CURRENT MICROBIOLOGY Vol. 30 (1995), pp. 201–209

Current Microbiology
An International Journal

The Structure of Subtilisin ALP I from Alkalophilic *Bacillus* sp. NKS-21

Youhei Yamagata,[1] Toshihiro Sato,[1] Satoshi Hanzawa,[2] Eiji Ichishima[1]

[1]Laboratory of Molecular Enzymology, Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1-1, Tsuysumidori-Amamiyamchi, Aoba-ku, Sendai 981, Japan
[2]Laboratory of Biotechnology, Tosoh Co., Hayakawa, Ayase 252, Japan

Abstract. The gene for an alkaline serine protease from alkalophilic *Bacillus* sp. NKS-21 (subtilisin ALP I) was cloned, and its nucleotide sequence was determined. The gene (*aprQ*) contained an open reading frame of 1125 bp, encoding a primary product of 374 amino acids. The mature protease, composed of 272 amino acids, was preceded by a putative signal sequence of 37 amino acids and a pro-sequence of 65 amino acids. The mature protease conserved the catalytic triad, Asp, His, and Ser, as subtilisin BPN′ or other subtilisins, and the subtilisin ALP I might belong to the subtilisin super family. The primary structure of subtilisin ALP I was compared and discussed with those of 13 subtilisins, 5 subtilisins from alkalophilic *Bacillus*, and 8 from neutrophiles. Low homology was shown between subtilisin ALP I and subtilisins from alkalophiles or subtilisins from neutrophiles. Forty-five amino acid residues of the mature protein of subtilisin ALP I were entirely independent of other subtilisins. According to the homology of ALP I with other subtilisins, subtilisin ALP I might be in the middle point between alkaline subtilisins and neutral ones.

An alkalophilic *Bacillus* sp. NKS-21 produces two serine proteases, subtilisin ALP I and subtilisin ALP II (they were designated as ALPase I and ALPase II previously) [19, 22]. Though these enzymes share certain similarities—the optimum pH of these enzymes is pH 10 when milk casein is used as a substrate, and their P_1 preference is Phe than Tyr with fluorogenic substrates—there are some differences between the two. The isoelectric points of subtilisin ALP I and ALP II are 8.2 and 2.8, respectively. In comparison with the activity against synthetic oligomeric substrates, ALP I shows less than 1/50 of the activity of ALP II. Subtilisin ALP I also has unique characteristics compared with other well-known subtilisins. Although ALP I is produced from alkalophilic *Bacillus*, which can not grow in a neutral pH region but is able to grow only in an alkaline environment, the substrate specificity of the enzyme against the oxidized B-chain of insulin is different from those of the enzymes from alkalophilic bacilli but similar to those of the subtilisins from neutrophilic ones [19]. The fluorogenic substrate succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosyl-4-methyl-coumaryl-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) is preferable to succinyl-L-alanyl-L-alanyl-L-prolyl- L-phenylalanyl-4-methyl-coumaryl-7-amide (Suc-Ala-Ala-Pro-Phe-MCA) for typical subtilisins from either neutrophilic bacilli or alkalophilic one, but subtilisin ALP I preferred the latter substrate over Suc-Leu-Leu-Val-Tyr-MCA [22].

We think these enzymatic properties of subtilisin ALP I must be caused by its peculiar structure and tried to obtain the structural gene for the enzyme and to determine the nucleotide sequence to clarify the structural originality. To obtain the information about the secondary structure we also measured the CD spectrum of subtilisin ALP I.

The nucleotide sequence data reported in this paper appear in the GSDB, DDBJ, EMBLE, and NCBI nucleotide sequence databases with accession number D29736.

