observed to be low in the case of the PH20 variant, and it is determined that the activation level of CD8+ T cells by PH20 is higher than the activation level of CD8+ T cells by the PH20 variant. The conclusions drawn from the above results are as follows:

[360] 1) the activation levels of CD4+ T cells and CD8+ T cells by PH20 and the PH20 variant are relatively low; and

[361] 2) the possibility of activation of CD4+ T cells and CD8+ T cells by the PH20 variant is lower than that by PH20.

[362] From these results, it is expected that the PH20 variant will have a lower possibility of triggering an immunogenic response in clinical processes than PH20.

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Table 13. Stimulating Index (SI) measured from *in vitro* immunogenicity assay results

PBMC No.	CD4				CD8			
	PH20 (ng/mL)		PH20 variant (ng/mL)		PH20 (ng/mL)		PH20 variant (ng/mL)	
	1.5	15	1.5	15	1.5	15	1.5	15
1	1.03	0.859	0.728	1.176	1.38	1.42	1.22	1.60
2	1.05	0.09	0.596	0.922	1.20	1.11	1.00	1.07
3	1.90	1.44	1.56	1.12	1.43	1.31	1.59	1.35
4	**2.51**	**2.79**	0.788	1.82	1.28	1.90	0.579	0.974
5	1.27	1.47	1.09	1.14	0.987	0.932	0.972	1.02
6	0.825	0.834	0.998	0.946	0.904	1.13	1.09	0.986
7	**2.07**	**2.07**	1.43	1.90	**2.08**	**2.34**	1.30	**2.46**
8	0.898	1.079	0.967	0.822	0.926	1.06	0.896	0.813
9	0.882	0.957	0.895	0.941	0.853	0.993	0.950	1.09
10	0.983	0.970	1.12	1.13	1.15	1.08	1.15	1.24

ADVANTAGEOUS EFFECTS

[366] The PH20 variants or fragments thereof according to the present invention have increased protein expression levels and show an increase in protein aggregation temperature of 4-11.5°C or so when expressed in CHO (ExpiCHO) cells so that they can be efficiently produced while having high thermal stability, compared to the mature wild-type PH20.

[367] Further, as the result of substrate-gel assay, one of tests to measure the activity of hyaluronidase, the PH20 variants or fragments thereof according to the present

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invention have improved protein refolding so that they are re-natured faster than that of the mature wild-type PH20, and the original enzymatic activity is maintained regardless of the C-terminal cleavage position.

[368] Furthermore, the PH20 variants or fragments thereof according to the present invention have low immunogenicity so that they can be repeatedly administered to the human body.

[370]~[376]

Reference

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Sequence List (Free Text)

The electronic file is attached.

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【CLAIMS】

【Claim 1】

An PH20 variant or fragment thereof, which comprises one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, T341G, S343E, M345T, K349E, L353A, L354I, N356E and I361T in wild-type PH20 having the amino acid sequence of SEQ ID NO: 1.

【Claim 2】

The PH20 variant or fragment thereof of claim 1, which comprises one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, L354I and N356E.

【Claim 3】

The PH20 variant or fragment thereof of claim 1, which further comprises one or more amino acid residue substitutions in the region corresponding to an alpha-helix region and/or its linker region in wild-type PH20 having the amino acid sequence of SEQ ID NO: 1.

【Claim 4】

The PH20 variant or fragment thereof of claim 3, wherein the alpha-helix region is an alpha-helix 8 region (S347 to C381), and its linker region is a linker region (A333 to R346) between alpha-helix 7 and alpha-helix 8.

【Claim 5】

The PH20 variant or fragment thereof of claim 4, wherein the region corresponding to the alpha-helix region and the its

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linker region is T341 to N363, T341 to I361, L342 to I361, S343 to I361, I344 to I361, M345 to I361, or M345 to N363.

【Claim 6】

The PH20 variant or fragment thereof of claim 4, wherein the alpha-helix 8 region (S347 to C381) and/or the linker region (A333 to R346) between alpha-helix 7 and alpha-helix 8 are/is substituted with one or more amino acid residues of the corresponding region of Hyal1.

【Claim 7】

The PH20 variant or fragment thereof of claim 1, which comprises amino acid residue substitutions of L354I and/or N356E, and further comprises an amino acid residue substitution at one or more positions selected from the group consisting of T341, L342, S343, I344, M345, S347, M348, K349, L352, L353, D355, E359, I361 and N363.

【Claim 8】

The PH20 variant or fragment thereof of claim 7, which comprises amino acid residue substitutions of L354I and/or N356E, and further comprises one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, T341D, T341G, T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, D355K, E359D, I361T and N363G.

【Claim 9】

The PH20 variant or fragment thereof of claim 7, which comprises M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T.

【Claim 10】

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The PH20 variant or fragment thereof of claim 9, which further comprises one or more amino acid residue substitutions selected from the group consisting of T341A, T341C, T341D, T341G, T341S, L342W, S343E, I344N and N363G.

【Claim 11】

The PH20 variant or fragment thereof of claim 10, which comprises any one amino acid residue substitution selected from the following amino acid residue substitution groups:

(a) T341S, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(b) L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(c) M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D, I361T and N363G;

(d) T341G, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(e) T341A, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(f) T341C, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(g) T341D, L342W, S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T;

(h) I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T; and

(i) S343E, I344N, M345T, S347T, M348K, K349E, L352Q, L353A, L354I, D355K, N356E, E359D and I361T.

【Claim 12】

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The PH20 variant or fragment thereof of any one of claims 1 to 11, wherein further one or more of the N-terminal or C-terminal amino acid residues are deleted.

【Claim 13】

The PH20 variant or fragment thereof of claim 12, wherein cleavage occurs before an amino acid residue selected from the group consisting of M1 to P42 at the N-terminus, so that one or more amino acid residues at the N-terminus are deleted.

【Claim 14】

The PH20 variant or fragment thereof of claim 13, wherein cleavage occurs before an amino acid residue L36, N37, F38, R39, A40, P41, or P42 at the N-terminus, so that one or more amino acid residues at the N-terminus are deleted.

【Claim 15】

The PH20 variant or fragment thereof of claim 12, wherein cleavage occurs after an amino acid residue selected from the group consisting of V455 to L509 at the C-terminus, so that one or more amino acid residues at the C-terminus are deleted.

【Claim 16】

The PH20 variant or fragment thereof of claim 15, wherein cleavage occurs after an amino acid residue selected from the group consisting of V455 to S490 at the C-terminus, so that one or more amino acid residues at the C-terminus are deleted.

【Claim 17】

The PH20 variant or fragment thereof of claim 16, wherein cleavage occurs after an amino acid residue V455, C458, D461, C464, I465, D466, A467, F468, K470, P471, P472, M473, E474,

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T475, E476, P478, I480, Y482, A484, P486, T488, or S490 at the C-terminus, so that one or more amino acid residues at the C-terminus are deleted.

