PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/48, 15/57, 15/18 | A1

(11) International Publication Number: **WO 98/38290**

(43) International Publication Date: 3 September 1998 (03.09.98)

(21) International Application Number: PCT/KR98/00032

(22) International Filing Date: 16 February 1998 (16.02.98)

(30) Priority Data:
1997/6756 28 February 1997 (28.02.97) KR

(71) Applicant *(for all designated States except US)*: LG CHEMICAL LTD. [KR/KR]; 20, Yoido-dong, Youngdeungpo-ku, Seoul 150-010 (KR).

(72) Inventors; and

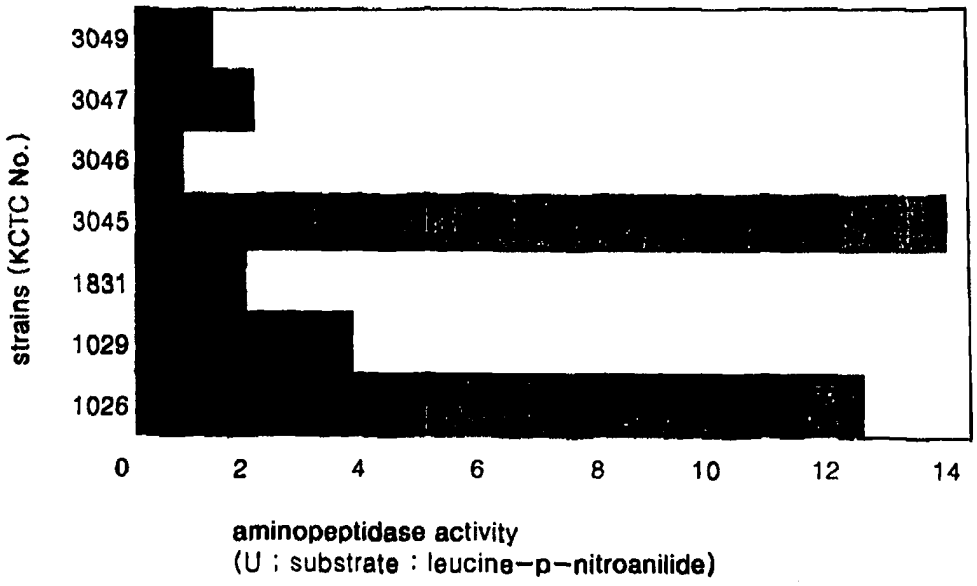
(75) Inventors/Applicants *(for US only)*: LEE, Young-Phil [KR/KR]; #6-401 LG Chemical Apt., 381-42, Doryong-dong, Yusong-ku, Taejon-si 305-340 (KR). HAN, Kyuboem [KR/KR]; #102-1002 Lucky Hana Apt., Shinsung-dong, Yusong-ku, Taejon-si 305-345 (KR). KIM, Se-Hoon [KR/KR]; #7-303 LG Apt., 381-42, Doryong-dong, Yusong-ku, Taejon-si 305-340 (KR). PARK, Soon-Jae [KR/KR]; #101-1503 Luckyhana Apt., Shinsung-dong, Yusong-ku, Taejon-si 305-345 (KR). LEE, Seung-Joo [KR/KR]; #126-1201 Kyoungsung Keunmareul Apt., 1418, Kalma-dong, Suh-ku, Taejon-si 302-171 (KR).

(74) Agent: LEE, Won-Hee; Sung-Ji Heights II, Suite 805, 642-16, Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: AMINOPEPTIDASE DERIVED FROM *BACILLUS LICHENIFORMIS* AND PROCESS FOR PREPARATION OF NATURAL TYPE PROTEINS



(57) Abstract

The present invention relates to a process for removing methionine (Met) residue at N-terminus of proteins specifically. Particularly, the present invention relates to an aminopeptidase which is purified from *Bacillus licheniformis* removes a methionine residue from N-terminus of peptide and proteins. And the present invention relates to a process for preparing a natural type protein from proteins produced in microorganisms by recombinant DNA technology. Various kinds of natural type proteins such as human growth hormone (HGH) can be prepared massively and easily by the process of the present invention.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Aminopeptidase derived from *Bacillus licheniformis* and process for preparation of natural type proteins

Field of the Invention

The present invention relates to a process for removing a methionine (Met) residue at the N-terminus of peptides and proteins specifically.

Particularly the present invention relates to a aminopeptidase which are derived from *Bacillus licheniformis* removes a methionine residue from the N-terminus of peptides and proteins. And the present invention relates to a process for preparing a natural type protein from recombinant proteins produced in microorganism. Various kinds of natural type proteins such as human growth hormone (HGH) can be prepared massively and easily by the process of the present invention.

Background of Invention

Recombinant DNA technology permits large scale production of eukaryotic proteins in bacteria. However the proteins so produced are frequently characterized by the addition of an extra methionine residue at their N-terminus. The proteins containing the N-terminal

methionine may induce immunogenic reaction in human and animal bodies and may not express effectively their original function. Therefore the process for preparing natural type proteins should be developed (Nature, 326, 315, 1987; *J. Bacteriol.*, 169, 751-757, 1987; *Bio/Technology*, 8, 1036-1040, 1990; *Appl. Microbiol. Biotechnol.*, 36, 211-215, 1991) to overcome the problems.

Especially human growth hormone (HGH) is a polypeptide composed of 191 amino acids, having 22,125 kDa of molecular weight and containing Phe-Pro-Thr sequence at the N-terminus. And HGH is produced in human pituitary gland and used mostly to improve dwarfism (Raben, M. S., *J. Clin. Endocr.*, 18, 901, 1958). Initially HGH has been extracted and purified from human pituitary gland to be utilized for medicinal treatment. However HGH has not been provided sufficiently to satisfy all the patients since the above process limits the HGH amount produced. And it is also reported that 4 children inoculated with HGH purified from hypothalamus have been infected with virus and died. Thus FDA has prohibited administration of HGH produced by the process for the treatment of patients (*Science*, 234, 22, 1986). Recently many researchers have attempted to produce HGH in *Escherichia coli* or yeast by using recombinant DNA

technology and to utilize this HGH clinically (in *E. coli*, Korean Patent Publication No. 89-1244 and No. 87-701, Patent Laid-open No. 87-2258 and No. 84-8695; in yeast, Patent Publication No. 92-99 and Patent Laid-open No. 90-9973 and No. 90-9976).

Generally recombinant HGH has an extra methionine residue which is added at the N-terminus during protein synthesis by initiation codon although natural type HGH is composed of 191 amino acids without the methionine. Thus the recombinant HGH is produced as a form of methionyl HGH which is composed of 192 amino acids and contains Met-Phe-Pro-Thr sequence at the N-terminus. In respect of biological activity, methionyl HGH exhibits the same activity with that of natural type HGH (Moore, J. A., *Endocrinology*, 122, 2920-2926, 1988). Any other side-effect which an extra methionine induces has not been reported. But it is reported that antibodies against an extra methionine may be generated, of which the rate is higher than that of natural type proteins (Lancet, March 29, 697, 1986).

Therefore, many researchers have attempted to produce natural type HGH without the N-terminal methionine. In particular a method has been developed to obtain natural type HGH in culture broth, which comprises fusing the N-terminus of HGH to a C-terminus

of other proteins and cutting the fusion protein with specific protease (PCT WO 89/12678; EP 20209; EP 321940). And a method also has been attempted, which comprises expressing HGH in cell cytoplasm, secreting it out of cell and removing a methionine residue during secretion (EP 008832; US 4755465; JP 01273591; EP 306673; Korean Patent Appl. No. 92-10932). Unfortunately these methods have some difficulties to perform complicated manipulation such as preparing expression vectors and transforming host cells. In addition fermentation condition should be optimized in each case.

And a method has been exploited in case of recombinant proteins containing an extra amino acid at the N-terminus, which uses aminopeptidases to remove the N-terminal amino acid and prepares natural type proteins by simple process. For example natural type HGH can be prepared from methionyl HGH when specific aminopeptidase removing the N-terminal methionine selectively is used. At that time methionyl HGH can be prepared in mass by the process already established (PCT WO 86/04609; WO 86/204527 A1).

Hitherto, several aminopeptidases which can be used to prepare natural type HGH have been reported, such as aminopeptidase purified from *Aeromonas proteolytica* (PCT WO 86/01229; EP 0489711 A3, B.T.G.

Co.; Prescott and Wikes, *Method in Enzymology*, 44, 530-543, 1976), aminopeptidase from pig kidney (PCT 86.204527 A1; *Bio/Technology*, 5, 824-827, 1987, Takeda Co.), dipeptidyl aminopeptidase from *Dictyostelium discoidem* (EP 557076 A1; US 5126249, Eli LiIly Co.) and aminopeptidase from *Streptomyces thermonitrificans* (EP 6296695; US 5569598; Lucky Co.).

However the aminopeptidase should react with only extra amino acid residues of proteins at the N-terminus, not with naturally occurring sequences of amino acids in order to prepare natural type proteins. That is, when N-terminal amino acid sequences are X-Y-Z in natural type protein and Met-X-Y-Z in recombinant protein respectively, aminopeptidase which removes the methionine residue without reacting other amino acids, such as X-Y-Z-, is preferred to prepare the same protein with natural type proteins. Thus substrate specificity of aminopeptidase is important to satisfy the above conditions. Since methionyl HGH has Met-Phe-Pro-Thr-Ile amino acid sequence at the N-terminus, aminopeptidase for the use should recognize X-Pro sequence and stop cutting reaction in front of X amino acid to remove the N-terminal methionine selectively. For industrial use the enzyme also exhibits higher specific activity outstandingly.

Various kinds of aminopeptidase have been purified from microorganisms. Most of aminopeptidases require Ca^{2+}, Zn^{2+} or other metal ions for their activities and remove amino acid residues at the N-terminus commonly. But aminopeptidases purified from different microorganisms have different enzymatic properties in respect of molecular weight, requirements of metal ions, optimal condition of reaction and substrate specificity (*FEMS Microbiol. Rev.*, 18, 319-344, 1996). These aminopeptidases are classified into exo-peptidase removing amino acid from the N-terminus of substrate proteins gradually.

Presently aminopeptidases which have been purified from *Bacillus* genus are from *Bacillus subtilis* (*Arch. Biochem. Biophys.*, 197, 63-77, 1979; *Arch. Biochem. Biophys.*, 202, 540-545, 1980; *J. Biochem.*, 107, 603-607, 1994; JP 03285684, Diacel-Chem Co.), *Bacillus stearothermophilus* (*Meth. Enzymol.*, 19, 544-552, 1970; *Biochem. Biophys. Acta*, 438, 212-220, 1976; EP 101653, Unitika), *Bacillus thuringensis* (Biokhimiya, 49, 1899-1907, 1984), *Bacillus licheniformis* (*Arch. Biochem. Biophys.*, 186, 383-391, 1978; *Microbiol.* Zh., 51, 49-52, 1989) and so forth.

In references cited above, aminopeptidases were analyzed to examine their enzymatic properties by using several substrates such as leucine-p-nitroanilide,

dipeptide and the like. But oligopeptide and protein containing Met-X-Pro sequence at the N-terminus have not been used to examine aminopeptidase activity. Thus it has not been expected that aminopeptidase can be used for removal of only an extra methionine from recombinant proteins containing Met-X-Pro sequence at the N-terminus.

In order to prepare natural type proteins by using recombinant DNA technology, we have investigated and purified aminopeptidases of *Bacillus licheniformis* strains which remove only a methionine residue at the N-terminus of proteins. Aminopeptidases derived from *Bacillus licheniformis* can remove a methionine residue from synthetic substrate, oligopeptide, protein and so on properly and recognize Met-X-Pro sequence (X indicates any amino acid available) specifically. Thus we have demonstrated that aminopeptidases of the present invention can be used to prepare natural type HGH efficiently.

Summary of Invention

The object of the present invention is to provide aminopeptidases derived from *Bacillus licheniformis* which removes a methionine residue at the N-terminus of

peptide and protein.

The aminopeptidases of the present invention recognize Met-X-Pro sequence at the N-terminus of peptide and protein to remove the methionine residue. Preferably X is phenylalanine.

The aminopeptidases contain amino acid sequences of SEQ ID. NO: 3 (Lys-Phe-Ser-Lys-Lys-Phe-Asn-Glu-Asn-Arg sequence) and SEQ ID. NO: 4 (Lys-Phe-Ser-Lys-Phe-Asn-Glu-Asn-Arg-Ala-Tyr-Gln-Tyr-Ile-Tyr-His-Leu sequence), at the N-terminus or sequence which are deleted and substituted in amino acids from the N-terminus of the above sequences.

