ベトナム産魚醤より分離されたBacillus sp.11-4が産生するプロテアーゼIの部分アミノ酸配列

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Partial amino acid sequence of protease I produced by *Bacillus* sp. 11-4 isolated from Vietnamese fish sauces

NISHIYAMA Yoshitaka[*1], MAEDA Haruko[*2], NAGASHIMA Toshio[*3]
WATANABE Toshihiro[*3] and MURA Kiyoshi[*1]

*1 *Department of Nutritional Science, Faculty of Applied Bioscience, Tokyo University of Agriculture 1-1-1, Sakuragaoka, Setagaya-ku, Tokyo 156-8502*

*2 *Department of Applied Biology and Chemistry, Faculty of Applied Bioscience, Tokyo University of Agriculture 1-1-1, Sakuragaoka, Setagaya-ku, Tokyo 156-8502*

*3 *Department of Food Science and Technology, Faculty of Bioindustry, Tokyo University of Agriculture 196, Yasaka, Abashiri-shi, Hokkaido 099-2493*

Bacillus sp. 11-4 metalloprotease (protease I), which shows collagenase-like specificity, was purified from the culture supernatant of the strain. The N-terminal amino acid sequence of the protease I was determined up to 43 residues from its N-terminus. Further, five derivative peptides were obtained by V8 protease digestion and their amino acid sequences were analyzed. Of the fragments, three derivatives showed identical N-terminal sequence with the parent protease I. On the other hand, the remaining two derivatives showed sequences, which seemed to match the internal region of parent protease I. Adapting the partial amino acid sequence information to a BLAST database search, the protease I showed significant similarity with the neutral metalloproteases, which possess the zinc-binding motif HEXXH, produced by some clostridia, bacilli, streptococci and staphylococci. The listed metalloproteases commonly possessed the hidden Markov model domain for thermolysin family protease. The functional features of the protease I determined previously, such as molecular masses, isoelectric points and pH optimums, showed agreement with those of thermolysin family proteases. Consequently the partial amino acid sequence analysis suggests that the protease I is defined as a thermolysin-like metalloprotease.

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Fish sauce is a popular seasoning in Southeast Asia, typified by Nuoc mam in Vietnam, Nam pla in Thailand and Patis in the Philippines. In Japan, fish sauces are also used in some provinces, such as Shottsuru in Akita, Ishiru in Ishikawa and Ikanago in Kagawa prefecture. Because fish sauce has a distinctive flavor, it is rarely used in unprocessed form. But fish sauce came into widespread usage as a hidden flavor in snack foods, basting for beef broil and fish sausages, for their idiosyncratic flavor. The flavors of fish sauces might be enhanced due to amino acids and peptides derived from fish proteins[1]. In general, fish sauce is produced from raw fish by adding 15~20% (w/w) concentration of salts and then matured for a half to one year. In fish sauce production, it was thought that the degradation of proteins was due to fish-produced protease (s) in muscles or digestive tracts. On the other hand, some protease-productive bacteria are known to exist in fish sauce. CHAIYANAN *et al.*[2] isolated a novel halobacillus, which grew in a medium containing 10~20% salt, from a fish sauce production line. The strain produced three types of proteases with molecular masses of 100, 42 and 17 kDa. Of the proteases, the 100-and 17-kDa proteases were defined as serine protease, whereas the 42-kDa protease was identified as metalloprotease. The halobacillus has been successfully employed to improve fermentation in industrial production of fish sauce in Thailand.

Previously we isolated some protease-productive bacilli from fish sauces collected in factories and markets in Vietnam[3]. The strains grew in medium containing a high concentration of NaCl ranging from 0 to 2.5 M, indicating that the bacilli was able to grow in the Vietnamese fish sauce. The