Correspondence to: E. Ichishima

Materials and Methods

Materials. Lysylendopeptidase was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Restriction endonucleases were from Takara Shuzo Co. Ltd. (Kyoto, Japan). T4 DNA ligase was

Table 1. N-Terminal amino acid sequences of N-terminus and lysylendopeptidase-digested fragments of subtilisin ALP I

Fragments	Sequences	Sequence position
1	QTVPWGIPYIYSDVVHRQGYFGNGV	N-terminus
2	VAVLDTGVAPHPDLHIRGGVSFIS	28–52[a]
3	VLDRNGSGSHASIAQGIEWAMNNGM	95–119[a]
4	YASVMAVGAVDQNGNRANFSSYGSELEIMAP	171–201[a]
5	HPHLTAAQIRNRMNQTAIPLGNSTYYGNGL	238–267[a]

[a] Numbering of subtilisin BPN'.

from Gibco BRL (Gaithersburg, Maryland). Prep-A-Gene was from Bio-Rad Laboratories (Hercules, California). Sequenase was from United States Biochemical Corp. (Cleveland, Ohio). Taq Dye Primer Cycle Sequencing Kit was purchased from Applied Biosystems, Inc. (Foster City, California).

Bacterial strains and plasmids. Alkalophilic *Bacillus* sp. NKS-21 strain [19] was used for the enzyme and chromosomal DNA source. *Escherichia coli* DH5α (*supE*44, Δ*lac*U169 (ϕ80*lacZ*ΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1) was used for routine transformation. Plasmids pUC118 and pUC119 [20] were used for cloning into *E. coli* DH5α cells. Alkalophilic *Bacillus* sp. NKS-21 was grown in 1% casein, 1% meat extract, 1% polypeptone, and 1% Na_2CO_3 at pH 10 [19].

DNA techniques. The following procedures were carried out by standard methods described by Ausubel et al. [1]: preparation of plasmid DNA, restriction endonuclease digestion, agarose gel electrophoresis, DNA ligation, bacterial transformation, colony hybridization, and Southern blot hybridization. Chromosomal DNA was prepared from alkalophilic *Bacillus* sp. NKS-21 as described by Saito and Miura [12]. DNA sequencing was carried out by using Taq Dye Primer Cycle Sequencing Kit and 373A DNA sequencer and software version 1.30 (Applied Biosystem). Oligonucleotides were synthesized with Model 391 PCR-MATE EP DNA synthesizer (Applied Biosystems) and then purified by Oligo-Pak™ EX cartridge column (Millipore).

Amino acid sequence analysis. Subtilisin ALP I was purified as reported previously [19]. One milligram of the purified protease was applied to a reverse-phase column, TSK-gel TMS-250 (ϕ4.6 × 75 mm) equipped with a Hitachi model L-6200 delivery system for further purification. Chromatographic recording was performed at 220 nm with a Hitachi L-4000 UV detector. The peak was collected and pooled, and then CH_3CN was added to the fraction up to 50%. The enzyme was inactivated overnight at −20°C and then dried in vacuo. The dried preparation was used for determination of the amino acid sequences of the amino terminal region and lysylendopeptidase digests. Five hundred micrograms were dissolved with 50 μl of 100 mM Tris-HCl, pH 9.0, containing 1% SDS, and then the preparation was treated at 100°C for 5 min. Tenfold H_2O and 5 μg of lysylendopeptidase were added to it. The mixture was incubated at 30°C for 24 h. The reaction mixture was applied to a reverse-phase column, TSK-gel ODS-120™ (ϕ4.6 × 250 mm) equipped with a same Hitachi model L-6200 delivery system. The amino terminal region and the purified fragments of subtilisin ALP I were sequenced with an Applied Biosystems 377A protein sequencer by the method of Hewick et al. [4]. The phenylthiohydantoin (PTH)-amino acid derivatives were identified by the high performance liquid chromatography (HPLC) system attached to the sequencer.