The aminopeptidases have a molecular weight at the range of 43-47 kDa on SDS-PAGE.

The aminopeptidases are active at the range of pH 7.5-10.5 preferably and more preferably at the range of pH 8.5-9.5.

The aminopeptidases are active at the range of 40-70°C preferably and more preferably at the range of 50-60°C.

The aminopeptidases are thermostable and maintains 50% activity even though it is incubated at 60°C for 4 hours.

The aminopeptidase activity is inhibited by dithiothreitol, iodoacetic acid and ortho-penanthroline and inhibited partially by ethylenediaminetetraacetic

acid (EDTA).

Another object of the present invention is to provide processes for preparation of the aminopeptidases, which comprise culturing *Bacillus licheniformis*, centrifuging the culture broth to obtain supernatant and performing gel filtration chromatography with the concentrated supernatant. Preferably *Bacillus licheniformis* strain is KCTC 3058, KCTC 12759, KCTC 3045 and KCTC 1030 in the above description.

The present invention provides a process for preparation of the aminopeptidases, which comprises preparing aminopeptidase-producing cells by using recombinant DNA technology, culturing the recombinant cells and purifying the enzyme.

Another object of the present invention is to provide a process for preparing natural type proteins, which uses recombinant proteins produced by using recombinant DNA technology with aminopeptidase and purifying proteins.

Precisely the present invention provides a process for preparing natural type HGH from methionyl HGH containing Met-X-Pro sequence at the N-terminus.

The process for preparation comprises using aminopeptidase at the range of pH 7.0-9.5 and at the range of 20-60°C and purifying natural type HGH through

ion exchange resin chromatography and so forth. At that time NaCl is added to 30-230mM into reaction mixture and enzymes is added at the range of 0.2-20U per 1mg of methionyl HGH preferably.

Brief Description of the Drawings

Fig. 1 shows the quantity of aminopeptidase which is produced from culture broth of alkaline protease-producing *Bacillus licheniformis* strains such as KCTC 1026, KCTC 1029, KCTC 1831, KCTC 3045, KCTC 3046, KCTC 3047 and KCTC 3049. As substrate, leucine-p-nitroanilide is used to measure the enzyme activity.

Fig. 2 shows the quantity of aminopeptidase which is produced from culture broth of α-amylase-producing *Bacillus licheniformis* strains such as KCTC 1030, KCTC 2215 and KCTC 3006. As substrate, leucine-p-nitroanilide is used to measure the enzyme activity.

Fig. 3 shows the quantity of aminopeptidase which is produced from culture broth of bacitracin-producing *Bacillus licheniformis* strains such as KCTC 3056, KCTC 3057 and KCTC 3058. As substrate, leucine-p-nitroanilide is used to measure the enzyme activity.

Fig. 4 shows the aminopeptidase activity of *Bacillus licheniformis* ATCC 12759 to compare with that of *Bacillus licheniformis* KCTC 3045, KCTC 1030 and KCTC

3058 having higher aminopeptidase activity. As substrate, leucine-p-nitroanilide is used to measure the enzyme activity.

Fig. 5 shows aminopeptidase activity of *Bacillus licheniformis* ATCC 12759 to compare with that of *Bacillus licheniformis* KCTC 3045, KCTC 1030 and KCTC 3058 having higher aminopeptidase activity. As substrate, oligopeptide containing Met-Phe-Pro-X sequence, the N-terminal sequence of HGH, is used to measure the enzyme activity.

Fig. 6 shows purity and molecular weight of aminopeptidase purified from *Bacillus licheniformis* ATCC 12759 strain, which is depicted by SDS-PAGE.

Fig. 7 shows aminopeptidase activities according to pH variation, which are measured by using leucine-p-nitroanilide as substrate.

Fig. 8 shows aminopeptidase activities according to temperature variation, which are measured by using leucine-p-nitroanilide as substrate.

Fig. 9 shows thermostability of aminopeptidase, which is measured by using leucine-p-nitroanilide as substrate.

Fig. 10 shows patterns removing methionine according to pH variation for preparing natural type HGH from methionyl HGH.

Fig. 11 shows patterns removing methionine

according to temperature variation for preparing natural type HGH from methionyl HGH.

Fig. 12 shows patterns removing methionine according to NaCl concentration variation for preparing natural type HGH from methionyl HGH.

Fig. 13 shows natural type HGH which is prepared from methionyl HGH by the process performing Mono-Q chromatography and SDS-PAGE.

Fig. 14 shows results analyzing the N-terminal amino acid of natural type HGH prepared by the above process.

Fig. 15 shows results analyzing amino acid sequence of natural type HGH prepared by the above process.

Fig. 16a shows the chromatogram of aminopeptidase which is purified from *Bacillus licheniformis* by the process performing Mono-S chromatography.

Fig. 16b shows aminopeptidase which is purified from *Bacillus licheniformis* by the process performing Mono-S chromatography and SDS-PAGE.

Practical and presently preferred embodiments of the invention are illustrative as shown in the following examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may

make modifications and improvements within the spirit and scope of the present invention.

Detailed Description of the Invention

Aminopeptidases of this invention, which removes a methionine residue at the N-terminus of peptides and proteins, is derived from *Bacillus licheniformis*. Approximately 20 strains of *Bacillus licheniformis* deposited in Korean Collection for Type Culture (KCTC) are examined and their aminopeptidase activities existing in culture broth are compared respectively.

Considering references reported previously, *Bacillus licheniformis* strains are divided as alkaline protease (containing detergent-resistant alkaline protease)-producing strain, α-amylase-producing strain and bacitracin-producing strain.

In detail 7 alkaline protease-producing strains of *Bacillus licheniformis* such as KCTC 1026 (ATCC 21415), KCTC 1029 (ATCC 21418), KCTC 1831 (ATCC 21424), KCTC 3045 (ATCC 21415), KCTC 3046 (ATCC 21416), KCTC 3047 (ATCC 21417), KCTC 3049 (ATCC 21424) (see Fig. 1), 3 α-amylase-producing strains KCTC 1030 (ATCC 27811), KCTC 2215 (ATCC 27811), KCTC 3006 (ATCC 39326) (see Fig. 2), and 3 bacitracin-producing strains such as KCTC 3056 (ATCC 10716, ATCC 11944), KCTC 3057 (ATCC 11945), KCTC

3058 (ATCC 11946) (see Fig. 3) are cultured. And in the supernatant of culture broth aminopeptidase activities are measured by the process described in Reference Example 1.

As a result, 6-25U range of aminopeptidase activities is observed in *Bacillus licheniformis* KCTC 3045, KCTC 1030, KCTC 3058 and ATCC 12759 strains by using leucine-p-nitroanilide as substrate (see Fig. 4). Among these *Bacillus licheniformis* strains ATCC 12759 strain, has been already reported by Rodriquez et. al., to produce an aminopeptidase (Rodriguez-Absi, J. and Prescott, J. M., *Arch. Biochem. Biophys.*, 186(2), 383-391, 1978).

<Reference Example 1> Measurement of aminopeptidase activity by using leucine-p-nitroanilide as substrate.

Aminopeptidase activities of this invention were measured by the method of Pfleiderer (Pfleiderer, *Meth. Enzymol.*, 19, 514-521, 1970). Precisely, 1U of enzyme activity defines the enzyme quantity changing 1 absorbance at 405nm which is measured by the process adding aminopeptidase in 1ml solution of 100mM Tris-Cl (pH 8.0) and 2mM leucine-p-nitroanilide (Sigma, USA), incubating at 37°C for 1min, then adding 100μl of 70% acetic acid to stop the reaction. At that time

leucine-p-nitroanilide was dissolved in dimethylsulfoxide (DMSO) to make 100mM solution.

And the aminopeptidase activities of *Bacillus licheniformis* strains described above are also measured by using methionyl oligopeptide as substrate in order to detect exo-peptidase activity removing methionine residues of peptides and proteins containing N-terminal methionine (see Fig. 5). Precisely oligopeptide containing amino acid sequence of SEQ ID.NO: 1 (Met-Phe-Pro-Thr-Glu-Pro-Ser) is used as substrate to measure aminopeptidase activity by the process described in Reference Example 2.

<Reference Example 2> Measurement of aminopeptidase activity by using oligopeptide as substrate

In order to measure aminopeptidase activities, reaction mixture containing substrate and aminopeptidase with known concentrations in buffer solution of 0.1M Tris.Cl, 0.1M NaCl was prepared. As substrate oligopeptide having amino acid sequence of SEQ ID.NO: 1 was used. The reaction proceeded at 37°C for predetermined time and equal volume of 70% acetic acid was added to stop it. Then high performance

liquid chromatography (HPLC) was performed to analyze the reaction product. Oligopeptide was dissolved in DMSO to make 30mM solution, diluted in 2.5mM by using DW and added into above reaction mixture with proper concentration.

Chromatography was performed to analyze the reaction product in the reaction condition as follows. Precisely YMC protein RP column (4.6 * 250mm, YMC Co.), solvent A containing 0.05% trifluoroacetic acid (TFA) and solvent B containing 0.05% trifluoroacetic acid and 80% acetonitrile were utilized for elution. The above mixture was injected into column and eluted with solvent B having linear concentration gradient of 10%-30%. Then area which is recording absorbance at 214nm was calculated. As a result substrate which was not reacted was eluted at about 9.5min and reaction product at about 7.0min in above reaction condition. Eluting substrate and reaction product peptide in such condition, area of each peak was proportionate to number of amino acids of peptide. Thus the proportion of area in substrate (6 peptide bonds) and product (5 peptide bonds) of the same concentration was 6:5 approximately. Therefore the concentration of product peptide produced in the above reaction was obtained by the formula as shown below.

$$[P] = (PA/5) / (SA/6 + PA/5) * [S]$$

PA : peak area of product peptide

SA : peak area of substrate peptide

[S] : initial concentration of substrate peptide

[P] : concentration of product peptide produced by the process

Since oligopeptide mentioned above contains a N-terminal methionine and adjacent X-Pro sequence, aminopeptidase which reacts only with the N-terminus, not with X-Pro sequence can be selected easily by using the oligopeptide. Precisely, *Bacillus licheniformis* strains such as KCTC 3045, KCTC 1030, KCTC 3058 and ATCC 12759 are assayed to produce aminopeptidase having properties mentioned above. Thus these aminopeptidase derived from these strains can be adopted usefully to remove the N-terminal methionine from peptides and proteins containing Met-X-Pro sequence at the N-terminus.

In addition, the aminopeptidase activity is also examined in order to identify the removal of N-terminal methionine from protein having higher molecular weight. At that time, aminopeptidase has been purified highly in order to diminish the additional reaction prompted

by other endoproteases.

Precisely aminopeptidases of *Bacillus licheniformis* ATCC 12759 and KCTC 3058 are purified and their enzymatic properties are examined. The aminopeptidase indicates the highest reactivity with oligopeptide referring to the reactivity with leucine-p-nitroanilide.

The aminopeptidases of the present invention is analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and have a molecular weight at the range of 43-47 kDa (see Fig. 6 and Fig. 16).

And it is identified that the aminopeptidase have the amino acid sequence of SEQ ID. NO: 3 (Lys-Phe-Ser-Lys-Lys-Phe-Asn-Glu-Asn-Arg) and SEQ ID. NO: 4 (Lys-Phe-Ser-Lys-Lys-Phe-Asn-Glu-Asn-Arg-Ala-Tyr-Gln-Thr-Ile-Tyr-His-Leu sequence) at the N-termini.

The aminopeptidase purified from *Bacillus licheniformis* ATCC 12759 coexists with deleted forms of enzyme without 1 or 2 amino acids at the N-terminus and the aminopeptidase purified from *Bacillus licheniformis* KCTC 3058 coexists with lysine-deleted form or with substituted form at the 9th amino acid (from asparagine to aspartic acid).

Generally, the aminopeptidases of the present invention can be classified into leucine aminopeptidase since it has specific and higher reactivity in leucine

than that in methionine.

Detaily the enzymatic properties of aminopeptidases are examined by using leucine-p-nitroanilide as synthetic substrate. The aminopeptidases of this invention are preferably active at the range of pH7.5-10.5 and more preferably at the range of pH8.5-9.5 (see Fig. 7).