【Claim 18】

The PH20 variant or fragment thereof of any one of claims 1 to 17, wherein the N-terminus comprises a human growth hormone-derived signal peptide having an amino acid sequence MATGSRTSLLLAFGLLCLPWLQEGSA of SEQ ID NO: 3, a human serum albumin-derived signal peptide having an amino acid sequence MKWVTFISLLFLFSSAYS of SEQ ID NO: 4, or a human Hyal1-derived signal peptide having an amino acid sequence MAAHLLPICALFLTLLDMAQG of SEQ ID NO: 5.

【Claim 19】

The PH20 variant or fragment thereof of any one of claims 1 to 11, wherein the PH20 variant or fragment thereof is selected from the group consisting of amino acid sequences of SEQ ID NOs: 60 to 115.

【Claim 20】

The PH20 variant or fragment thereof of claim 19, which has an amino acid sequence of SEQ ID NO: 99.

【Claim 21】

A composition for treating cancer, which comprises the PH20 variant or fragment thereof of any one of claims 1 to 20.

【Claim 22】

The composition for treating cancer of claim 21, wherein the composition is used for combined treatment with other anticancer drugs.

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【Claim 23】

The composition for treating cancer of claim 22, wherein the other anticancer drugs are immuno-oncologic agents.

【Claim 24】

The composition for treating cancer of claim 23, wherein the immuno-oncologic agents are immune checkpoint inhibitors.

【Claim 25】

A nucleic acid encoding the PH20 variant or fragment thereof of any one of claims 1 to 20

【Claim 26】

A recombinant expression vector comprising the nucleic acid of claim 25.

【Claim 27】

A host cell transformed with the recombinant expression vector of claim 26.

【Claim 28】

The host cell of claim 27, wherein the host cell is selected from the group consisting of animal cells, plant cells, yeasts, *E. coli*., and insect cells.

【Claim 29】

A method for producing a PH20 variant or fragment thereof, the method comprising a step of culturing the host cell of claim 28.

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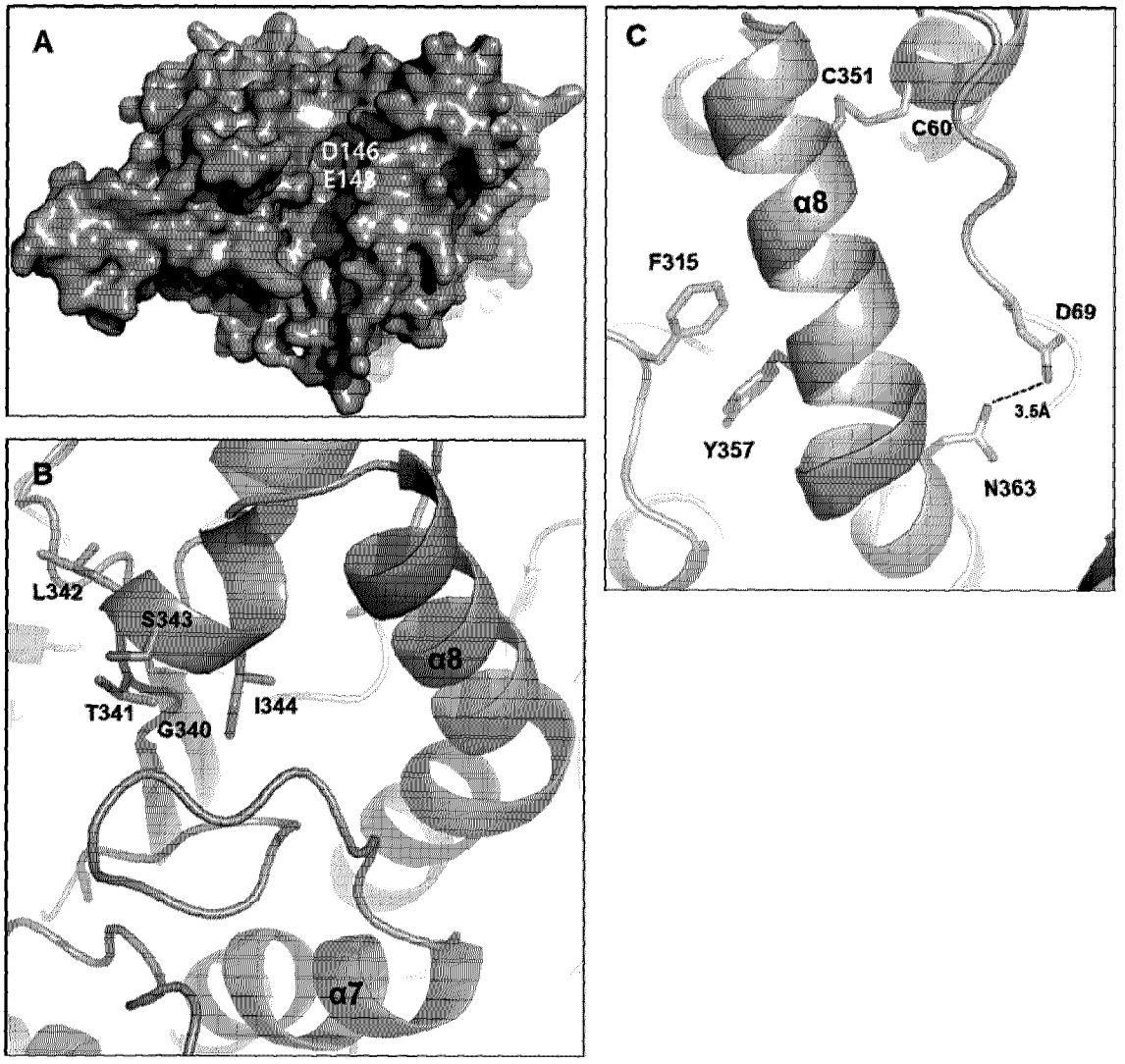
【Figures】

【Fig. 1】



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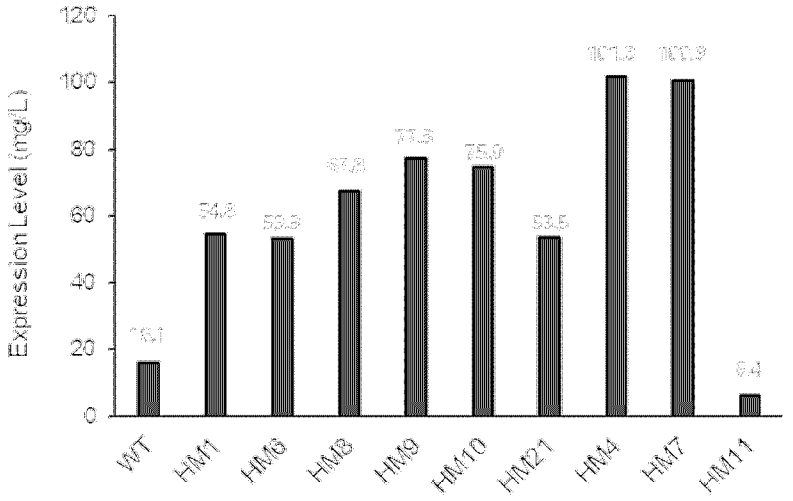
【Fig. 2】