*1 E-mail : NISHIYAMA Yoshitaka ; nishiyama@nodai.ac.jp, MURA Kiyoshi ; mura@nodai.ac.jp
*3 E-mail : NAGASHIMA Toshio ; t-nagash@bioindustry.nodai.ac.jp, WATANABE Toshihiro ; t-watana@bioindustry.nodai.ac.jp

major extracellular neutral protease (protease I) was purified from one of the bacilli, *Bacillus* sp. 11-4 isolated from Vietnamese fish sauce[4]. The molecular mass of the protease I was determined as 33.5 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Because the protease I required calcium ions and was inhibited by chelator as EDTA and 1, 10-phenantrolin, the protease I was defined as metalloprotease. Further, the protease I hydrolyzed native collagen, and specific substrates for collagenase such as FALGPA and z-Gly-Pro-Leu-Pro-H_2O · AcOEt, consequently the protease I seemed to be collagenase-like protease. However, some properties of the protease I differed from those of general collagenases. In this study, the purified protease I was applied to partial amino acid sequence analysis. The sequences exhibited significant similarity with the amino acid sequences of some metalloproteases belonging to the thermolysin family, but not with those of collagenases. To identify the protease purified from *Bacillus* sp. 11-4, the properties of protease I were compared with those of the metalloprotease obtained by a database homology search.

Materials and Methods

1. Strain

Bacillus sp. 11-4 was used. The strain was isolated from commercial fish sauces obtained from the market at Fue City in Vietnam as previously reported[3].

2. Purification of protease I

The protease I was purified as previously reported[4]. *Bacillus* sp. 11-4 was incubated in Luria-Bertani (LB) medium at 37℃ for 12 hrs with shaking. Four mℓ of the culture of the strain was inoculated into 400mℓ of LB medium, and then the medium was incubated at 37℃ for 18 hrs with shaking. The culture supernatant was collected by centrifugation (9,000 × g, 10min, 4℃) was concentrated by ultrafiltration using hollow fiber (with a molecular size of 10,000 Amicons) into a twentieth. The concentrate was fractionated by ammonium sulfate saturation at 90%. The precipitate was dissolved in 20 mM Tris-HCl (pH 7.2) containing 1 mM $CaCl_2$, dialyzed against the same buffer, and then applied to a CM-Sepharose CL-6B column equilibrated with the same buffer. The absorbed protease I was eluted by a linear gradient of NaCl ranging from 0 to 250 mM. Based on the protease assay described as the previous report[4], the fractions containing protease I were collected and concentrated into a minimum volume for the next step.

3. N-terminal and internal amino acid sequence analysis

The N-terminal amino acid sequences of the protease I and derivative peptides from the protein were determined with the direct protein sequencing method described by Hirano and Watanabe[5]. The protease I and derivative peptides were separated by SDS-PAGE using the method described by Laemmli[6]. The peptides on the gels were electroblotted onto polyvinylidine difluoride (PVDF) membrane using semidry blotting apparatus (Nippon Eido, Tokyo, Japan). The derivative peptides of the protease I were prepared by proteolytic digestion with *Staphylococcus aureus* V8 protease (Wako Pure Chemical, Osaka, Japan), during electrophoresis according to the methods described by Cleveland *et al.*[7] After the membrane was stained, the visible bands were cut out. The N-terminal amino acid sequences of the protease I and derivative peptides were determined using a pulsed liquid phase protein sequencer (Model 492, Applied Biosystems, Foster City, USA).

4. Database homology search

The N-terminal and internal amino acid sequences found were applied to a database homology search using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The Clustal W alignments[8] of the amino acid

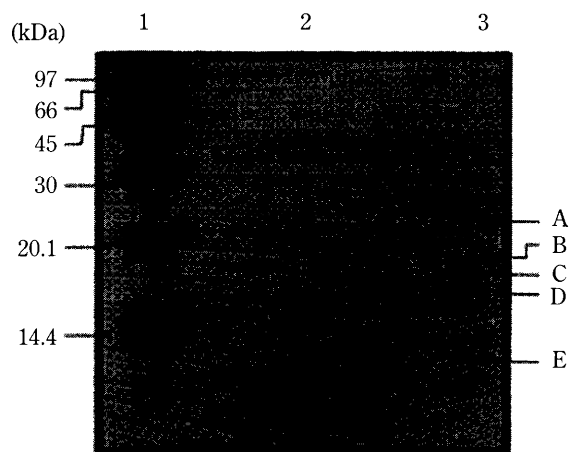

Fig. 1 SDS-PAGE banding profiles of purified *Bacillus* sp. 11-4 protease I and the V8 protease-derivative fragments

The proteolytic digestion of the protease I was performed in the stacking gels as described in Materials and Methods. Lane 1, molecular weight standard proteins ; 2, parent protease I ; 3, V8-protease digested fragments of the protease I. The fragment names are indicated in right hand of the gel.

sequences were created with MacVector nucleotide and protein analysis software. The hidden Markov model protein family database search was performed using Pfam[9] (http://www.sanger.ac.uk/Software/Pfam/).