And the aminopeptidases are preferably active at the range of 40-70°C and more preferably at the range of 50-60°C (see Fig. 8). And aminopeptidases are thermostable and maintain more than 50% activity even though it is incubated at 60°C for 4 hours (see Fig. 9). The aminopeptidase activities are inhibited by dithiotreitol, iodoacetic acid, ortho-penanthroline and inhibited partially by ethylene diamine tetraacetic acid(EDTA).

The present invention provides processes for preparing aminopeptidases derived from *Bacillus licheniformis*.

Precisely in order to prepare the aminopeptidase, culture broth of *Bacillus licheniformis* strain is obtained, centrifuged and then supernatant of the culture broth is concentrated to perform gel permeation chromatography and so forth. At that time, *Bacillus licheniformis* ATCC 12759, KCTC 3058, KCTC 3045 and KCTC

1030 strains can be used preferably.

In addition, aminopeptidase-producing cells is prepared by using recombinant DNA technology, cultured and then the aminopeptidase can be purified.

The present invention provides a process for preparing natural type proteins from recombinant proteins.

In addition, the process of the present invention for preparing natural type proteins comprises treating proteins produced by recombinant DNA technology with the aminopeptidase and purifying products which is exactly same with naturally occurring protein.

Precisely natural type HGH in which methionine is free at the N-terminus is prepared from methionyl HGH containing Met-X-Pro sequence and so forth by the process described above. At that time 0.2-20U of aminopeptidase per 1mg of methionyl HGH is treated, preferably at the range of pH 7.0-9.5 (see Fig. 10), at the range of 20-60°C, more preferably 37°C (see Fig. 11). And NaCl is added to 30-230mM preferably, 100mM more preferably (see Fig. 12). And ion exchange resin chromatography is performed after treating aminopeptidase to purify natural type HGH obtained by the process mentioned above (see Fig. 13).

Examples

<Example 1> Activity measurement of aminopeptidase from alkaline protease-producing *Bacillus licheniformis*

7 *Bacillus licheniformis* strains, KCTC 1026 (ATCC 21415), KCTC 1029 (ATCC 21418), KCTC 1831 (ATCC 21424), KCTC 3045 (ATCC 21415), KCTC 3046 (ATCC 21416), KCTC 3047 (ATCC 21417) and KCTC 3049 (ATCC 21424), which have been known to produce alkaline protease were inoculated into 100ml of broth containing ampicillin in autoclaved 500ml flask (AP-broth) and incubated at 30°C for more than 50 hours with shaker of 150rpm.

1ml of culture broth obtained above was centrifuged and in the supernatant aminopeptidase activity was measured by using leucine-p-nitroanilide as substrate. The result were shown in Fig. 1. In most strains of *Bacillus licheniformis* aminopeptidase activities were assayed and the exact values were 12.3U, 3.62U, 1.87U, 13.6U, 0.67U, 1.98U and 1.25U respectively.

<Example 2> Activity measurement of aminopeptidase from -amylase-producing *Bacillus licheniformis*

3 *Bacillus licheniformis* strains, KCTC 1030 (ATCC

27811), KCTC 2215 (ATCC 27811) and KCTC 3006 (ATCC 39326), which have been known to produce α-amylase, were incubated in flask (AP-broth) by the same method of Example 1. Then the supernatant of culture broth was obtained, in which aminopeptidase activity was measured by using leucine-p-nitroanilide as substrate. The result were shown in Fig. 2. As shown in Fig. 2 aminopeptidase activities were assayed and the exact values were 1.04U, 0.08U and 0.09U respectively. These enzymes activities measured by using leucine-p-nitroanilide were much lower than that of other *Bacillus licheniformis* strains.

<Example 3> Activity measurement of aminopeptidase from bacitracin-producing *Bacillus licheniformis*

3 *Bacillus licheniformis* strains, KCTC 3056 (ATCC 10716, ATCC 11944), KCTC 3057 (ATCC 11945) and KCTC 3058 (ATCC 11946), which have been known to produce bacitracin, were incubated in flask (AP-broth) by the same method of Example 1. Then the supernatant of culture broth was obtained, in which aminopeptidase activity was measured by using leucine-p-nitroanilide as substrate. The results were shown in Fig. 3. The exact values of aminopeptidase activities were calculated as 4.40U, 11.8U and 13.3U respectively.

<Example 4> Activity measurement of aminopeptidase from aminopeptidase-producing *Bacillus licheniformis* ATCC 12759

Bacillus licheniformis ATCC 12759 which has been known to produce aminopeptidase was incubated in flask by the same method of Example 1. Then the supernatant of culture broth was obtained, in which aminopeptidase activity was measured by using leucine-p-nitroanilide as substrate. The result were shown in Fig. 4. The exact values of aminopeptidase activities were 4.16U which was intermediate comparing with that of other *Bacillus licheniformis* strains.

<Example 5> Activity measurement of aminopeptidase in large-scale culture broth by using leucine-p-nitroanilide as substrate

With reference to the results of Example 1, 2 and 3, *Bacillus licheniformis* strains which have high aminopeptidase activities such as KCTC 3045 (ATCC 21415), KCTC 1030 (ATCC 27811) and KCTC 3058 (ATCC 11946) were selected. These 3 *Bacillus licheniformis* strains and *Bacillus licheniformis* ATCC 12759 were inoculated into 50ml of LB broth (yeast extract 5g/l, bacto-tryptone 10g/l, NaCl 5g/l) in autoclaved 50ml

flask and incubated at 30°C for 20 hours with shaker of 150rpm. Then the culture broth was inoculated into 5L of AP broth (glucose 10g/l, potassium phosphate mono-basic 10g/l, di-basic 10g/l, NaCl 5g/l, yeast extract 5g/l, bacto-peptone 10g/l, magnesium sulfate 0.01g/l, $FeSO_4.7H_2O$ 1mg/l, $ZnSO_4.7H_2O$ 1mg/l, $MnSO_4.H_2O$ 1mg/l) in autoclaved 5L fermented and incubated at 37.5°C for more than 50 hours with shaker of 300-400rpm. 1ml of culture broth were obtained and centrifuged.

Then in the supernatant aminopeptidase activities were measured by using leucine-p-nitroanilide as substrate. The results were shown in Fig. 4. In each *Bacillus licheniformis* strain aminopeptidase activities were assayed and the exact values were 14.6U, 7.86U, 24.4U and 6.10U respectively. Among these strains *Bacillus licheniformis* ATCC 12759 has lowest aminopeptidase activity and *Bacillus licheniformis* KCTC 3058 has 4 times higher activity than that of ATCC 12759 strain.

<Example 6> Activity measurement of aminopeptidase in large-scale culture broth by using oligopeptide as substrate

Aminopeptidase activity was measured by the process using culture broth obtained in Example 5 and

oligopeptide as described below. Into reaction buffer (0.1M Tris-Cl, pH8.0, 0.1M NaCl, 1.25mM methionyl oligopeptide of SEQ ID. NO: 1 (Met-Phe-Pro-Thr-Glu-Pro-Ser sequence)) culture broth having 0.2U of enzyme activity in leucine-p-nitroanilide substrate was added, adjusted to 0.3ml of final volume and incubated at 37°C for 10min. Then 0.3ml of 70% acetic acid was added to stop the reaction. Contaminant was removed by centrifuging at 14,000rpm, for 5min and filtering with 4μm membrane sequentially. The reaction mixture was analyzed by HPLC and aminopeptidase activity in oligopeptide substrate was measured by the method of Reference Example 2.

The aminopeptidase activity were examined in *Bacillus licheniformis* KCTC 1030 (ATCC 27811), KCTC 3058 (ATCC 11946), ATCC 12759 and the exact values were 2.11, 1.46, 2.14 and 2.76μmole/min respectively. Since these aminopeptidase removed only methionine residue of oligopeptide at the N-terminus without proceeding reaction, it can be used to remove a methionine residue in both oligopeptide and protein containing Met-X-pro sequence at the N-terminus. Since the enzyme activity measured by using oligopeptide and leucine-p-nitroanilide has some difference in spite of the same unit of culture broth, the enzyme activities may have no relation in two substrates as described.

As a result, it is proved that the aminopeptidase derived from *Bacillus licheniformis* can be used properly to remove a methionine residue at the N-terminus by using methionyl oligopeptide as substrate. In respect of substrate specificity, aminopeptidase did not react with X-Pro sequence of the N-terminus although it can remove a methionine residue of the N-terminus.

It is also identified that the aminopeptidase derived from *Bacillus licheniformis* can be used properly to remove a methionine residue by using protein substrate as described in Examples below. As protein substrate methionyl HGH containing Met-X-Pro sequence at the N-terminus (initiated with Met-Phe-Pro-Thr-Ile sequence at N-terminus) was attempted for the use. When protein was utilized as substrate, contaminating proteases such as endo-proteases induced additional reactions. Thus aminopeptidase produced in *Bacillus licheniformis* strains should be purified highly before the reaction.

As shown in Fig. 5, the aminopeptidase purified from *Bacillus licheniformis* ATCC 12759, KCTC 3058 and so forth has the highest aminopeptidase activity in oligopeptide substrate which is compared with that of *Bacillus licheniformis* strains of the present

invention. At that time the aminopeptidase activity in leucine-p-nitroanilide was used as standard. Therefore aminopeptidase purified in mass was adopted usefully in the process removing a methionine residue from methionyl HGH.

<Example 7> Cultivation of *Bacillus licheniformis* ATCC 12759

In order to obtain *Bacillus licheniformis* ATCC 12759 in mass, *Bacillus licheniformis* strains were inoculated into 100ml of autoclaved LB broth and incubated at 37°C for 20 hours for seed culture. The culture broth was inoculated into 10L of autoclaved LB broth in fermenter of 10L volume and incubated for 22-24 hours maintaining pH 7.0.

<Example 8> Purification of aminopeptidase from *Bacillus licheniformis* KCTC 12759

Culture broth obtained in Example 7 (approximately 9L) was centrifuged at 8000rpm for 20min. The resulting supernatant was exploited, added 1mM PMSF (phenylmethylsulfonylfluoride) and concentrated into about 1.5L by using concentrator (Amicon Spiral Cartridge, MWCO 10000). Into above concentrating

solution acetic acid was added to control pH 5.0 and ammonium sulfate was added to make 40% of final concentration (weight/volume). And the same volume of ter-butanol was added, mixed for 1 hour, then centrifuged to recover lower aqueous layer by using separating funnel (1st TTP). Then pH was adjusted to 6.0 by adding 0.5N NaOH and ammonium sulfate was also added to make 85% solution of final concentration. Then the same volume of ter-butanol was added, mixed for 1 hour, then centrifuged for 10min to recover protein precipitation layer in middle band (2nd TTP).

Precipitated protein was dissolved in 20mM PBS (pH 6.8) and centrifuged at 10,000rpm for 20 min to remove contaminating precipitate. Considering conductivity of the solution, salt concentration was adjusted below 0.3M approximately. Then 1.5L of SP-Sepharose (Pharmacia) was filled into INdeX 220/500 and equilibrated with same buffer. The above protein sample was injected, then the column was washed with PBS buffer containing 0.3-0.4M NaCl (flow rate 50ml/min). Aminopeptidase was eluted with linear concentration gradient of 0.3-0.4M NaCl to 1M NaCl (flow rate 25ml/min).

Each fraction eluted from SP-Sepharose column was examined to measure enzyme activity of aminopeptidase. And fractions indicating the enzyme activity were

collected and concentrated into 20ml by ultrafiltration (Amicon stirred cell type). Then gel filtration chromatography was performed by using packed column of 1800ml Sephacryl S-200 and 20mM PBS buffer (pH 6.8) containing additional 0.25M NaCl was used for elution.

Above fractions obtained by using gel filtration chromatography was concentrated and diluted with 20mM phosphate buffer (pH 6.8). Then the diluted sample was adsorbed into FPLC mono-S column (0.5cm * 5cm) equilibrated 3 times with the buffer and proteins were eluted with linear gradient of 0.3 to 1M NaCl for obtaining pure aminopeptidase. Although aminopeptidase eluted had several peaks on the column chromatography, all the peaks had same molecular weights measured by SDS-PAGE as shown in Fig. 6. In addition all the peaks indicated also same enzyme activities. And it was proved by N-terminal sequencing of the aminopeptidases that one or two amino acids deleted from N-terminus of the aminopeptidase coexisted. The processes of the chromatography described above was summarized in table 1.

Table 1.