A



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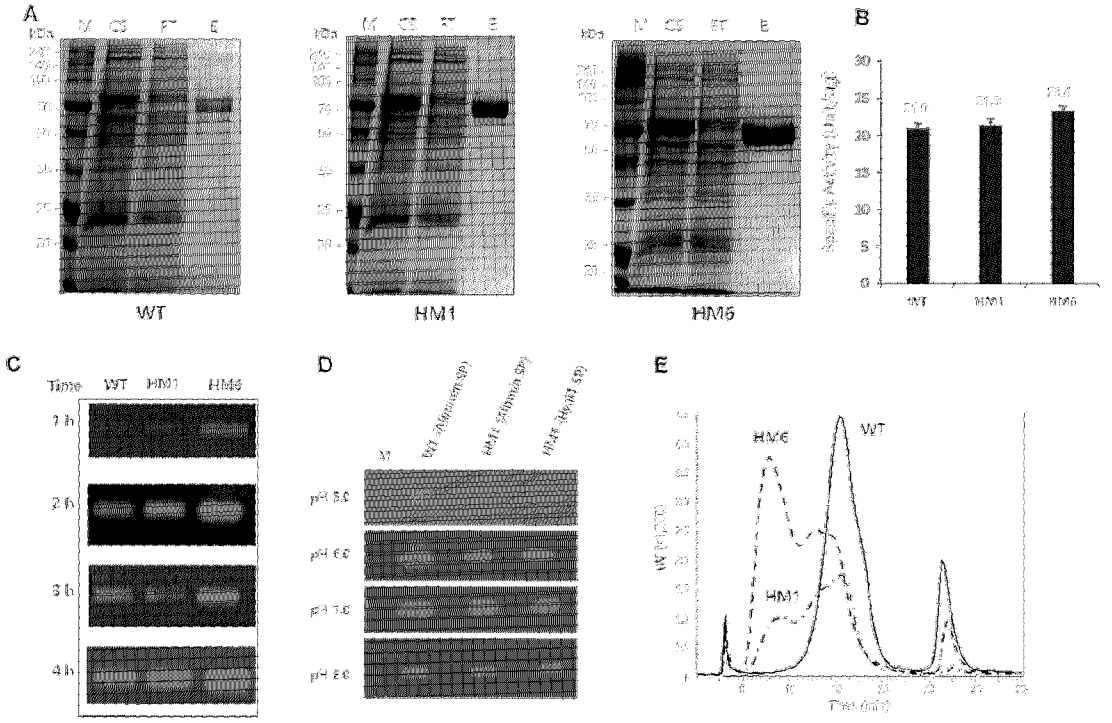
【Fig. 3】



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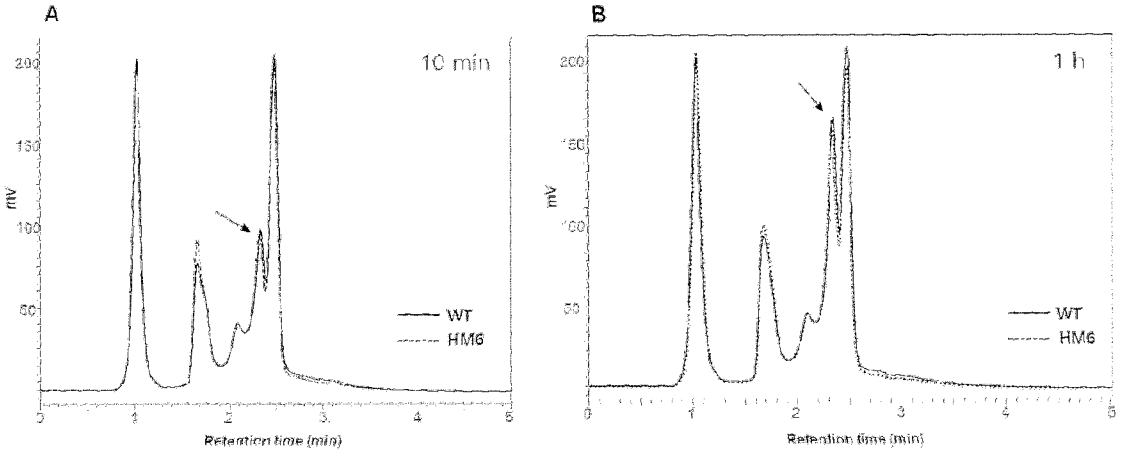
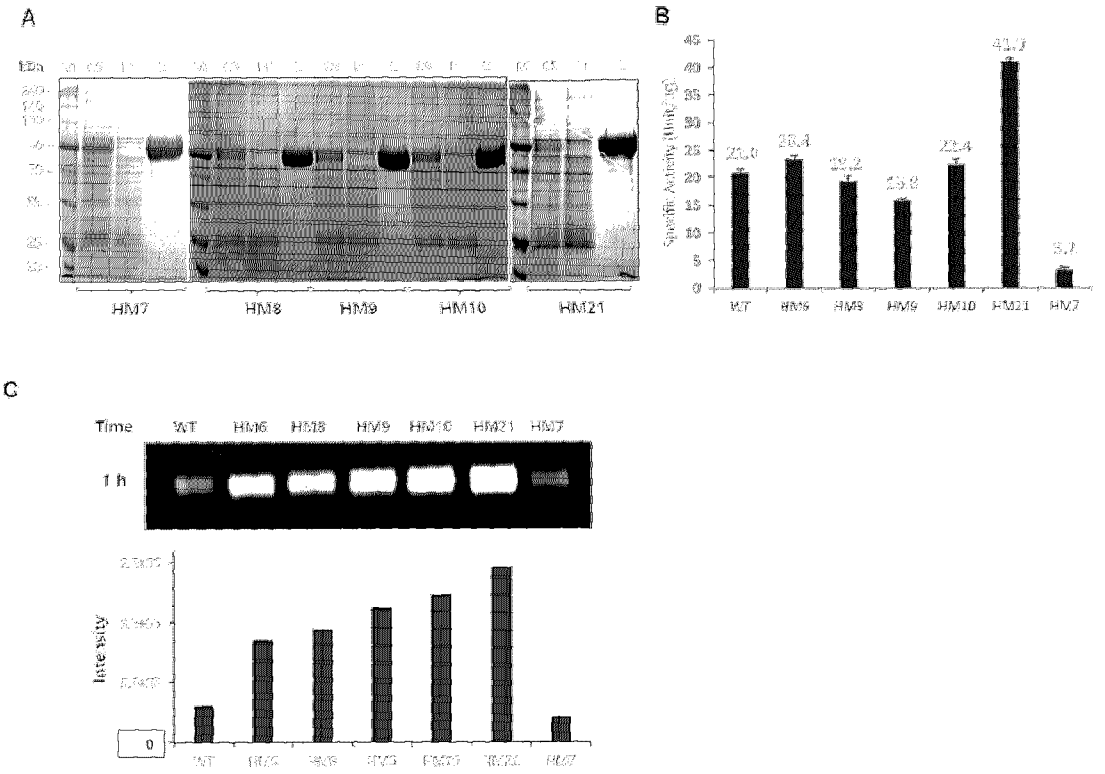
【Fig. 4】