Results and Discussion

The purified protease I demonstrated a peptide band with a molecular mass of approximately 33.5 kDa on SDS-PAGE as shown in Fig. 1. The peptide was electroblotted onto PVDF membrane and then applied to N-terminal amino acid sequence analysis. As shown in Table 1, the N-terminal amino acid sequence of parent protease I was determined as A-A-T-T-G-S-G-Y-G-V-L-D-D-Y-K-T-L-N-T-Y-S-S-N-G-T-Y-Y-L-Y-D-V-T-K-P-M-N-G-V-I-E-T-F-T. The database search for the determined sequence with the BLAST program enumerated *Clostridium perfringens* lambda toxin and some metalloproteases such as elastase, bacillolysin and aureolysin (Table 2). According to the sequence of listed

Table 1 N-terminal amino acid sequences of purified protease I and derived fragments obtained by V8 protease digestion developed by SDS-PAGE

Peptide band	N-terminal amino acid sequences determined
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43
Parent	A A T T G S G Y G V L D D Y K T L N T Y S S N G T Y Y L Y D V T K P M N G V I E T F T
	1 2 3 4 5 6 7 8 9 10
A	A A T T G S G Y G V
	1 2 3 4 5 6 7 8 9 10
B	A A T T G S G Y G V
	1 2 3 4 5 6 7 8 9 10
C	A A T T G S G Y G V
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
D	S F S D V F G Y F L D P G D Y L M G E D
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
E	H M N N Y V N T S S D N G G V H T N S G

The band names A through E are corresponded to Fig. 1.

Table 2 List of the proteins obtained by BLAST search using determined partial amino acid sequences

Sequence	Source	Protein	Accession No.
N-terminal	*Clostridium perfringens*	lambda toxin	D 45904
	Clostridium acetobutylicum	extracellular neutral metalloprotease, NPRE	NC 003030
	Staphylococcus epidermidis	extracellular elastase precursor (SEPP1)	P 43148
	Staphylococcus chromogenes	metalloprotease	AF 218055
	Bacillus megaterium	bacillolysin precursor (neutral protease)	Q 00891
	Bacillus megaterium	neutoral proteinase	I 40227
	Bacillus thuringiensis	neutoral protease A	L 77763
	Bacillus cereus	bacillolysin precursor (neutral protease)	P 05806
	Staphylococcus aureus	zinc metalloproteinase aureolysin precursor	P 81177
Fragment D	*Staphylococcus epidermidis*	extracellular elastase precursor (SEPP1)	P 43148
	Staphylococcus aureus	zinc metalloproteinase aureolysin precursor	P 81177
	Staphylococcus chromogenes	metalloprotease	AF 218055
	Listeria monocytogenes	bacillolysin-like proteinase	A 60280
	Bacillus amyloliquefaciens	bacillolysin precursor (neutral protease)	P 06832
	Bacillus subtilis	bacillolysin precursor (neutral protease)	P 06142
Fragment E	*Pectobacterium carotovorum*	extracellular protease (prt)	AAA 24858.1
	Clostridium acetobutylicum	extracellular neutral metalloprotease, NPRE	NC 003030
	Staphylococcus epidermidis	extracellular elastase precursor (SEPP1)	P 43148
	Serratia marcescens	extracellular protease	M 59854
	Staphylococcus aureus	zinc metalloproteinase aureolysin precursor	P 81177
	Streptomyces coelicolor	putative metalloprotease	AL 136534

metalloproteases, the molecular masses of the nascent translational products were estimated as 56 ~62 kDa. The N-terminal 25~30 residues of these proteases exhibited typical signal-peptide sequence. The products could primarily convert into pro-protease by eliminating the signal peptide, and finally mature into active metalloenzyme by additional excision of the pro-regions[10),11)]. Consequently, the molecular masses of the mature-form proteases were approximately 33~35 kDa, and the values coincided with the molecular mass of *Bacillus* sp. 11-4 protease I. As shown in Fig. 2, the N-terminal amino acid sequence of *Bacillus* sp. 11-4 protease I showed a good match with those of matured analogues. From these data, the protease I might be primary synthesized as a precursor having the enhanced peptide at their N-terminus.