Purification process of the aminopeptidase of *Bacillus licheniformis* ATCC 12759

purification stage	vol. of sample	amino-peptida-se(U/ml)	total amino-peptida-se(U/ml)	endo-peptida-se(U/ml)
culture broth	6250	8.7	54000	0.43
2nd TPP	1520	32.95	50081	0.701
SP-Sepharose	1650	24.95	43600	0.008
Sephacryl S-200 (conc.)	35	307	10700	0.023
Mono-S (peak I)	10.5	345	3620	0.003
Mono-S (Peak II)	6.5	243	1580	0.002

purification stage	total endo-peptida-se(U)	protein (mg/ml)	specific amino-peptida-se(U/mg)	specific endo-peptida-se(U/mg)
culture broth	2365	0.94	9.26	0.46
2nd TPP	1065	1.07	30.79	0.66
SP-Sepharose	14	0.07	356.4	0.11
Sephacryl S-200(conc.)	0.8	0.25	1228	0.09
Mono-S (peak I)	0.032	0.232	1490	0.011
Mono-S (Peak II)	0.013	0.149	1630	0.01

<Example 9> Structural properties of aminopeptidase

(1) Measurement of molecular weight

To determine the molecular weight of aminopeptidase, SDS-PAGE was performed with above sample as shown in Fig. 6. In Fig. 6, lane M is standard protein as molecular weight marker, lane 1 and lane 2 is aminopeptidase of this invention. Molecular weight of the aminopeptidase was identified to be 43-47 kDa approximately.

(2) Amino acid sequencing

In order to determine amino acid sequences of N-terminus of aminopeptidase, automated Edman degradation analysis (Geoffrey Zubay, Biochemistry, 2nd Ed., 47-48, 1988) was performed by using amino acid analyzer (Model 471A, Applied Biosynthesis). And phenylthiohydantoin attached amino acid was separated by HPLC column chromatography (220nm * 2.1mm Model PHT-222, Applied Biosystems, USA) and the retention time was compared. As a result, N-terminul sequence of amino acid was identified to have SEQ. ID. No: 3 (Lys-Phe-Ser-Lys-Lys-Phe-Asn-Glu-Asn-Arg). And aminopeptidase purified above coexisted with forms in which one and two amino acids were deleted at the N-terminus. These enzymes

can be separated by increasing salt concentration of linear gradient in FPLC mono-S-chromatography slowly and had same molecular weights and enzyme activities in the results of SDS-PAGE.

<Example 10> Enzymatic properties of aminopeptidase

(1) Measurement of optimal pH

In order to measure optimal pH range of aminopeptidase, enzyme activities of aminopeptidase were examined at the range of pH6.0-11.5. 0.1M concentration of sodium phosphate (pH 6-7.5), Tris-Cl (pH 7.0-9.0), Boric acid (pH 8.5-10.0), CAPS (3-Cyclohexylamino-1-propanesulfonic acid) buffers were used to control the pH of enzyme reaction. And leucine-p-nitroanilide as synthetic substrate was used to measure the enzyme activities. As a result aminopeptidase of this invention had higher activity at the range of pH 7.5-10.5 and the highest activity at the range of pH 8.5-9.5 as shown in Fig. 7.

(2) Measurement of optimal temperature

In order to examine the optimal temperature range, enzyme activities of aminopeptidase were measured at the range of 30-90°C at every 10°C interval by using 0.1M of Tris-Cl buffer (pH 8.0) And leucine-p-

nitroanilide was used as synthetic substrate. As a result aminopeptidase of this invention had higher activity at the range of 40-70°C and the highest activity at the range of 50-60°C as shown in Fig. 7.

(3) Thermostability

In order to examine thermostability of aminopeptidase, purified aminopeptidases was incubated at 60°C and 80°C for 1, 2, 3, 4 and 6 hours respectively, then used to measure the enzyme activities. And the results were compared with that of aminopeptidase which did not treat heat. At that time leucine-p-nitroanilide was used as synthetic substrate.

Since aminopeptidase incubated at 60°C for 6 hours maintained more than 50% of its activity, the enzyme of this invention is proved to be thermostable as shown in Fig 9. Thermostability is closely related with stability of protein molecules, so that stable aminopeptidase is not inactivated during purification process and enzyme reaction. Thus enzyme reaction can be performed for long time and the enzyme maintains initial activity continuously. Therefore aminopeptidase of this invention can be utilized very usefully in enzyme reaction requiring long time such as removing a methionine residue from recombinant

proteins.

(4) Effect of chemical materials

In order to examine chemical materials affecting enzyme activity of aminopeptidase, enzyme activities of aminopeptidase treated for 2 hours with dithiotreitol, iodoacetic acid, ortho-phenanthroline and EDTA as 1, 5 and 20mM respectively were measured. At that time leucine-p-nitroanilide was used as synthetic substrate and the results were shown in table 2.

Precisely aminopeptidase of this invention is identified as metalloprotease since it was inhibited by ortho-phenanthroline more strongly than EDTA. Since the aminopeptidase was inhibited by dithiothreitol reacting with disulfide bond and iodoacetic acid reacting with sulfhydryl group, cysteine residue of the aminopeptidase may perform important role in enzyme reaction.

Table 2

Effect of chemical materials affecting enzyme activity

chemical materials	relative activity (%)			
	0mM	1mM	5mM	20mM
EDTA	100	93.8	83.4	66.3
ortho-penanthroline	100	91.9	86.8	4.7

dithiotreitol	100	0.8	0	0
iodoacetic acid	100	98.1	104.2	0

<Example 11> Substrate specificity of the aminopeptidase and measurement of chemical kinetic constant

(1) Preparation of substrate

In order to compare substrate specificity of aminopeptidase of this invention, 2 kinds of substrate were used. In detail, one was synthetic substrates of p-nitroanilide derivative and purchased from Sigma, USA and the other was oligopeptide such as methionyl oligopeptide of SEQ. ID. NO: 1 which have amino acid sequence similar to N-terminus of methionyl HGH and leucyl oligopeptide of SEQ. ID. NO: 2. The oligopeptides of this invention were synthesized.

In order to synthesize oligopeptide mentioned above, solid-phase peptide synthesis was performed automatically in 0.1mM scale by use of peptide synthesizer (Applied Biosystems 431A, USA). Peptide-resin complex produced in above reaction was cut to obtain oligopeptide prepared as sample by the process

of purification and drying. The composition of amino acid in the oligopeptides above was analyzed by amino acid analyzer and amino acid sequence of synthetic peptide was determined exactly by gas chromatography-mass analyzer. The oligopeptides were dissolved in DMSO to 30mM concentration and diluted with DW to 2.5mM. Then the oligonucleotides diluted was added to reaction mixture at predetermined concentration.

(2) Reaction condition

Enzyme reaction of aminopeptidase was performed by the process described in Reference Examples. At that time p-nitroanilide derivative was used as synthetic substrate. Leucine-p-nitroanilide was used as substrate to perform the reaction in concentration of 0.03125, 0.0625, 0.125, 0.25, 0.5, 1mM and methionine-p-nitroanilide in 0.125, 0.25, 0.5, 1mM and aminopeptidase in 0.5U respectively. Above reactions using leucine-p-nitroanilide proceeded for 2min and methionine-p-nitroanilide for 40min. Then 70% acetic acid was added to stop the reactions and absorbance at 405nm was measured. The product concentration was measured by adopting absorbance constant ϵ_{405} = 9450$M^{-1}cm^{-1}$.

In addition oligopeptide was also used as substrate to perform enzyme reaction by using substrate

of known concentration and aminopeptidase in buffer containing 0.1M Tris-Cl, 0.1M NaCl to make 500μl reaction volume. The enzyme reaction was performed at 37°C and after 5 and 10min 250μl of reaction mixture was adopted and added 70% acetic acid to stop the reaction. The reaction mixture was analyzed by performing HPLC chromatography. At that time substrate was used in concentration of 0.125, 0.160, 0.25, 0.75, 1.25mM regardless of kinds of substrate and aminopeptidase was used in 0.01U in case of leucyl peptide, 0.2U in case of methionyl peptide.

(3) Kinetic parameters of aminopeptidase

In reaction condition mentioned above, initial velocity of enzyme reaction was measured with varying the substrate concentration. Lineweaver-Burk plot was used to determine Km value and Maximum reaction velocity of aminopeptidase from *Bacillus licheniformis*. In addition kcat and kcat/Km was determined by using Km value and maximum reaction velocity described above. These results were depicted in Table 3 and compared with that of *Aeromonas proteolytica (Vibrio proteolytica)* aminopeptidase (Korean Patent Publication No. 94-14804) measured by same experiment.

According to general classification method, both the aminopeptidases were classified as leucine

aminopeptidase since it reacted with substrate containing N-terminal leucine effectively. Aminopeptidase from *Bacillus licheniformis* had similar kinetic parameters in p-nitroanilide derivative substrate as that of aminopeptidase from *Aeromonas proteolytica*. But in oligopeptide substrate aminopeptidase from *Bacillus licheniformis* had still higher kcat value. Since aminopeptidase from *Bacillus licheniformis* has lower Km value than that from *Aeromonas proteolytica* already reported, it showed higher affinity with substrate. Therefore it was identified that aminopeptidase of the present invention can remove a methionine and leucine residue at N-terminus efficiently.

Table 3

Comparison of kinetic parameters of aminopeptidase in various substrate

aminopeptidase	substrate	Km (mM)	kcat (sec^{-1})	Kcat/Km ($mM^{-1}sec^{-1}$)
Bacillus licheniformis aminopeptidase	leucine-p-nitroanilide	0.739	130	176
	methionine-p-nitroanilide	1.004	2.59	2.58
	leucyl oligopeptide	0.172± 0.035	275.6± 33.9	1626± 141
	methionyl oligopeptide	0.387± 0.100	21.08± 3.79	56.13± 12.35

Aeromonas proteolytica aminopeptidase	leucine-p-nitroanilide	0.240	48	200
	methionine-p-nitroanilide	0.942	2.64	2.80
	leucyl oligopeptide	0.336± 0.045	70.29± 4.37	211.2± 2.4
	methionyl oligopeptide	0.956± 0.680	3.23±1.16	3.76± 1.50

<Example 12> Preparation of natural type HGH

(1) Optimization of reaction removing N-terminal methionine from methionyl HGH

In order to prepare natural type HGH by using aminopeptidase from *Bacillus licheniformis*, optimal condition of reaction removing N-terminal methionine of protein was examined as described below. In principle reaction mixture containing 1mg/ml methionyl HGH (final 0.1mg), 50mM Tris-Cl(pH8.0), 0.1M NaCl, 1mM PMSF was prepared and 0.5U of aminopeptidase was added to make 100ul of final volume. Then the reaction was performed at 37°C for 30min and 11μl of 100% TCA was added to stop the reaction. The reaction mixture was centrifuged to precipitate proteins by small-scale centrifuge at 14ooorpm for 5min. The precipitate of protein was washed twice with acetone and was dried. Automatic Edman degradation was performed by amino acid sequence analyzer (Model 471A, Applied Biosystems, USA)

with dried sample.

In order to examine removal rate of methionine residue, methionine versus phenylalanine ratio detected at 1st cycle was measured. In order to examine optimal pH of above reaction, the removal rate of methionine from methionyl HGH was measured in pH range of 7.0-11.5. As buffer solution, Tris-Cl(pH7.0-9.0), boric acid(pH8.5-10.0) and CAPS(pH9.5-11.5) was used in 50mM to regulate pH in above reaction.

As a result, optimal pH range which can be used to prepare natural type HGH by using aminopeptidase is 7.5-9.5 as shown in Fig. 10. In order to examine optimal temperature, the removal rate of methionine residue at N-terminus was measured at the range of 20-70°C and the results are shown in Fig. 11. Optimal temperature range removing methionine was 50-60°C. But at high temperature removal rate of methionine became low due to precipitation of HGH. Considering stability of HGH, low temperature below 37°C is more preferable than high temperature of 50-60°C. As shown in Fig 12, NaCl can be added to 430mM in reaction mixture, preferably about 100mM.

(2) Preparation of natural type HGH

About 1mg of methionyl HGH and reaction buffer (50mM Tris-Cl, pH8.0, 100mM NaCl, 1mM PMSF) containing

10U of aminopeptidase were mixed (final volume 1ml) in dialysis tube and reacted at 37°C for 24 hours in water bath dialyzing with same buffer. After 24 hours the above reaction mixture was dialyzed again with Tris-Cl (pH8.0) at 4°C sufficiently to complete the reaction. Then Mono-Q ion exchange chromatography (HPLC chromatography) was performed to elute HGH with 20mM Tris-Cl containing 0.1M NaCl. At that time HGH recovered above was absolutely pure without aminopeptidase and other contaminants and the results were identified by SDS-PAGE as shown in Fig. 13.