【Fig. 5】



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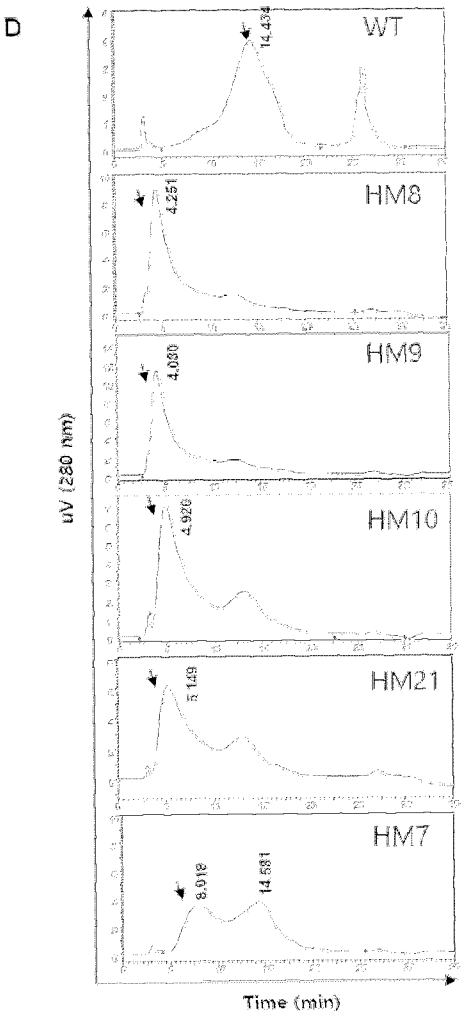

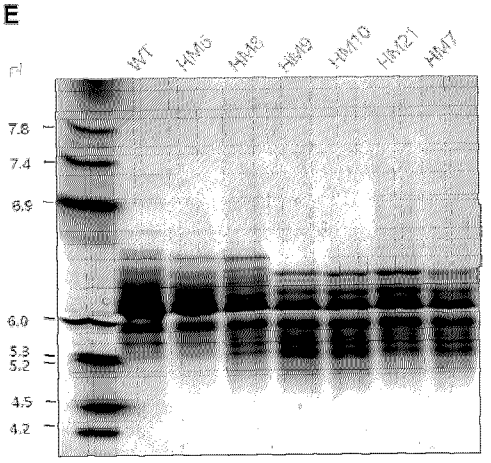

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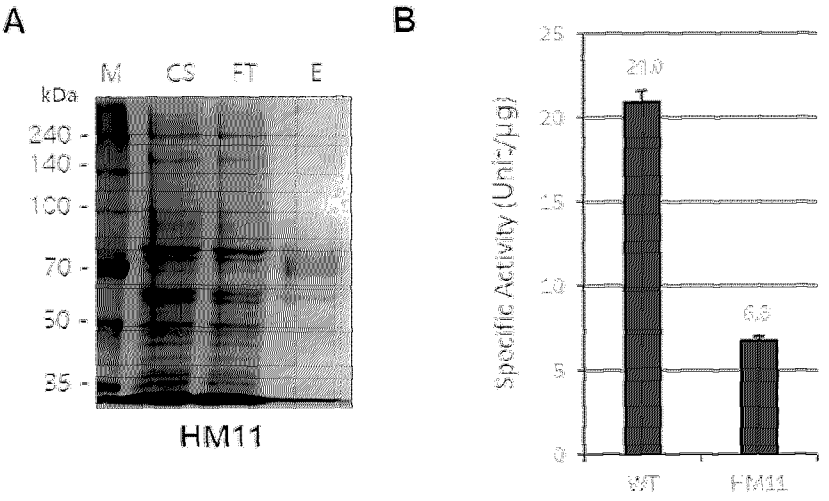
【Fig. 6】



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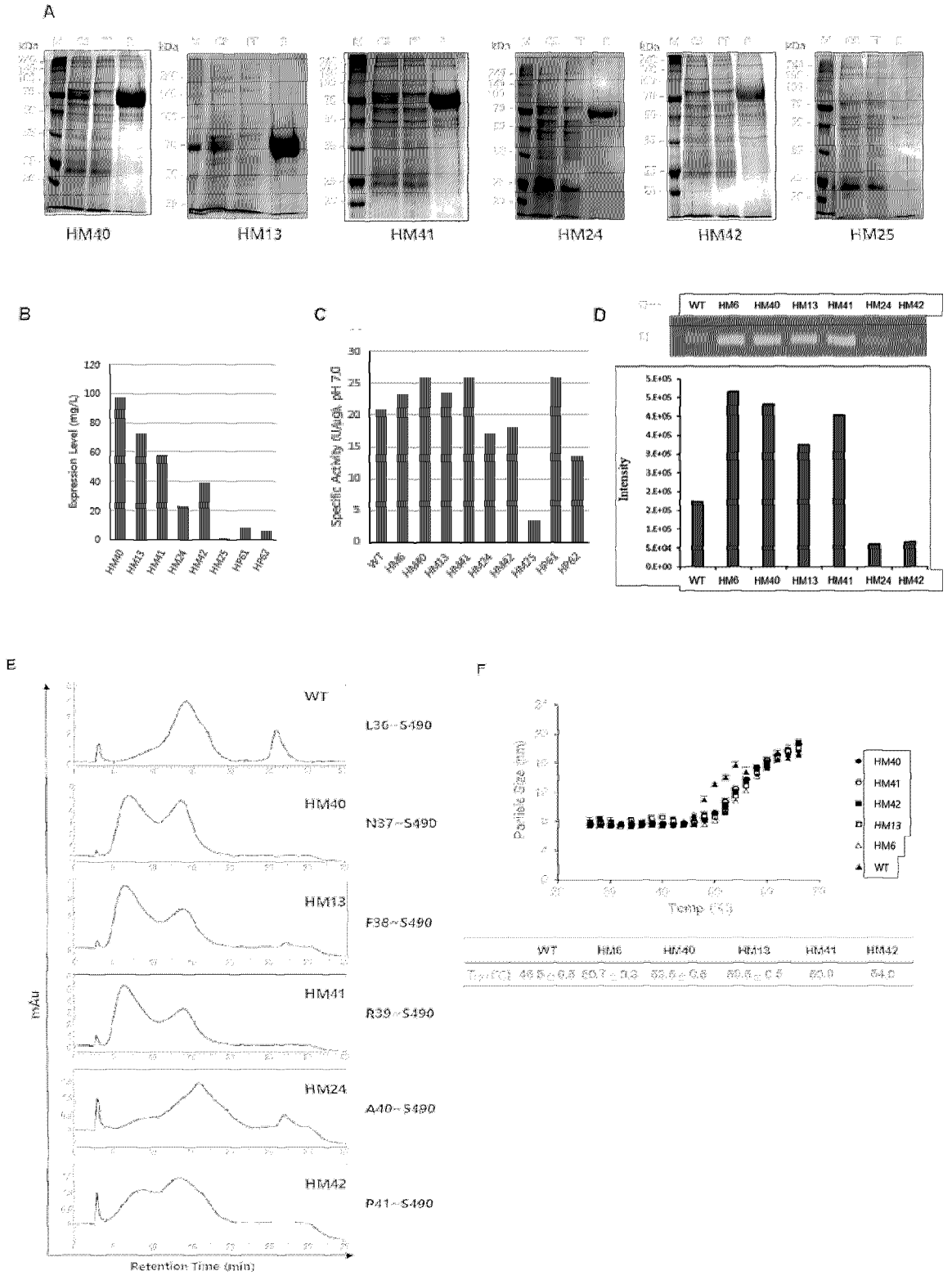
【Fig. 7】