To obtain more primary structure information, the protease I separated by SDS-PAGE was digested with V8 protease and then the derivative peptides were sequenced. As shown in Fig. 1, five derivatives were obtained. Of the five derivatives, the amino acid sequences of the fragment A, B and C were exactly identical to the N-terminal amino acid sequence of the parent protease I (Table 1), indicating that the fragments obtained were lacking their C-terminal regions. On the other hand, the sequences of fragment D and E were different from that of the N-terminus of the parent peptide. The sequences were significantly similar with those of some metalloproteases, likewise the N-terminal sequence of the parent peptide, based on the results of the BLAST searches (Table 2). Figure 2 shows multiple alignments of the amino acid sequences of *Clostridium perfringens* lambda toxin, *Staphylococcus epidermidis* elastase, *Bacillus subtilis* bacillolysin and *Staphylococcus aureus* aureolysin, with determined partial amino acid sequence of *Bacillus* sp. 11-4 protease I. The amino acid sequences of the homologous metalloproteases with the protease I were applied to the hidden Markov model protein family searches using Pfam[9)]. As a result, the N-terminal third part of the proteases matched with the consensus domain sequences for thermolysin metallopeptidase propeptide regions. The prediction agreed with the previous reports about signal-and pro-peptides in N-terminus of each protease[10),11)]. Further, the domain searches lined up the candidates for the centermost third part of the proteases as thermolysin metallopeptidase α-helical domain, and for the C-terminal third part as thermolysin metallopeptidase catalytic domain. More than half of the metalloproteases possessed the HEXXH motif, and the triad (two histidine and one glutamic acid residues) could be acting as catalytic residues. The motif HEXXH sequences were also observed in the C-terminal third part domain of listed proteins as shown in Fig. 2. This observation could indicate that the protease I also possessed a zinc-binding site in the molecule. Furthermore, the thermolysin family proteases contain four calcium-binding sites, which provide thermal stability for the enzyme[12),13)]. Previously Maeda *et al.*[4)] reported that protease I was inactivated with zinc-specific chelator 1, 10-phenantorolin, further that active protease I required calcium ions. These observations support the prediction that the protease I would belong to the thermolysin family. The bacillolysin is known as the thermolysin family protease produced by bacilli. The primary structure information of the bacillolysin is available in six strains of bacilli. Although the determined partial sequences of the protease I showed some similarity with these sequences (data not shown), the identical sequence was not obtained from these data. Thus the protease I may be defined as a relative to bacillolysin, but could not be identified as bacillolysin. According to the previous report[4)], the protease I hydrolyzed collagen and a specific substrate to collagenase. Evidence for collagen digestion was also found in some thermolysin family proteases. A member of the family, pseudolysin, exhibits broader specificity, acting on large molecules such as elastin, laminin, proteoglycan, immunoglobrin A and G, and collagen[14)]. The broad specificity of the pseudolysin is considered to be attributable to a wider active site cleft. Furthermore, the *Clostridium perfringens* lambda toxin, which had been found to be a member of the thermolysin family proteases, also degrades some large molecules such as fibronectin, fibrinogen, immunoglobrin A and collagen, but not elastin.

Recently, supplements of protease-containing viscera[15)] or proteases[16),17)] to accelerate the fermentation process have been attempted for mass-production of fish sauce. Lopetchrat and Park[18)] described that the effective enzymes in fermentation of fish sauce were heat stable and salt tolerant, based on the data about the characterization of the fermentation stage of fish sauce production using Pacific Whiting. Thus

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lambda   1 MKKILISLLTVAAIVTMNSSHIVSAIEEGRQMKSKTEIIQQISEDTN 47
elast    1 MKNFSKFALTSIAALTVASPLVNTEVDAKDKVS----ATQNIDAKVT 43
bacill   1 MGLGKKLSVRVAASFMSLSISLPGVQAAEGHQLKEN----QTNFLSKKPI 46
aureo    1 MRKFSRYAFTSMATVTLLSSLTPAALASDTNHKP---ATSDINFEIT 44

lambda  48 GKEEIFMDESDGVQIFIKGNLDLNTGVSKDTVLSYLENNRSLFNFKNNDL 97
elast   44 QESQATD----ALKELPK-SENIKKHYKDYKVTDTE-------------- 74
bacill  47 AQSELSAPNDKAVKQFLKKNSNIFKGDPSKSVKLVEST------------ 84
aureo   45 QKSDAVK----ALKELPK-SENVKNHYQDYSVTDVK------------- 75