And amino acids at N-terminus of HGH recovered above was analyzed by amino acid sequence analyzer (model 471A, Applied Biosystems, USA) as shown in Fig. 14. As a result, it was identified that aminopeptidase of the present invention remove N-terminal methionine of methionyl HGH completely and replace N-terminus methionine with phenylalanine to produce new natural type HGH.

(3) Preparation of natural type HGH in large scale

50mg of aminopeptidase was treated in 50mg of methionyl HGH at 37°C for 24 hours in a condition mentioned above. HGH was obtained by the process wherein the sample was treated with aminopeptidase, diafiltrated to reduce the salt concentration,

proceeded to ion exchange chromatography using DEAE-Sepharose (Pharmacia) and recovered. N-terminal amino acid sequence of HGH recovered was determined by amino acid sequence analyzer as shown in Fig. 15. As a result, it was identified that HGH produced by the above process had phenylalanine at the N-terminus due to methionine removal.

<Example 13> Purification of aminopeptidase from *Bacillus licheniformis* KCTC 3058 (ATCC 11946)

Bacillus licheniformis KCTC 3058 was cultured by the same process described in Example 7. Culture broth (approximately 8L) was centrifuged at 8000rpm for 20min. The resulting supernatant was recovered, and concentrated into about 1.6L by using concentrator (Amicon Spiral Cartridge, MWCO 10000). Into above concentrating solution 10% of acetic acid was added to adjust pH 5.0 and ammonium sulfate was added to make 40% of final concentration (saturation concentration). And the same volume of ter-butanol was added, mixed for 1 hour, then centrifuged at 8000rpm for lawn to recover lower aqueous layer by using separating funnel (1st TTP). Then pH was adjusted to 6.0 by adding 0.5N NaOH and ammonium sulfate was also added to make 85% solution of final concentration (saturation

concentration). Then the same volume of ter-butanol was added, mixed for 1 hour, then centrifuged at 8000rpm for 10min to recover protein precipitation layer in middle layer (2nd TTP).

Precipitated protein was dissolved in 20mM PBS (pH 6.8) and centrifuged at 10,000rpm for 20 min to remove contaminating precipitate. Considering conductivity of the solution, salt concentration was diluted with same buffer below 0.3M approximately. Then 500ml of SP-Sepharose (Pharmacia) column was prepared and equilibrated with the same buffer. The above protein sample was injected, then the column was washed with PBS buffer containing 0.3-0.4M NaCl (flow rate 15ml/min). Aminopeptidase was eluted with linear concentration gradient of 0.3-0.4M NaCl to 1M NaCl (flow rate 10ml/min).

Each fraction eluted from SP-Sepharose column was examined to measure enzyme activity of aminopeptidase. And fractions showing the enzyme activity were collected and concentrated into 20ml by ultrafiltration (Amicon stirred cell type). Then gel filtration chromatography was performed to purify the aminopeptidase of this invention.

The purification process described above was performed by two different buffer systems. At first process, 20 mM Tris-Cl buffer (pH 7.5) containing 0.5

M NaCl and at second process 20 mM PBS buffer (pH 6.8) containing 0.5 M NaCl, 0.1% tween 20 were utilized to perform gel permeation chromatography. The processes of the chromatography described above was summarized in table 4 and table 5.

Above fractions obtained by using gel filtration chromatography was concentrated and diluted. Then the diluted sample was adsorbed into FPLC mono-S column (0.5cm * 5cm) and proteins were eluted with linear concentration gradient of 0.4-1M NaCl for obtaining pure aminopeptidase.

Table 4.

Purification process of the aminopeptidase of *Bacillus licheniformis* KCTC 3058 in which 20mM Tris-Cl (pH 7.5) was used in gel filtration.

purification stage	vol. of sample	protein	aminopeptidase activity		
		conc. (mg/ml), [total mmg]	U/ml	U/mg	total (U)
culture broth	10,280	12,181 [125,000]	13.67	1.12	140,528
conc. spt.	2,750	3.174 [8,700]	31.22	9.87	82,855
2nd TTP	1,000	1.664 [1,644]	69.9	42.5	69,900
SP-Sepharose	18	1.773 [31.9]	1401	790.7	25,222
S-200	24.5	0.394 [9.65]	442	1,122	10,829

purification stage	vol. of sample	protein	endopeptidase activity		
		conc. (mg/ml), [total mmg]	U/ml	U/mg	total (U)
culture broth	10,280	12,181 [125,000]	0.710	0.058	7,299
conc. spt.	2,750	3.174 [8,700]	0.778	0.246	2,140
2nd TTP	1,000	1.664 [1,644]	0.708	0.431	708
SP-Sepharose	18	1.773 [31.9]	0.056	0.032	1.01
S-200	24.5	0.394 [9.65]	0.031	0.079	0.76

Table 5.

Purification process of the aminopeptidase of *Bacillus licheniformis* KCTC 3058 in which 20mM PBS (pH 6.8) buffer containing 0.5M NaCl and 0.1% Tween 20 was used in gel filtration.

purification stage	vol. of sample	protein	aminopeptidase activity		
		conc. (mg/ml), [total mmg]	U/ml	U/mg	total (U)
culture broth	8,100	14.216 [115,000]	12.49	0.88	101,169
conc.spt.	16,000	11.214 [17,900]	83.63	7.48	133,813

2nd TTP	1,000	2.132 [2,132]	111.18	52.1	111,177
SP-Sepharose	32	2.753 [88.1]	3.636	1,321	116,362
S-200	12.2	1.765 [21.5]	1,520	862.5	18,544
S-200	13.3	1.715 [22.8]	1,824	1,064	24,259
S-200	11	0.317 [3.49]	648	2,042	7,128
S-200	12.4	0.468 [5.8]	834	1,783	10,342
S-200	11.5	0.879 [10.1]	1,660	1,890	19,090

purification stage	vol. of sample	protein	endopeptidase activity		
		conc. (mg/ml), [total mmg)]	U/ml	U/mg	total (U)
culture broth	8,100	14.216 [115,000]	0.266	0.019	2,155
conc. spt.	16,000	11.214 [17,900]	0.507	0.045	811
2nd TTP	1,000	2.132 [2,132]	0.612	0.287	612
SP-Sepharose	32	2.753 [88.1]	0.047	0.017	1.50
S-200	12.2	1.765 [21.5]	0.011	0.006	0.134
S-200	13.3	1.715 [22.8]	0.010	0.006	0.133

S-200	11	0.317 [3.49]	0.023	0.072	0.253
S-200	12.4	0.468 [5.8]	0.024	0.051	0.298
S-200	11.5	0.879 [10.1]	0.046	0.052	0.529

<Example 14> Structural characteristics of aminopeptidase from *Bacillus licheniformis* KCTC 3058

(1) Measurement of molecular weight

To determine the molecular weight of aminopeptidase, which was purified from *Bacillus licheniformis* KCTC 3058 (ATCC 11946) in Example 13, the fractions resolved on FPLC Mono-S column chromatography were analyzed in SDS-PAGE. As a result, all peaks of the aminopeptidase which showed different elution time was identified to have the same molecular weights in spite of the above chromatogram as shown in Fig. 16a and Fig. 16b. And aminopeptidase derived from *Bacillus licheniformis* KCTC 3058 also has the same molecular weights with that of aminopeptidase from *Bacillus licheniformis* ATCC 12759. Molecular weight of the aminopeptidase was identified to be 43-47 kDa approximately.

(2) Amino acid sequencing

In order to determine amino acid sequence of the N-terminus of aminopeptidase derived from *Bacillus licheniformis* KCTC 3058 (ATCC 11946), automated Edman degradation analysis (Geoffrey Zubay, Biochemistry, 2nd Ed., 47-48, 1988) was performed by using amino acid analyzer of Example 9. As a result, the N-terminul sequence of amino acid was identified to have SEQ. ID. No: 4 (Lys-Phe-Ser-Lys-Lys-Phe-Asn-Glu-Asn-Arg-Ala-Tyr-Gln-Thr-Ile-Tyr-His-Leu). And the aminopeptidase purified above coexisted with forms in which the N-terminal lysine was deleted at the N-terminus and 9th asparagine was substituted for aspartic acid. The amino acid sequence identified above was exactly same with that of aminopeptidase derived from *Bacillus licheniformis* ATCC 12759.

Summarizing the results described above, the aminopeptidases derived from different strains of *Bacillus licheniformis* are same, since aminopeptidases derived from *Bacillus licheniformis* KCTC 3058 and ATCC 12759 respectively has the same molecular weight and amino acid sequence at the N-terminal.

The aminopeptidases of the present invention is useful since they are purified from *Bacillus licheniformis* and remove the N-terminal methionine

residue specifically. Since aminopeptidases recognize Met-X-Pro sequence at the N-terminus, in addition to HGH all the proteins having X-Pro sequence at the N-terminus could be changed easily to natural type proteins having complete activity after mass production in bacterial cell. And the aminopeptidases of the present invention are also proper to be used in the reaction mentioned above due to their thermostability.

Sequence Listing

(1) GENERAL INFORMATION

(i) APPLICANT : LG CHEMICAL LTD.

LEE, Young-Phil

HAN, Kyuboem

KIM, Se-Hoon

PARK, Soon-Jae

LEE, Seung-Joo

(ii) TITLE OF INVENTION : Aminopeptidase derived from *Bacillus licheniformis* and process for preparation of natural type proteins

(iii) NUMBER OF SEQUENCES : 4

(2) INFORMATION FOR SEQ ID NO: 1 :

(i) SEQUENCE CHARACTERISTICS :

(A) LENGTH : 7 amino acids

(B) TYPE : amino acid

(C) STRANDEDNESS : single

(D) TOPOLOGY : linear

(ii) MOLECULAR TYPE : peptide

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 1

Met Phe Pro Thr Glu Pro Ser

1 5

(2) INFORMATION FOR SEQ ID NO: 2 :

(i) SEQUENCE CHARACTERISTICS :

(A) LENGTH : 7 amino acids

(B) TYPE : amino acid

(C) STRANDEDNESS : single

(D) TOPOLOGY : linear

(ii) MOLECULAR TYPE : peptide

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 2

Leu Phe Pro Thr Glu Pro Ser
1 5

(2) INFORMATION FOR SEQ ID. NO: 3 :

(i) SEQUENCE CHARACTERISTICS :

(A) LENGTH : 10 amino acids

(B) TYPE : amino acid

(C) STRANDEDNESS : single

(D) TOPOLOGY : linear

(ii) MOLECULAR TYPE : N-terminal amino acids

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 3

Lys Phe Ser Lys Lys Phe Asn Glu Asn Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 4 :

(i) SEQUENCE CHARACTERISTICS :

(A) LENGTH : 18 amino acids

(B) TYPE : amino acid

(C) STRANDEDNESS : single

(D) TOPOLOGY : linear

(ii) MOLECULAR TYPE : N-terminal amino acids

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 4

Lys Phe Ser Lys Lys Phe Asn Glu Asn Arg Ala Tyr Gln Thr Ile Tyr
1 5 10 15

His Leu

What is claimed is :

1. A aminopeptidase derived from *Bacillus licheniformis* strain which removes a methionine residue at the N-terminus of peptide and protein, and contains amino acid sequence of SEQ ID NO: 3 at the N-terminus, and has a molecular weight at the range of 43-47kDa.

2. The aminopeptidase according to Claim 1, which recognizes Met-X-Pro sequence at the N-terminus of peptide and protein to remove the methionine residue.

3. The aminopeptidase according to Claim 2, wherein preferably X is phenylalanine.

4. The aminopeptidase according to Claim 1, which contains sequence deleted and substituted in amino acids.

5. A process for preparing the aminopeptidase of Claim 1 which comprises culturing *Bacillus licheniformis*, centrifuging the culture broth and performing gel filtration chromatography with concentrated supernatant.

6. The process for preparing the aminopeptidase

according to Claim 5, wherein *Bacillus licheniformis* strains are ATCC 12759, KCTC 3058, KCTC 3045 and KCTC 1030.

7. A process for preparing the aminopeptidase of Claim 1, which comprises preparing aminopeptidase-producing cells by using recombinant DNA technology, culturing the recombinant cells and purifying the enzyme.

8. Process for preparing a natural type protein which comprises treating recombinant proteins produced by using recombinant DNA technology with the aminopeptidase of Claim 1 and purifying the resultant.

9. The process for preparing a natural type protein according to Claim 8, wherein recombinant protein contain Met-X-Pro sequence at the N-terminus.