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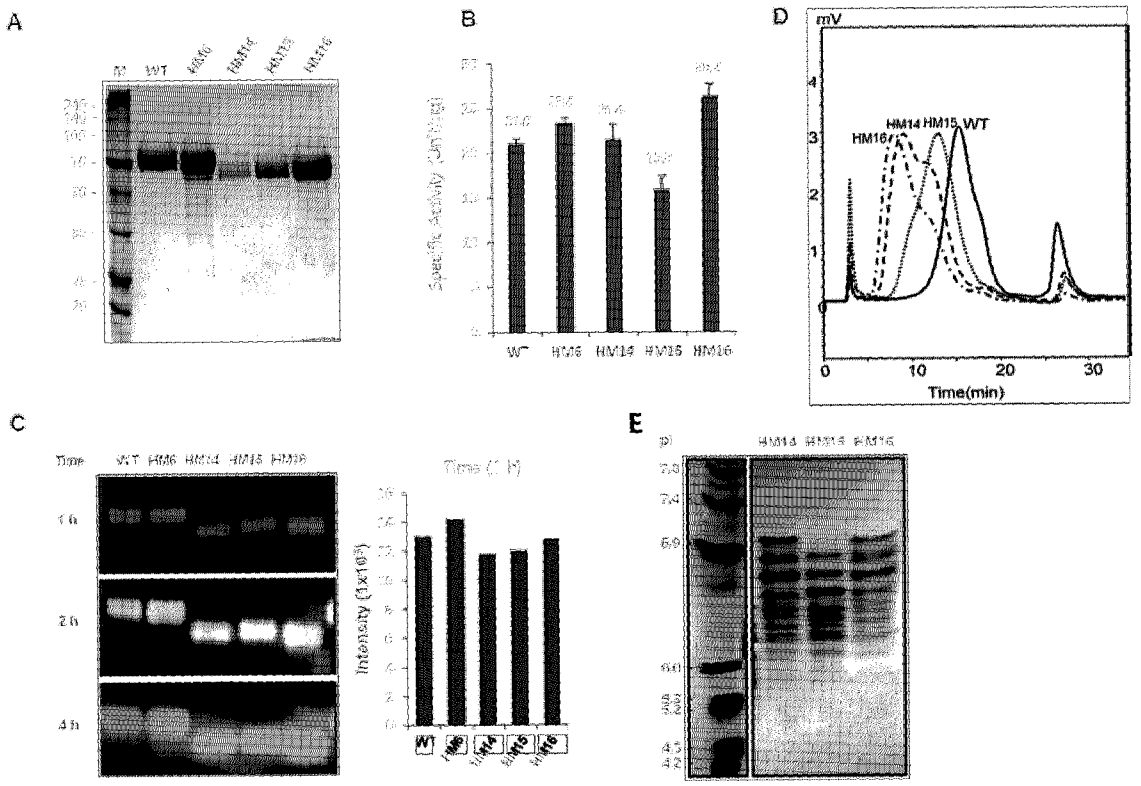
【Fig. 8】



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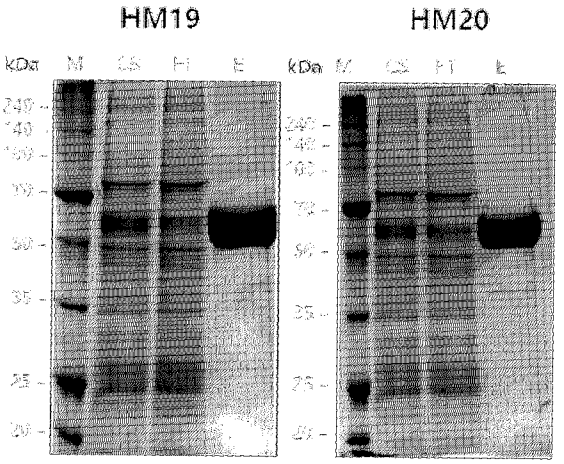
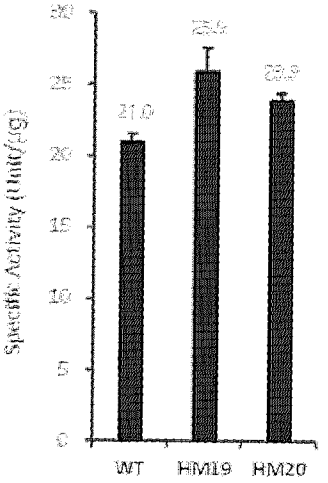
【Fig. 9】

A



B



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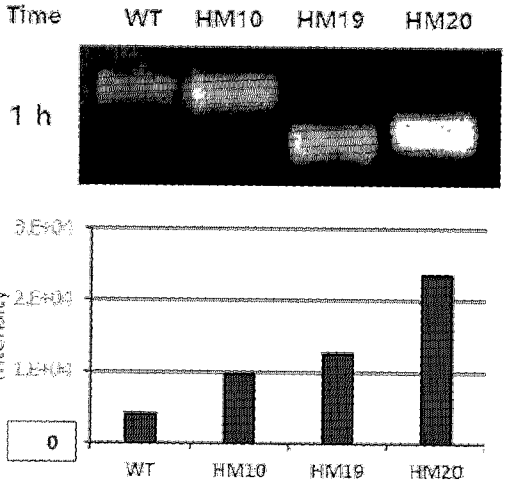