lambda  98 NFRIDKYETDDLGFTHVKLKETYKGKDVYGREMTVHFDKSGEINSITGTL 147
elast   75 --------KDNKGFTHYTLQPKVGNTYAPDKEVKVHTNKEGKVVLVNGDT 116
bacill  85 --------TDALGYKHFRYAPVVNGVPIKDSQVIVHVDKSDNVYAVNGEL 126
aureo   76 --------TDKKGFTHYTLQPSVDGVHAPDKEVKVHADKSGKVVLINGDT 117

lambda 148 EDRIQSITKKNTQAISSSSAIEIAKSSK-SYDILSE------EPKAENYI 190
elast  117 DAKKVQPTNKVS-ISKESATDKAFEAIKIDRQKAKN---LKSDVIKTNKV 162
bacill 127 HNQSAAKTDNSQKVSSEKALALAFKAIGKSPDAVSNGAAKNSNKAELKAI 176
aureo  118 DAKKVKPTNKVT-LSKDEAADKAFNAVKIDKNKAKN---LQDDVIKENKV 163

                                             Mature form ↓
lambda 191 YFKDGKAYDVYRVNIVYDTPEFASWEIFVDIYSGDIIEEKSLMREFNTTG 240
elast  163 EIDGEKNKYVYNIEIITTSPKISHWNVKIDAETGQVVDKLNMIKEAATTG 212
bacill 177 ETKDGSYRLAYDVTIRYVEPEPANWEVLVDAETGSILKQQNKVEHAAATG 226
aureo  164 EIDGDSNKYIYNIELITVTPEISHWKVKIDADTGAVVEKTNLVKEAAATG 213

                                         N-Terminal     AATTG

lambda 241 SGIAVNGDLTNLNVYKYGNKYYLQDRTKDMSGYINTYTANHRYTD-NGSL 289
elast  213 TGKGVLGDTKQININSVSGGYALQDLTQQGTLSAYNYDANTGQA----YL 258
bacill 227 SGTTLKGATVPLNISYEGGKYVLRDLSKPTGTQIITYDLQNRQSRLPGTL 276
aureo  214 TGKGVLGDTKDININSIDGGFSLEDLTHQGKLSAYNFNDQTGQA----TL 259

           SGYGVLDDYKTLNTYSSNGTYYLYDVTKPMNGVIETGT

lambda 290 IYNYTQDINDP---AAVSAHSYAGVVYDFYKNIFNRNSIDDNGMSIDSVV 336
elast  259 MQDKDRNFDDDEQRAGVDANYYAKETYDYYKNTFGRESYDNQGSPIISLA 308
bacill 277 VSSTTKTFTSSSQRAAVDAHYNLGKVYDYFYSNFKRNSYDNKGSKIVSSV 326
aureo  260 ITNEDENFVKDDQRAGVDANYYAKQTYDYYKNTFGRESYDNHGSPIVSLT 309

lambda 337 HYG-----SNYNNAYWNGYKMIYGDGDNVNFTMLSGDLDVVGHEMTHGVV 381
elast  309 HVNNFQGGQDNRNNAAWIGDKMIYGDGDGRTFTALSGANDVVAHEITHGVT 358
bacill 327 HYG-----TQYNNAAWTGDQMIYGDGDGSFFSPLSGSLDVTAHEMTHGVT 371
aureo  310 HVNHYGGQDNRNNAAWIGDKMIYGDGDGRTFTNLSGANDVVAHELTHGVT 359
                                                  * * * * *

lambda 382 TNTCNLNYENQSGALNESISDVFGVLIQTYEKYDVKNGGDWIFNPSDWVI 431
elast  359 QQTANLVYRSQSGALNESFSDVFGYFVDD---------------EDFLM 392
bacill 372 QETANLIYENQPGALNESFSDVFGYFNDT---------------EDWDI 405
aureo  360 QETANLEYKDQSGALNESFSDVFGYFVDD---------------EDFLM 393