10. The process for preparing a natural type protein according to Claim 9, wherein recombinant protein is methionyl human growth hormone (HGH).

11. The process for preparing a natural type protein according to Claim 10, wherein the aminopeptidase is treated at the range of pH 7.0-9.5 and at the range of 20-60°C and NaCl is added to 30-230mM into reaction

mixture.

12. The process for preparing a natural type protein according to Claim 10, wherein the aminopeptidase is used in 0.2-20 U per 1mg of methionyl HGH.

13. The process for preparing a natural type protein according to Claim 10, wherein after treating the aminopeptidase, natural type HGH is purified by performing ion exchange resin chromatography.

Fig. 1

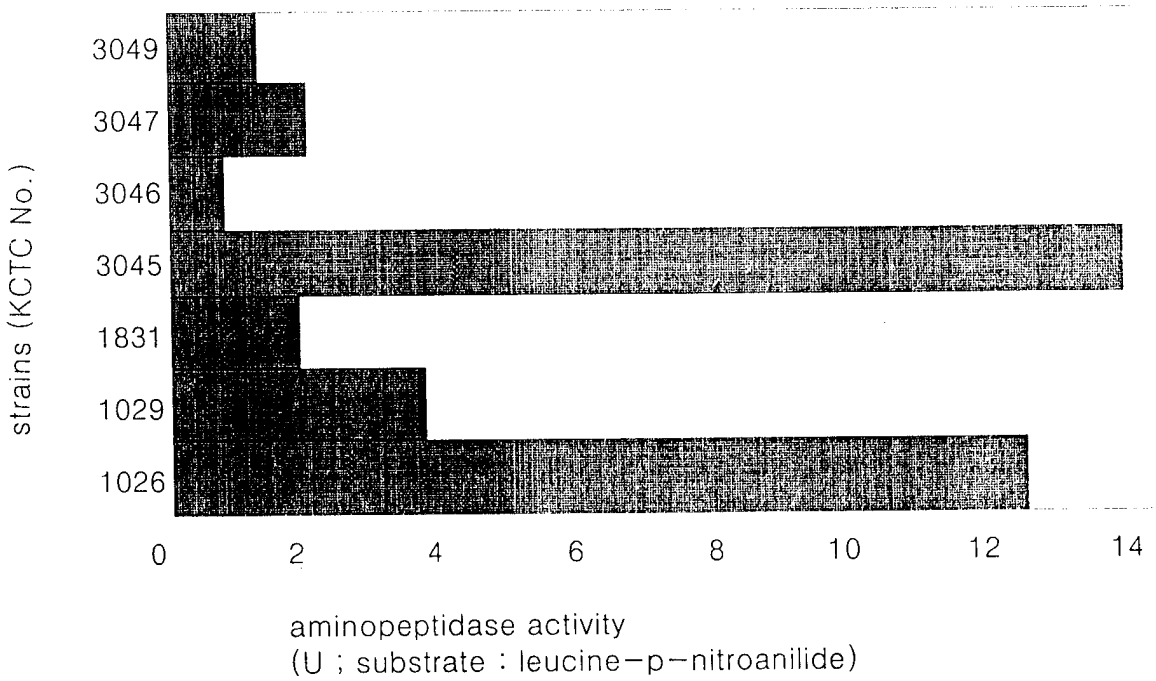

3049
3047
3046
3045
1831
1029
1026
strains (KCTC No.)
0
2
4
6
8
10
12
14
aminopeptidase activity
(U ; substrate : leucine-p-nitroanilide)

Fig. 2

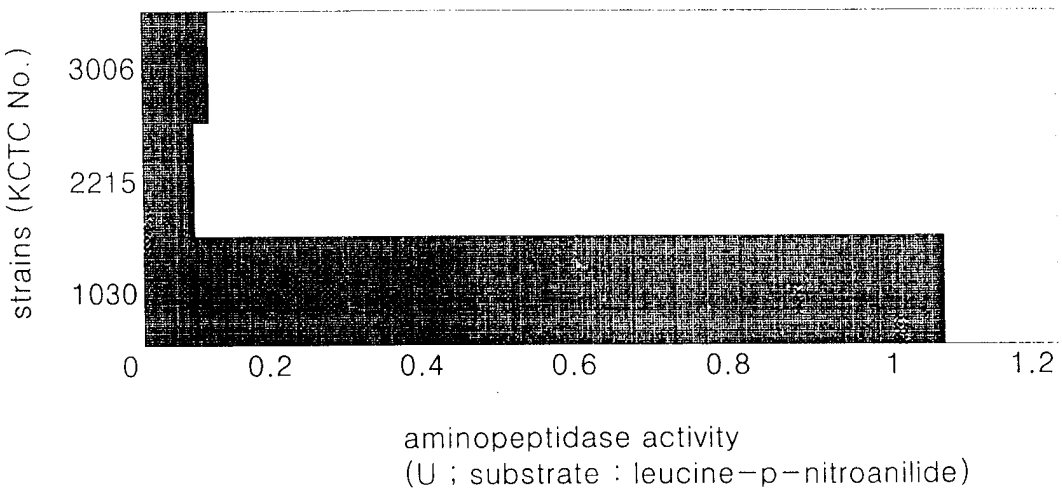

strains (KCTC No.)
3006
2215
1030
0
0.2
0.4
0.6
0.8
1
1.2
aminopeptidase activity
(U ; substrate : leucine-p-nitroanilide)

Fig. 3

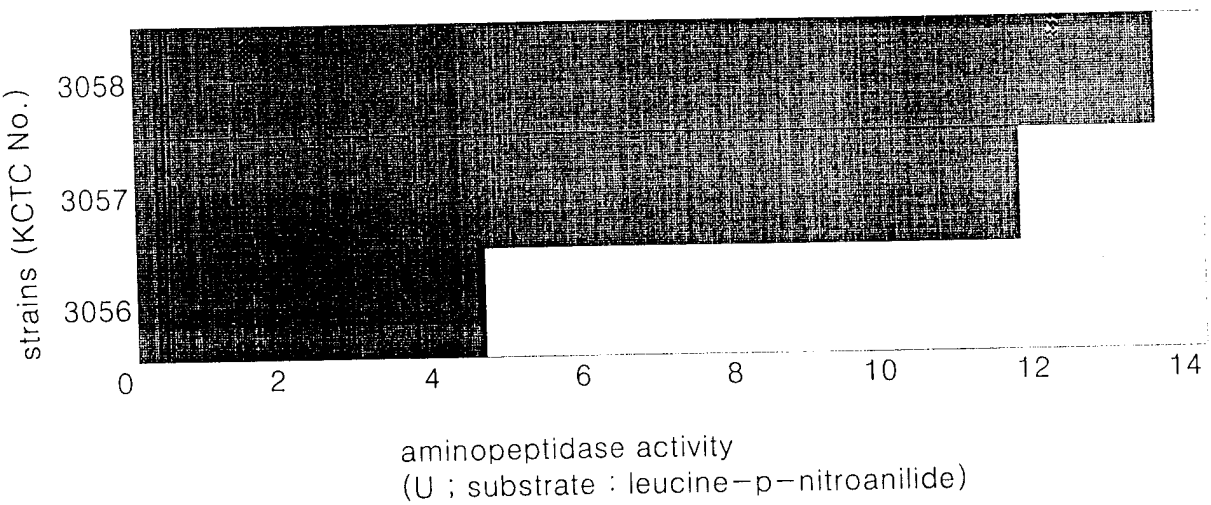

strains (KCTC No.)
3058
3057
3056
0
2
4
6
8
10
12
14
aminopeptidase activity
(U ; substrate : leucine-p-nitroanilide)

Fig. 4

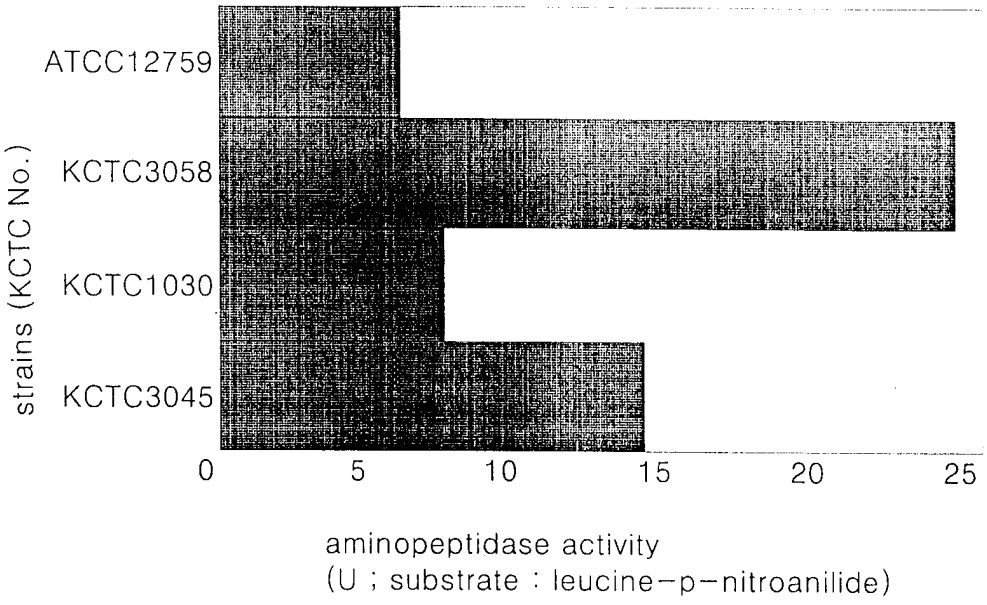

ATCC12759
KCTC3058
KCTC1030
KCTC3045
strains (KCTC No.)
0
5
10
15
20
25
aminopeptidase activity
(U ; substrate : leucine-p-nitroanilide)

Fig. 5

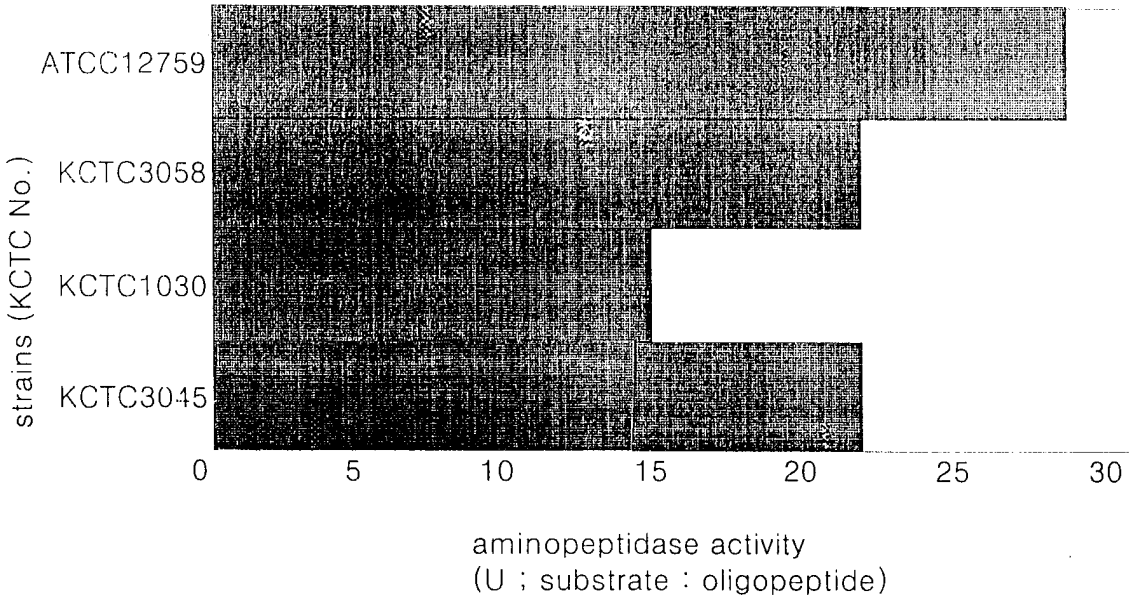

ATCC12759
KCTC3058
KCTC1030
KCTC3045
strains (KCTC No.)
0
5
10
15
20
25
30
aminopeptidase activity
(U ; substrate : oligopeptide)