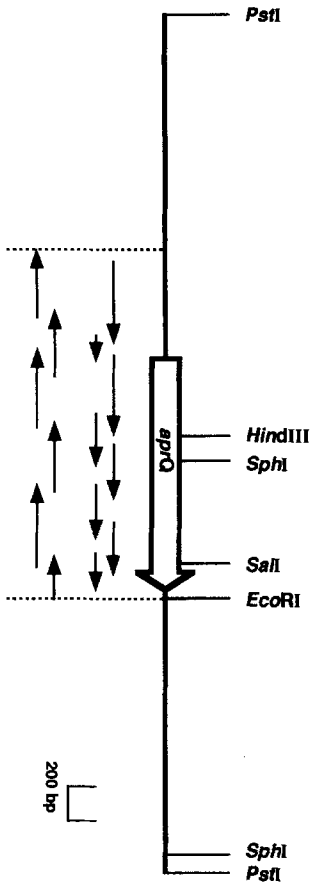


Fig. 1. Restriction map and sequence strategy for subtilisin ALP I gene. The arrows indicate the starting point and the direction of individual sequencing. The open arrow indicates the open reading frame and the direction of subtilisin ALP I coding gene (*aprQ*).

Circular dichroism (CD). CD measurements were done by the method described previously [24]. The contents of the α-helix and β-structure of the enzyme were calculated according to the SSE-338 program in [25].

Results

Amino acid sequence of subtilisin ALP I. N-Terminal sequence of subtilisin ALP I and four amino acid sequences of the peptide fragments digested with lysylendopeptidase were determined (Table 1). The N-terminal amino acid was Gln; it was different from other subtilisins.

Molecular cloning experiments to obtain the *aprQ* gene. A 56-mer oligonucleotide (5'-TGGGGIATIC-CITA(T/C)ATITA(T/C)I I I GA(T/C)GTIGTI-CA(T/C)IGICA(A/G)GGITA(T/C)TT(T/C)G-GIAA-3') was designed from an amino acid sequence (Trp[5]-Gly-Ile-Pro-Tyr-Ile[10]-Tyr-Ser-Asp-Val-Val-