           Fragment D  SFSDVFGYFLDP----------------GDYLM

lambda 432 GDEIYTPGIKGDALRSLANPTLYDQPDHMKNYYNLPNTENGDYGGVHINS 481
elast  393 GEDVYTPGVGGDALRSMSNPERFGQP---SHMNDFVYTNS-DNGGVHTNS 438
bacill 406 GEDITVS---QPALRSLSNPTKYNQPDNYANYRNLPNTDEGDYGGVHTNS 452
aureo  394 GEDVYTPGKEGDALRSMSNPEQFGQP---SHMKDYVYTEK-DNGGVHTNS 439

           GED                Fragment E   HMNNYYVNTSS-DNGGVHTNS

lambda 482 GIPNKAAYNLASTLGCEKTARIYYRATTQYFNSTTSFVEARLGLVQAAKD 531
elast  439 GIPNKAAYNTIRSIGKQRSEQIYYRALTVYLTSNSDFQDAKASLQQAALD 488
bacill 453 GIPNKAAYNTITKLGVSKSQQIYYRALTTYLTPSSTFKDAKAALIQSARD 502
aureo  440 GIPNKAAYNVIQAIGKSKSEQIYYRALTEYLTSNSNFKDCKDALYQAAKD 489

           G

lambda 532 LYGNNSLEAEAVGNAFSNVGIN 553
elast  489 LYG--EGIAQQVGQAWDSVGV 507
bacill 503 LYG--STDAAKVEAAWNAVGL 521
aureo  490 LYD--EQTAEQVYEAWNEVGVE 509
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Fig. 2 Multiple alignment of various thermolysin family metalloproteases and determined partial amino acid sequences of the *Bacillus* sp.11-4 protease I

The white letters indicate the identical or near relative residues. The shaded residues in the partial sequence of the protease I indicate the residues exhibiting the match with at least one of the other proteases. Fragments D and E correspond to the names defined in Fig. 1. Fragments A, B and C are omitted to describe, because they shows identical sequences with the N-terminus of parent protease I. The positions, which were predicted as cleavage sites with signal peptidases, are indicated with arrowheads. The N-termini of the mature proteins are indicated by the arrow. lambda, *Clostridium perfringens* lambda toxin ; elast, *Staphylococcus epidermidis* elastase ; bacillo, *Bacillus subtilis* bacillolysin ; aureo, *Staphylococcus aureus* aureolysin.

the heat-stable thermolysin family proteases could play a beneficial role in the industrial production of fish sauce. To determine their structural and functional features, the precise primary structure for the *Bacillus* sp. 11-4 protease I should be clarified. Moreover, the coding gene for protease I has to be cloned for use in mass-production of fish sauce.

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ベトナム産魚醤より分離された*Bacillus* sp.11－4が産生するプロテアーゼＩの部分アミノ酸配列

西山由隆[*1]・前田治子[*2]・永島俊夫[*3]
渡部俊弘[*3]・村　清司[*1]
＊1　東京農業大学応用生物科学部栄養科学科
（〒156-8502　東京都世田谷区桜丘１－１－１）
＊2　東京農業大学応用生物科学部生物応用化学科
（〒156-8502　東京都世田谷区桜丘１－１－１）
＊3　東京農業大学生物産業学部食品科学科
（〒099-2493　北海道網走市八坂196）

Bacillus sp.11－4培養液中からコラゲナーゼ様の特異性を示す金属プロテアーゼ（プロテアーゼI）を精製した。精製プロテアーゼIは，N末端アミノ酸配列分析を行い，そのN末端から43残基までのアミノ酸配列を決定した。さらに，Ｖ8プロテアーゼ処理によって5つのペプチド断片を調製し，そのアミノ酸配列を分析した。得られた断片のうち3つはプロテアーゼＩのN末端アミノ酸配列と一致したが，残る2つの断片は内部領域と考えられる配列を示した。得られた部分アミノ酸配列をBLASTデータベース検索した結果，クロストリジウム属，バチルス属，ストレプトコッカス属およびスタフィロコッカス属が産生する中性金属プロテアーゼと相同性を示した。これらのプロテアーゼは亜鉛結合モチーフHEXXE配列を持ち，サーモリシンファミリープロテアーゼに共通の隠れマルコフモデルドメインを有していた。また，さきに報告されたプロテアーゼＩの特性はサーモリシンファミリープロテアーゼのそれらと類似していた。以上の結果から，プロテアーゼIは部分アミノ酸配列の結果を基にサーモリシン様プロテアーゼであると推定された。

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