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【Fig. 10】

	$T_{agg.}$ (°C)
WT	46.5 ± 0.5
HM1	53.0 ± 1.2
HM4	56.5 ± 1.5
HM6	50.5 ± 0.5
HM7	58.0 ± 1.0
HM8	52.5 ± 0.5
HM9	53.0 ± 1.0
HM10	55.5 ± 0.5
HM13	50.5 ± 0.5
HM14	51.0 ± 1.0
HM15	49.0 ± 1.0
HM16	51.0 ± 1.0
HM19	53.0 ± 1.0
HM20	51.0 ± 1.0
HM21	51.7 ± 0.7

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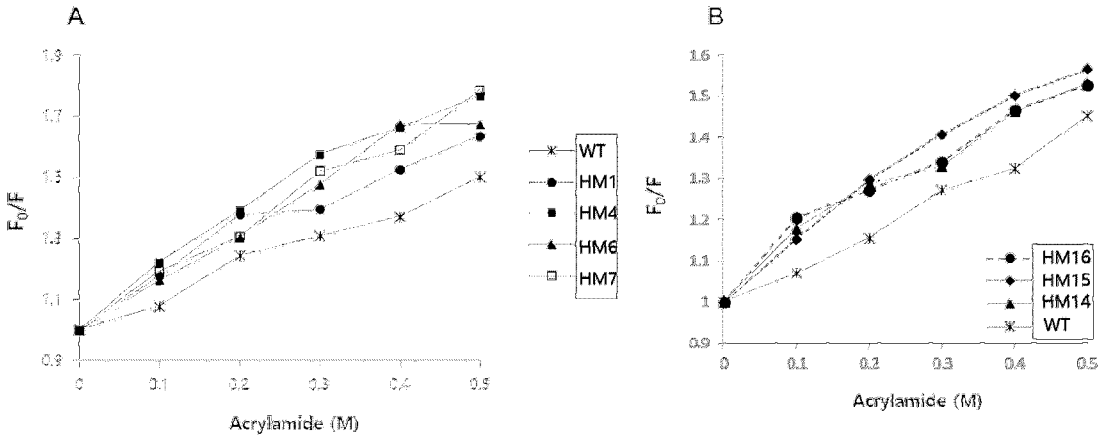
【Fig. 11】



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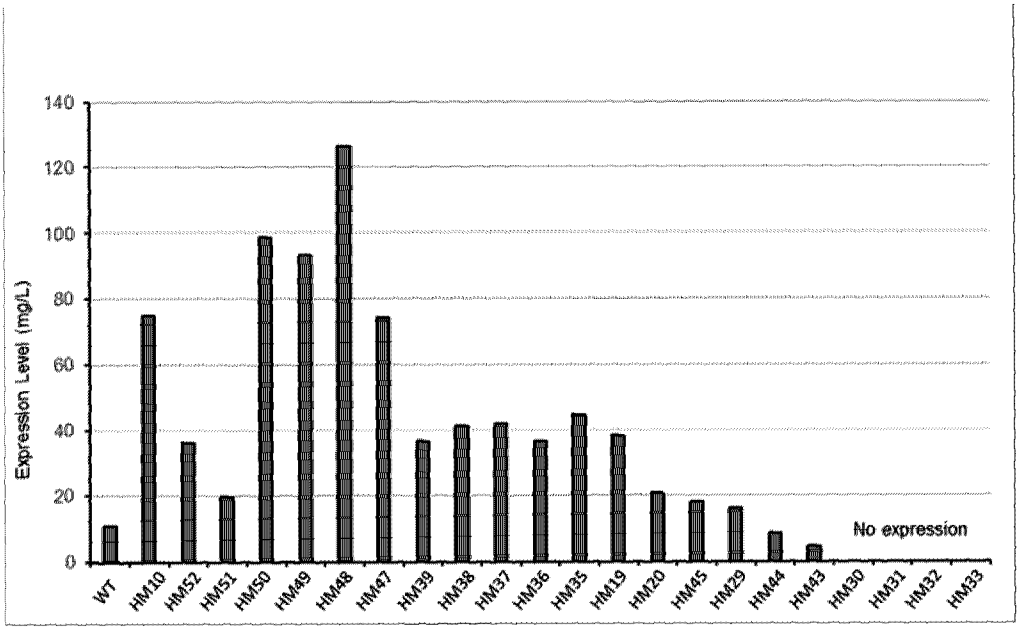
【Fig. 12】

A



B

	Total length	Expression level (mg/L)		Total length	Expression level (mg/L)		Total length	Expression level (mg/L)
WT	L36-S490	16.1	HM39	F38-E476	36.7	HM29	L36-A467	16.3
HM10	L36-S490	75.0	HM38	F38-T475	41.4	HM44	F38-D466	8.5
HM52	F38-T488	36.2	HM37	F38-E474	41.9	HM43	F38-H465	4.7
HM51	F38-P486	19.7	HM36	F38-M473	36.6	HM30	L36-C464	No
HM50	F38-A484	98.9	HM35	F38-P472	44.8	HM31	L36-D461	No
HM49	F38-Y482	93.4	HM19	F38-K470	38.3	HM32	L36-C458	No
HM48	F38-I480	126.8	HM20	F38-F468	20.8	HM33	L36-V455	No
HM47	F38-P478	74.4	HM45	F38-A467	17.9			

5

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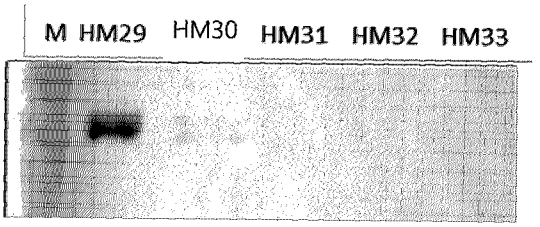
【Fig. 13】