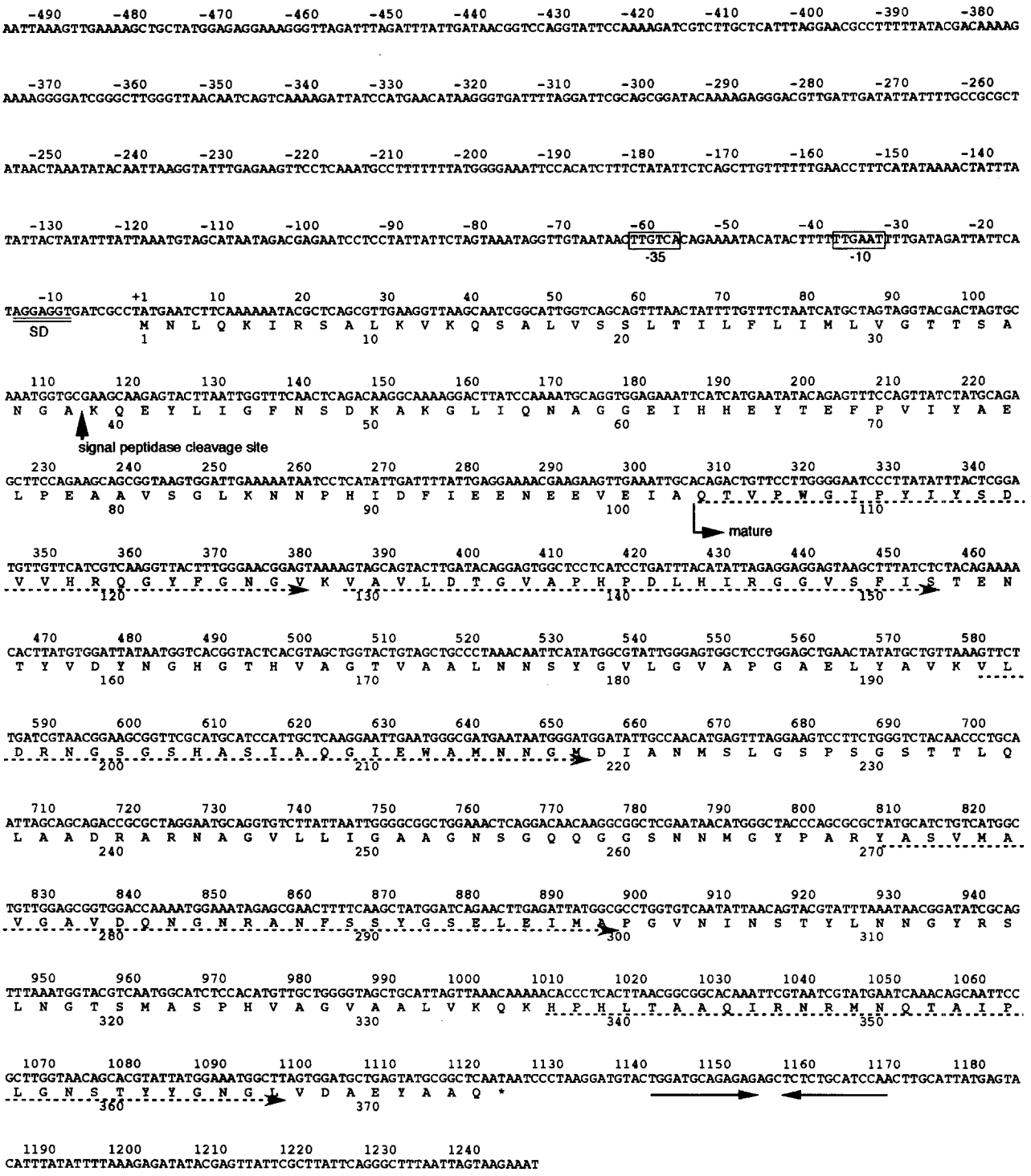


Fig. 2. Nucleotide sequence and deduced amino acid sequence of subtilisin ALP I. The putative ribosome binding site (SD sequence) is double-underlined. The putative promoters (−10 and −35 regions) are boxed. The putative transcriptional terminator is shown by inverted arrows. The asterisk indicates a stop codon. The N-terminal sequence of mature enzyme and amino acid sequences of the peptide-digested lysylendopeptidase are shown by dotted arrows.

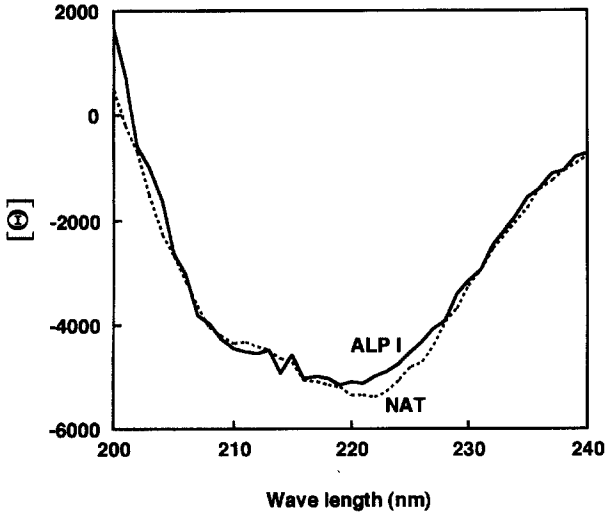


Fig. 3. CD spectra of subtilisin ALP I and NAT on the region 200–240 nm. Solid line (—), subtilisin ALP I; dotted line (···), subtilisin NAT.

His-Arg-Gln-Tyr-Phe[20]-Gly-Asn) that was determined from the amino terminal sequence of subtilisin ALP I (Table 1).

Chromosomal DNA of alkalophilic *Bacillus* sp. NKS-21 was digested to completion with some restriction enzymes. The reaction mixture was applied to agarose gel electrophoresis, and then the Southern blot analysis was performed with ^{32}P-labeled oligonucleotides. The probe hybridized to an approximately 5-kb *Pst*I and 4.5-kb *Hin*dIII. A fraction (5 kb) of genomic DNA from *Bacillus* sp. NKS-21 digested with *Pst*I was isolated from the electrophoresed agarose gel with Prep-A-Gene. It was ligated at *Pst*I site of plasmid vector pUC119. *Escherichia coli* DH5α was transformed with the ligated mixture and placed on Luria-Bertani agar plate containing 50 μg/ml of ampicillin sodium salt. After colony hybridization, 45 clones showing prominent reaction with the probe were found. Plasmid DNA was prepared from these clones, and all of the plasmids contained a 5-kb *Pst*I fragment.