Fig. 6

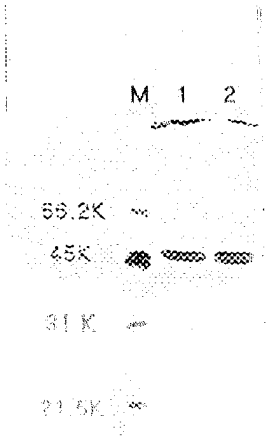


Fig. 7

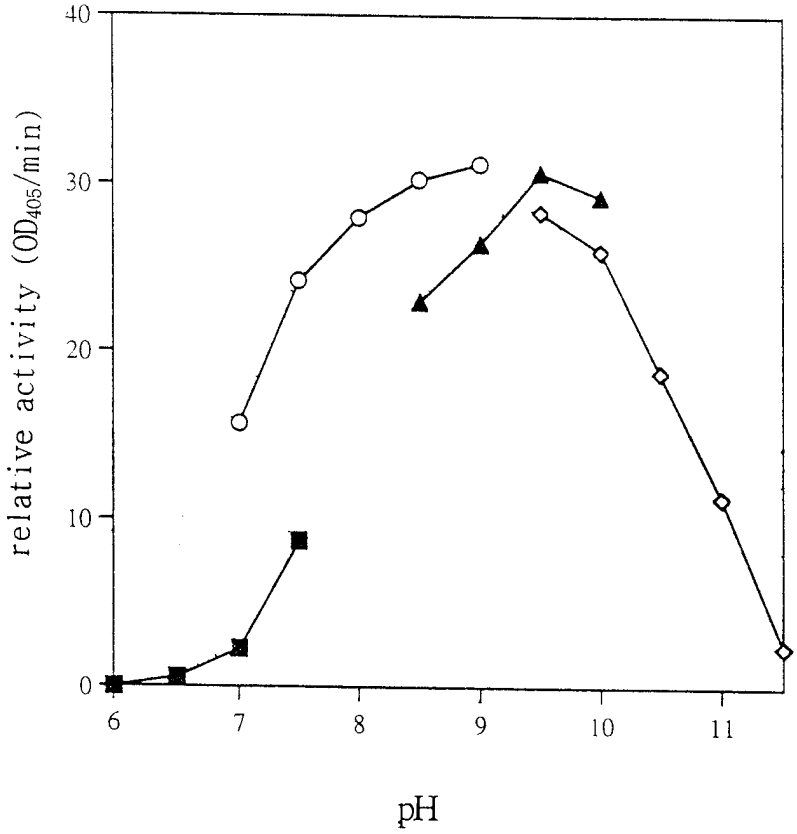


Fig. 8

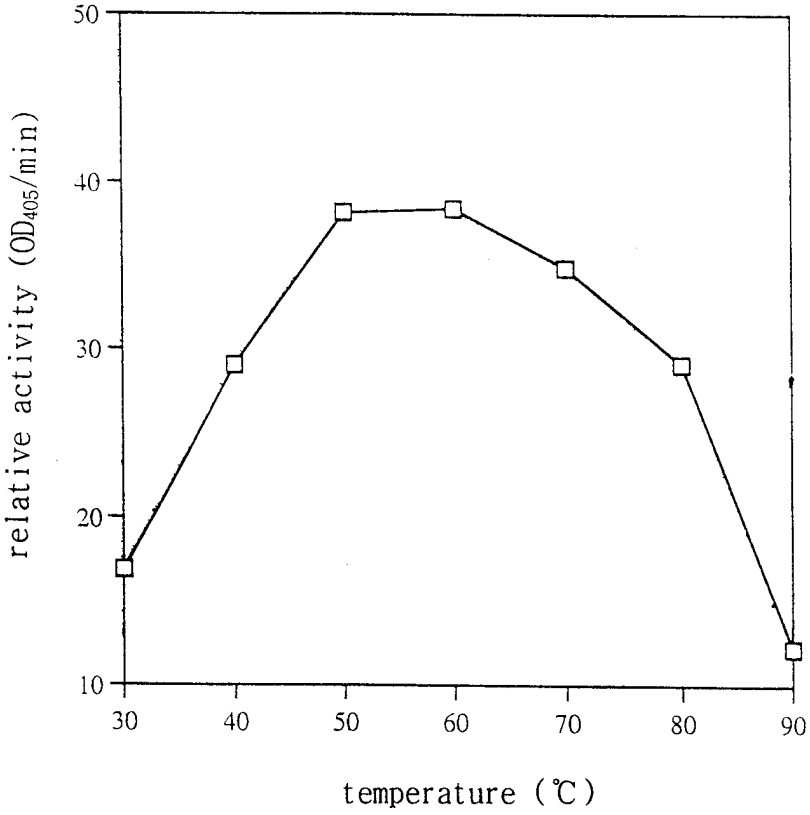

50
40
30
20
10
relative activity (OD_{405}/min)
30
40
50
60
70
80
90
temperature (℃)

Fig. 9

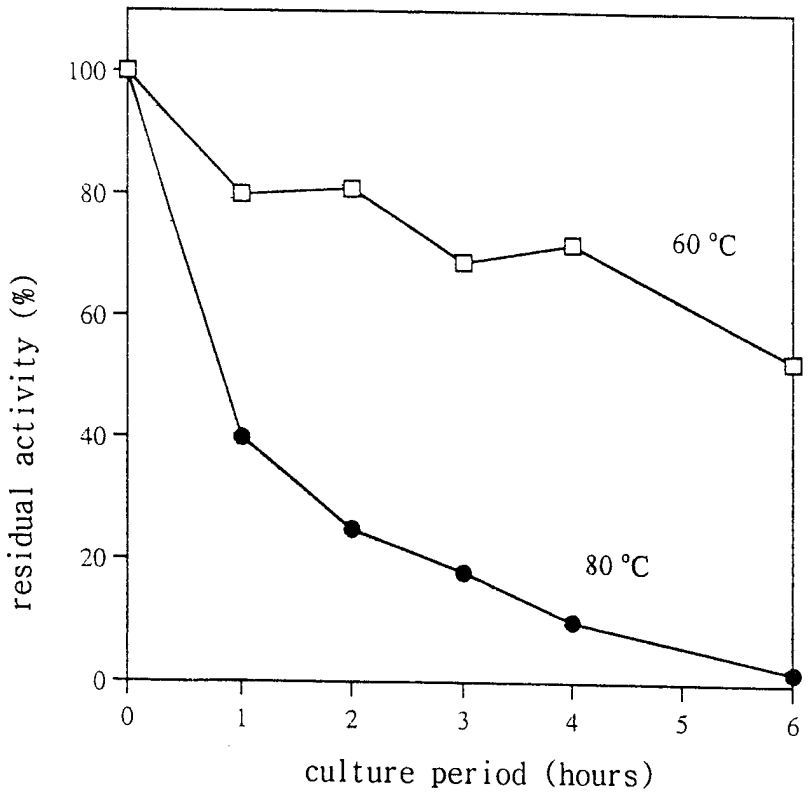

100
80
60
40
20
0
residual activity (%)
60 °C
80 °C
0
1
2
3
4
5
6
culture period (hours)

Fig. 10

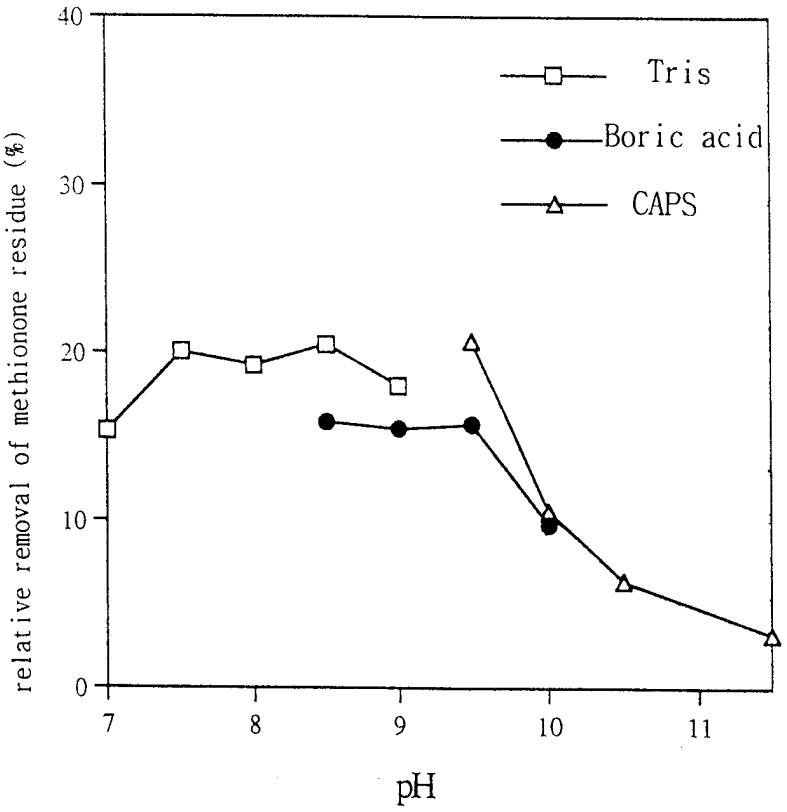

40
30
20
10
0
7
8
9
10
11
pH
relative removal of methionone residue (%)
Tris
Boric acid
CAPS

Fig. 11

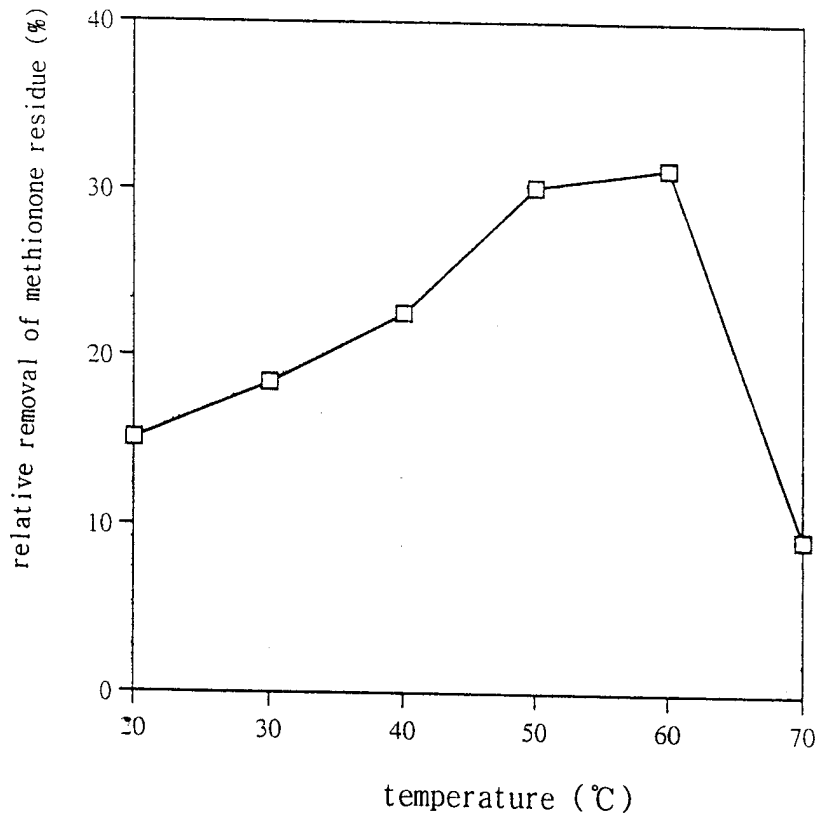

relative removal of methionone residue (%)
40
30
20
10
0
20
30
40
50
60
70
temperature (℃)

Fig. 12

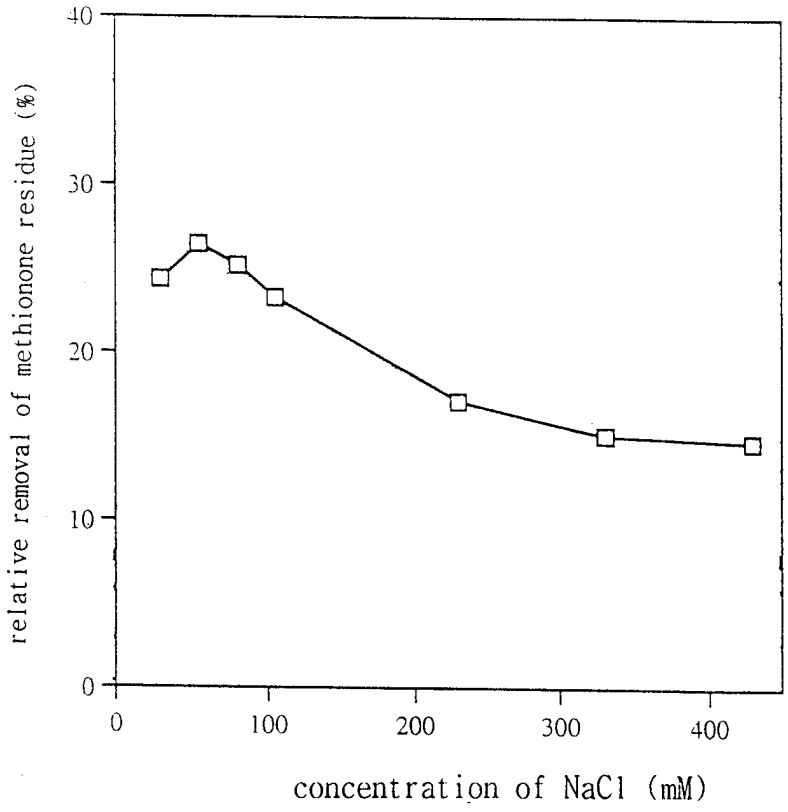

relative removal of methionone residue (%)
40
30
20
10
0
0
100
200
300
400
concentration of NaCl (mM)