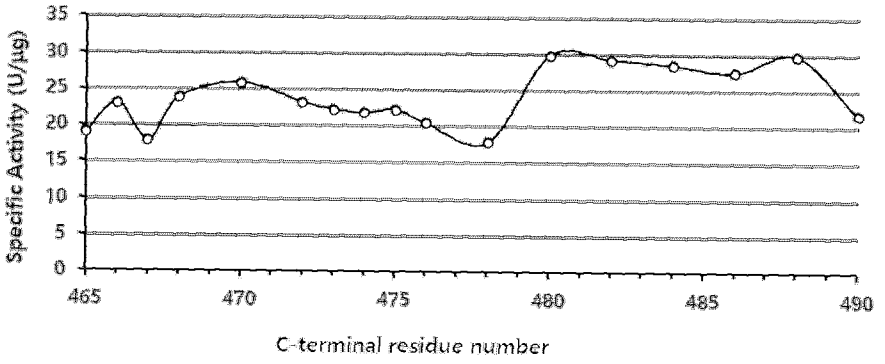


【Fig. 14】

A

	C-term residue number	Specific activity (U/μg), pH 7.0		C-term residue number	Specific activity (U/μg), pH 7.0
WT	490	22.3 ± 0.4	HM36	473	22.0 ± 0.3
HM10	490	21.7 ± 0.6	HM35	472	23.3 ± 0.2
HM52	488	29.5 ± 1.9	HM19	470	25.9 ± 1.6
HM51	486	27.5 ± 1.8	HP19	470	19.9 ± 0.6
HM50	484	28.5 ± 2.4	HM20	468	23.9 ± 0.5
HM49	482	29.3 ± 1.7	HP20	468	21.6 ± 0.6
HM48	480	29.9 ± 0.7	HM45	467	18.0 ± 2.5
HM47	478	17.8 ± 0.3	HM39	467	18.2 ± 0.2
HM39	476	20.4 ± 0.2	HM44	466	23.2 ± 0.2
HM38	475	22.3 ± 0.4	HM43	465	19.1
HM37	474	21.8 ± 0.5			

B



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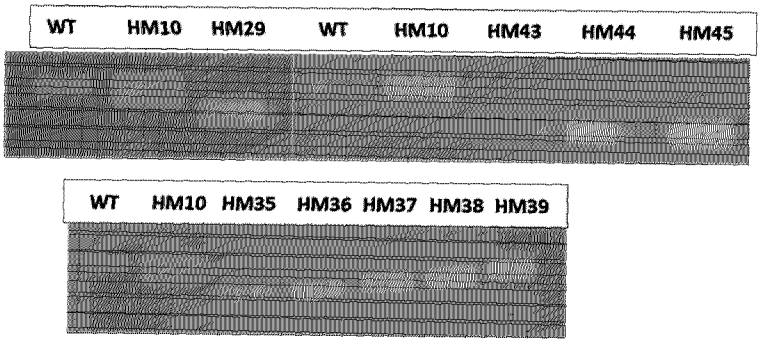

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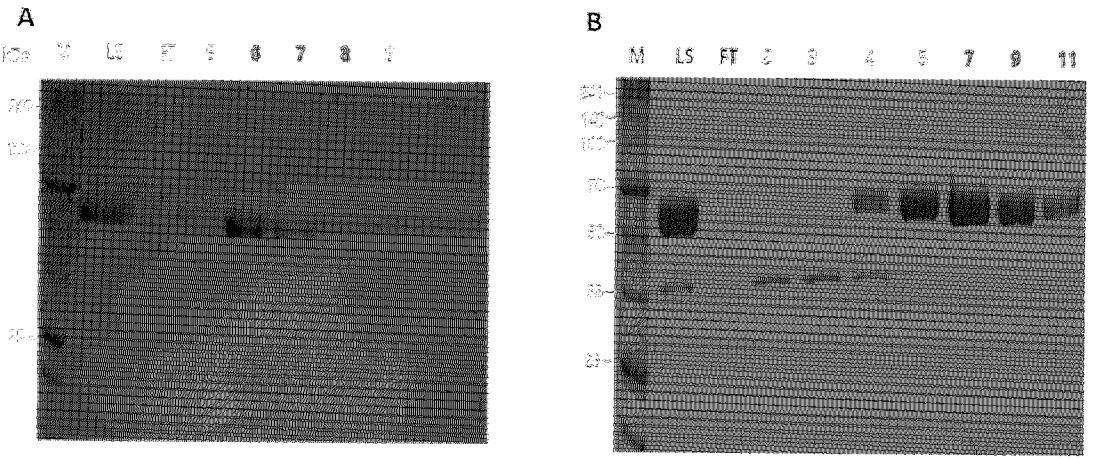
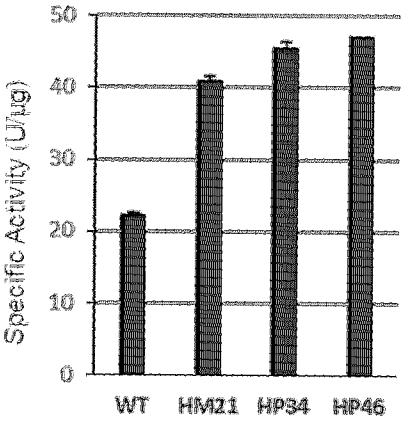
【Fig. 15】



【Fig. 16】

A

	Specific activity (U/μg), pH 7.0
WT	22.3 ± 0.4
HM21	41.0 ± 0.6
HP34	45.6 ± 0.9
HP46	47.2



B

	K_m (mg/mL)	k_{cat} (1/min)	k_{cat}/K_m (1/min/mg/mL)
HW2	2.29±0.20	0.72±0.01	0.32±0.03
HM21	1.65±0.10	0.88±0.12	0.53±0.06
HP34	1.94±0.18	1.03±0.13	0.54±0.08
HP46	1.78±0.04	1.31±0.10	0.73±0.04

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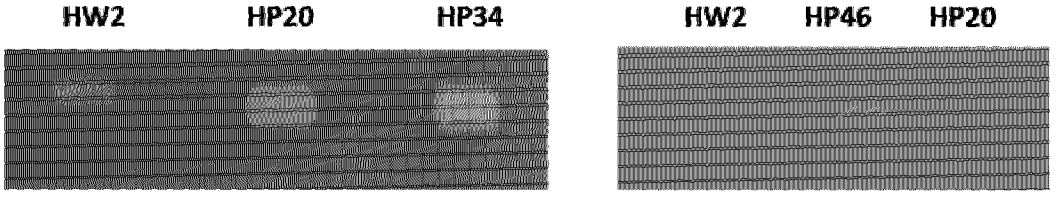
【Fig. 17】

A

	T_{agg} (℃)
HW2	46.5
HM21	51.7
HP34	51.5
HP46	51.0

B



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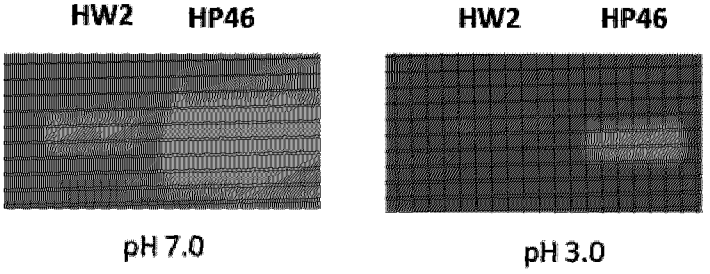

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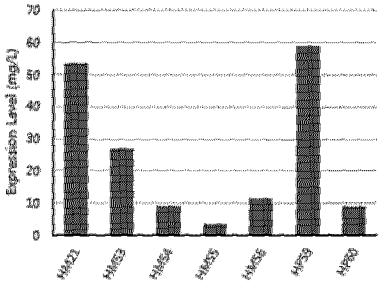