Nucleotide sequence of the subtilisin ALP I gene (*aprQ*) and its deduced amino acid sequence. Sequence strategy and a partial restriction map of *Pst*I fragment are shown in Fig. 1. The DNA sequence of the subtilisin ALP I gene (*aprQ*) is shown in Fig. 2. The gene *aprQ* was shown to be a single copy on the genomic DNA of the alkalophilic *Bacillus* sp. NKS-21 with Southern blot analysis (data not shown). The DNA sequence of adjacent fragment revealed an open reading frame that began at position 1 and proceeded through position 1,122 (374 amino acid residues) (Fig. 2). A potential Shine-Dalgarno (SD) sequence (AGGAGGT) [14] was found 10 bp upstream from a probable translation start codon (ATG). Putative promoter sequences (−35 region, TTGTCA; −10 region, TTGAAT) [9] were also found 42 bp and 18 bp from the SD sequence, respectively. An inverted repeat was found at positions 1,130 to 1,188 downstream of the termination codon (TAA).

Table 2. Secondary structure contents (%) of subtilisin ALP I

Enzyme	α-Helix	β-Structure	Turns	Random
ALP I	11.8	67.1	0.8	20.4
NAT	20.3	12.2	33.8	33.8
Sendai[a]	27.9	0.7	39.4	31.9

[a] Data from ref. [23].

The contents of secondary structure in subtilisin ALP I. The contents of each secondary structure in subtilisin ALP I was a variant from other subtilisins. Figure 3 shows the CD spectra of subtilisin ALP I from alkalophile and subtilisin NAT from neutrophile. The α-helix and the β-structure contents of subtilisin ALP I were ca. 11.8% and 67.1% respectively. The α-helix and β-structure contents of subtilisin NAT were about 20.3% and 12.2% respectively. Table 2 shows a comparison of these contents between subtilisins ALP I and NAT. Subtilisin ALP I had much more β-structure than subtilisin NAT and less α-helix.

Discussion

In Fig. 2, the first 37 amino acids following the Met were estimated as a signal sequence by using the weight-matrix approach [3]. After the putative signal peptidase cleavage site, a propeptide region consisting of 65 amino acid residues was followed by the beginning of the mature subtilisin ALP I.

The amino acid starting the mature enzyme was confirmed by the N-terminal amino acid sequence as Gln. Almost all subtilisins were started with Ala. Subtilisins started with Gln were not known as well. Only an alkaline elastase Ya-B [7] was known. The entire mature protein was deduced to contain 272 amino acids with a predicted molecular weight of 28,253. The deduced amino acid sequence was coincident with those determined by amino acid sequencing of the amino terminal region and four peptide frag-