Fig. 13

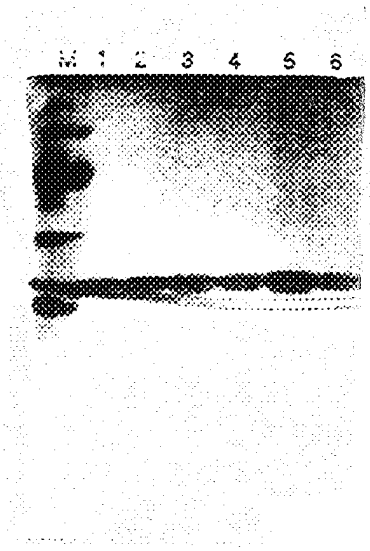


Fig. 14

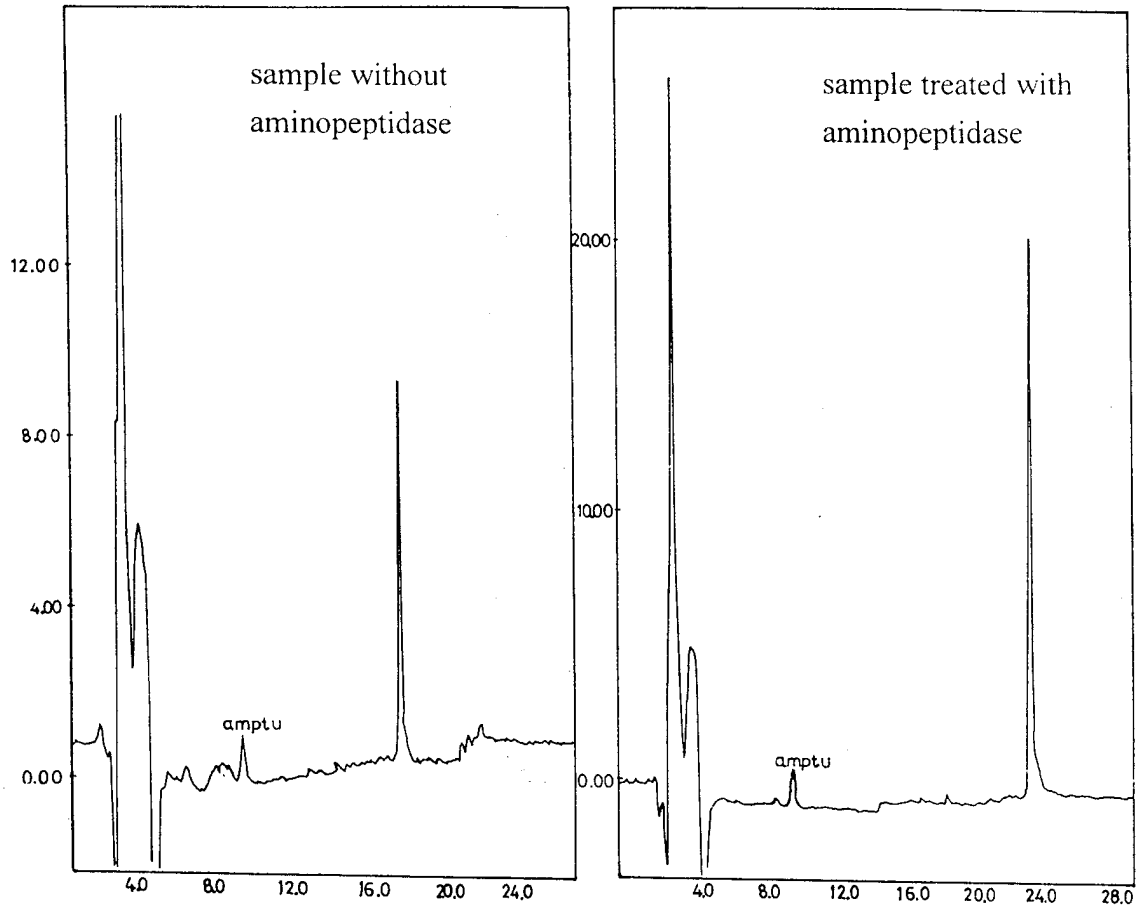

sample without
aminopeptidase
12.00
8.00
4.00
0.00
amptu
4.0
8.0
12.0
16.0
20.0
24.0
sample treated with
aminopeptidase
20.00
10.00
0.00
amptu
4.0
8.0
12.0
16.0
20.0
24.0
28.0

Fig. 15

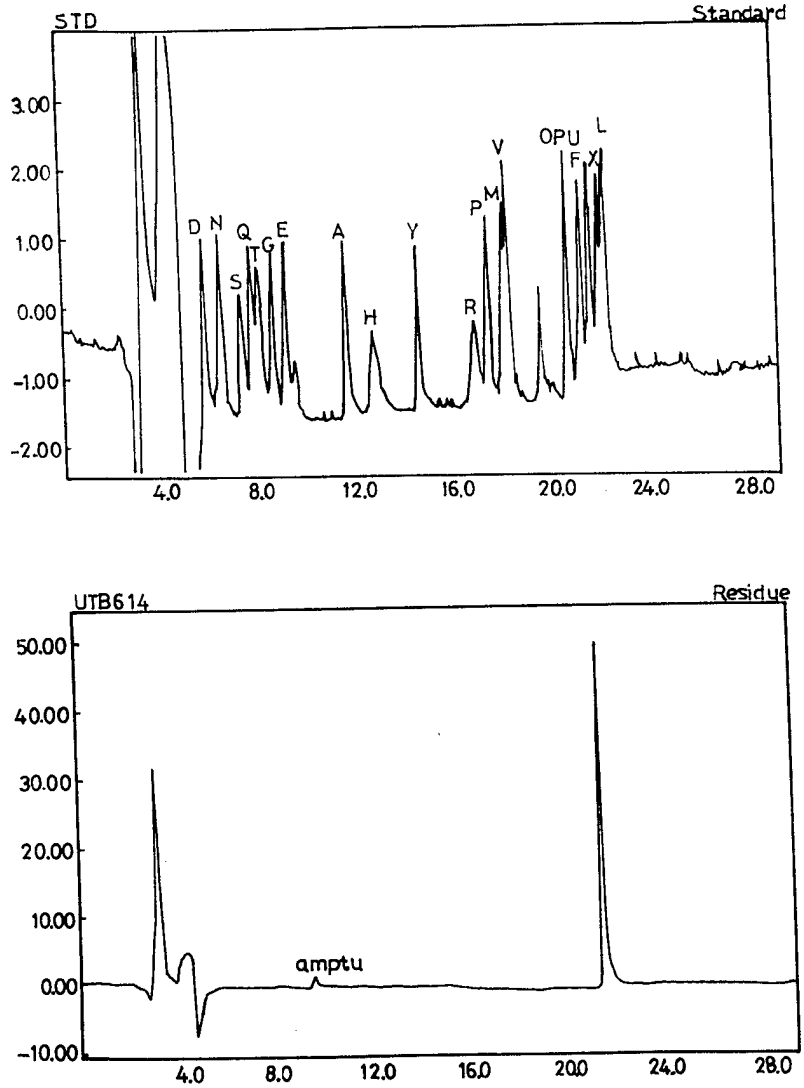

STD
Standard
3.00
2.00
1.00
0.00
-1.00
-2.00
D
N
Q
T
G
E
S
A
H
Y
R
P
M
V
OPU
F
X
L
4.0
8.0
12.0
16.0
20.0
24.0
28.0
UTB614
Residue
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
amptu
4.0
8.0
12.0
16.0
20.0
24.0
28.0

Fig. 16a

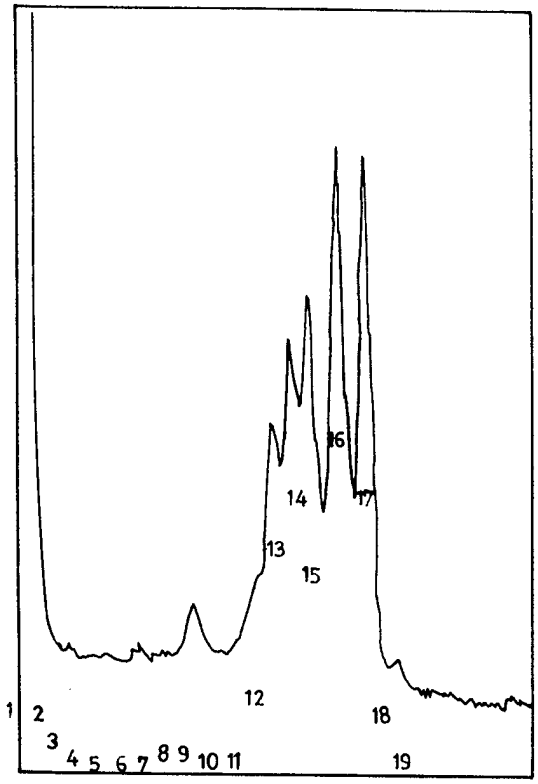
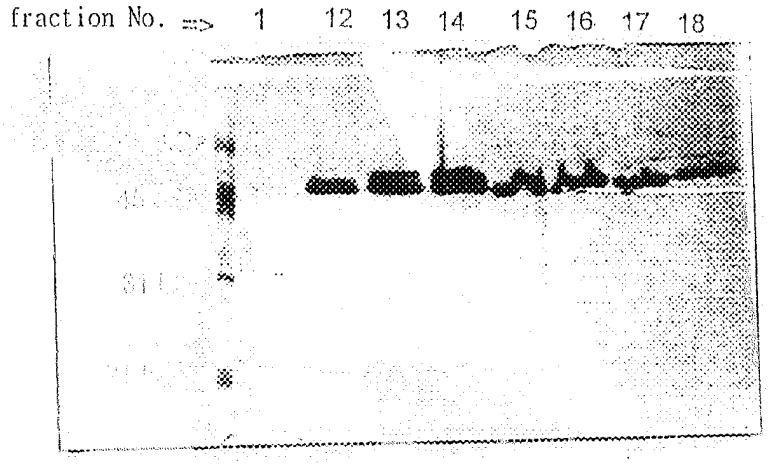


Fig. 16b



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00032

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 9/48, 15/57, 15/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 9/48, 15/57, 15/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, Vol.186, No.2, March 1978, J.R.ABSI et al.:"Isolation and Properties of an Aminopeptidase from Bacillus licheniformis", pages 383-391, totality.	1,5
A	Patent Abstracts of Japan, Vol.16, No.114 (C-921), 1992, JP 03-285684 A (PAICEL CHEM.IND.LTD.), abstract.	1,7,8
A	WO 86/01 229 A1 (BIO-TECHNOLOGY GENERAL CORP.) 27 February 1986 (27.02.86), claims 1,21,22,32.	1-3,7-10

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 May 1998 (08.05.98)	15 May 1998 (15.05.98)

Name and mailing address of the ISA/ AT	Authorized officer
AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna	Wolf
Facsimile No. 1/53424/535	Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 98/00032

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 8601229	27-02-86	AT E 81673	15-11-92
		AU A1 47739/85	07-03-86
		AU B2 591464	07-12-89
		CA A1 1318869	08-06-93
		DE C0 3586773	26-11-92
		DE T2 3586773	18-03-93
		EP A1 191827	27-08-86
		EP A2 489711	10-06-92
		EP A3 489711	07-10-92
		EP B1 191827	21-10-92
		HK A 1430/96	09-08-96
		IL A0 76107	31-12-85
		IL A1 76107	10-03-91
		JP T2 62500003	08-01-87
		JP B4 8022240	06-03-96
		ZA A 8506194	30-04-86