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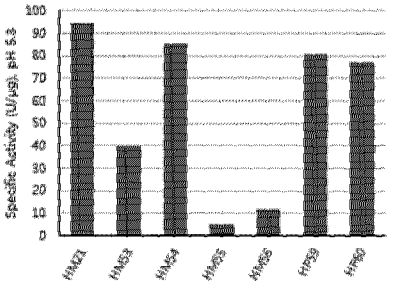

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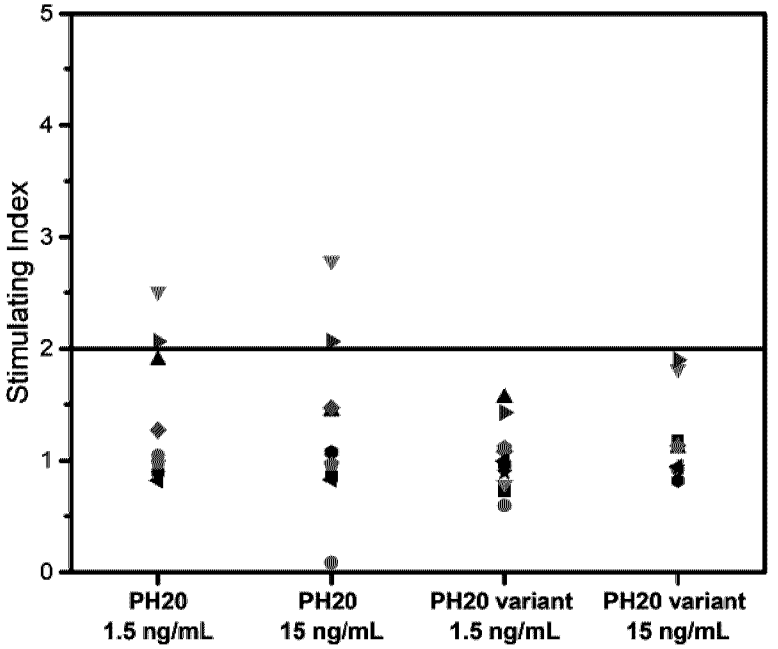
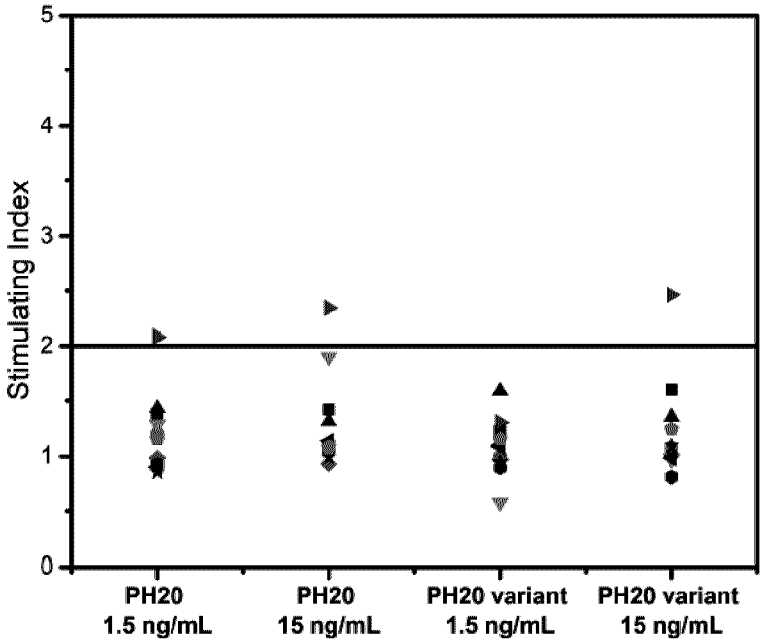
【Fig. 18】



【Fig. 19】



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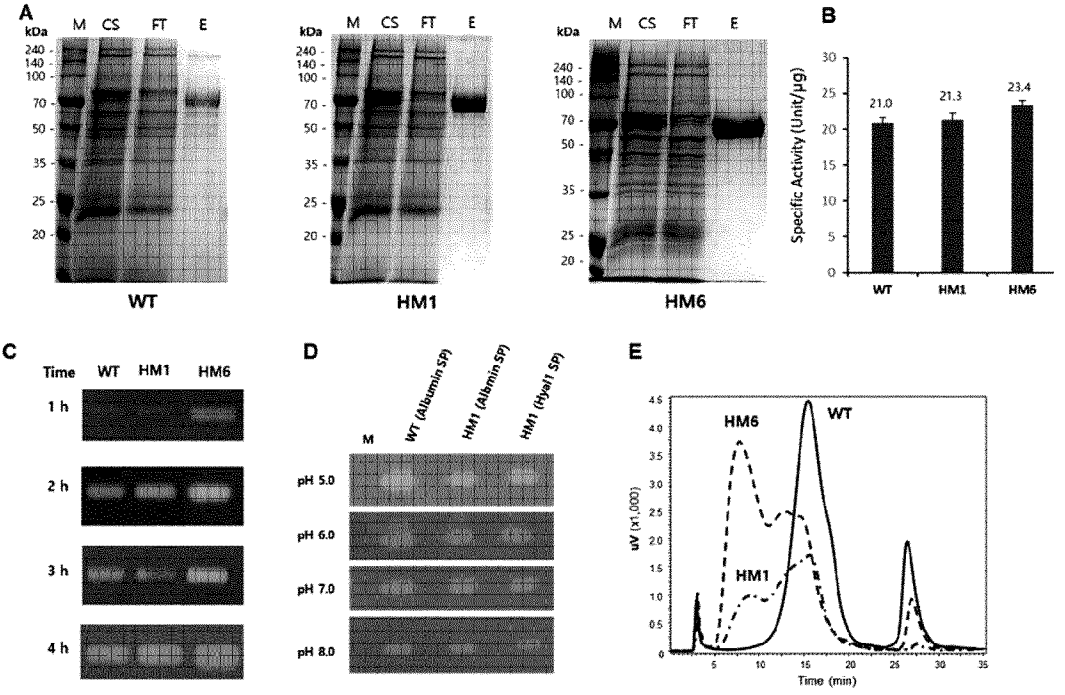
A
kDa
M CS FT E
240
140
100
70
50
35
25
20
WT
HM1
HM6
B
Specific Activity (Unit/μg)
21.0
21.3
23.4
WT
HM1
HM6
C
Time
WT HM1 HM6
1 h
2 h
3 h
4 h
D
M
WT (Albumin SP)
HM1 (Albumin SP)
HM1 (Hyal1 SP)
pH 5.0
pH 6.0
pH 7.0
pH 8.0
E
HM6
WT
HM1
uV (x1,000)
Time (min)