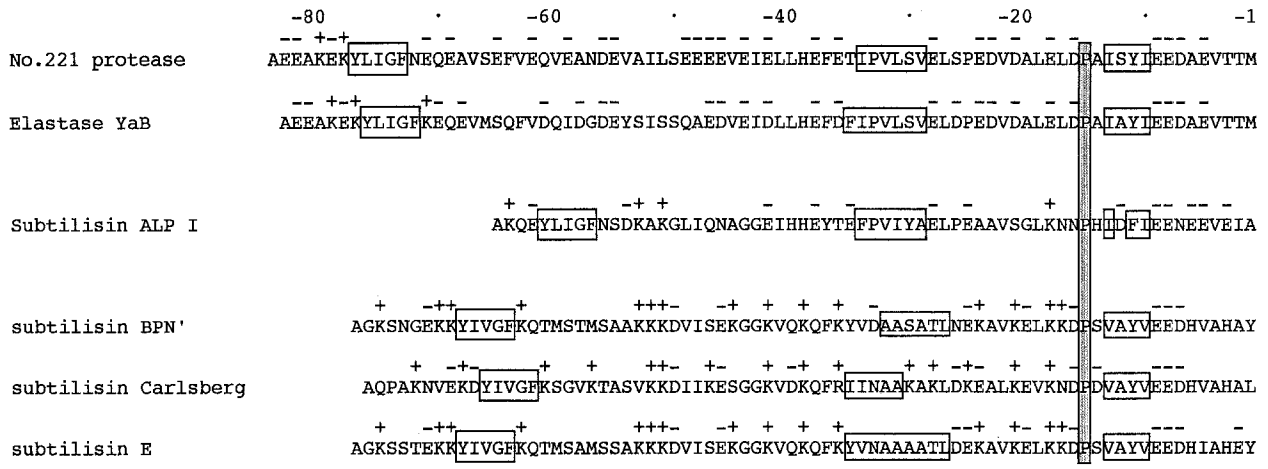


Fig. 4. Pro-sequence of subtilisin ALP I and other subtilisins. The open boxes (□) show hydrophobic clusters. The dark box (▩) shows a conserved Pro residue at position −15 from the cleavage site between the pro- and each mature enzyme.

ments obtained from digestion of the subtilisin ALP I with lysylendopeptidase.

There were some common structures in the propeptide region of subtilisins, but subtilisin ALP I showed some differences from them (Fig. 4). The pro-sequence of subtilisin ALP I was shorter than those of subtilisins from alkalophilic *Bacillus* (alkaline subtilisin) by about 20 amino acid residues or those of subtilisin from neutrophiles (neutral subtilisin) by about 12 residues. The first hydrophobic cluster, Tyr-Leu-Ile-Gly-Phe (YLIGF), in the pro-region of the enzyme was at position −60 from the cleavage site between the pro- and the mature enzyme. As a result, the distance between the first hydrophobic cluster and the second one, Phe-Pro-Val-Ile-Tyr-Ala (FPVIYA), was short, and there were only five charged amino acids in it. Subtilisins have many charged amino acids in their propeptide regions. The propeptide region of subtilisin was thought to be an intramolecular chaperone [15]. These charged amino acids were thought to interact with the α-helix of the mature region and to make folding subtilisin molecular [13]. Alkaline subtilisins have more than 20 negatively charged amino acid residues and a few positively charged ones. Their net charges were −26 of No. 221 protease [18] and −23 of alkaline elastase Ya-B [7]. On the other hand, neutral subtilisins, subtilisin BPN′ [21], Carlsberg [5], and E [16] have about 10 negatively charged amino acid residues and 15 positively charged ones. The net charge of the pro-region of subtilisin ALP I was −9. It was an intermediate value between those of alkaline subtilisins and neutral subtilisins. The third hydrophobic cluster, Ile-Asp-Phe-Ile (IDFI), of subtilisin ALP I was divided by a charged Asp residue. In spite of these differences, a Pro residue was conserved at position −15 in common, which might influence the secondary structure of the pro-sequence of subtilisins. The second hydrophobic cluster was also conserved around position −30 from the cleavage site. These two structural properties in the propeptide region might be important for the folding of the mature region of subtilisin ALP I.

The amino acid sequence of the mature subtilisin ALP I and other 13 subtilisins, 8 enzymes from neutrophilic bacilli (neutral subtilisins) and 5 from alkalophilic bacilli (alkaline subtilisins), are aligned in Fig. 5. The active site regions containing the catalytic triad composed of Asp^{32}, His^{64}, and Ser^{221} of subtilisin BPN′ [21] was well conserved in subtilisin ALP I as well as in other subtilisins. Only 18 amino acids of subtilisin ALP I were identical with all other alkaline subtilisins, and 13 amino acids of the enzyme were identical with all neutral subtilisins. Forty-five amino acids of the enzyme were different from all subtilisins.

The homologies between subtilisin ALP I and the other five alkaline subtilisins were about 60% (Fig. 6), but the other alkaline subtilisins were more homologous in any combination. Their homologies were more than 78% to each other. The homologies between ALP I and eight neutral subtilisins were 55% to 62%. The subtilisin family might be roughly divided into four subfamilies by their homologies. PB92 protease [8], No. 221 protease [18], Savinase [2], subtilisin Sendai [23], and alkaline elastase Ya-B [7] formed one group of alkaline subtilisins. Subtilisin Carlsberg [5] and DY [11] were in a same subfamily of

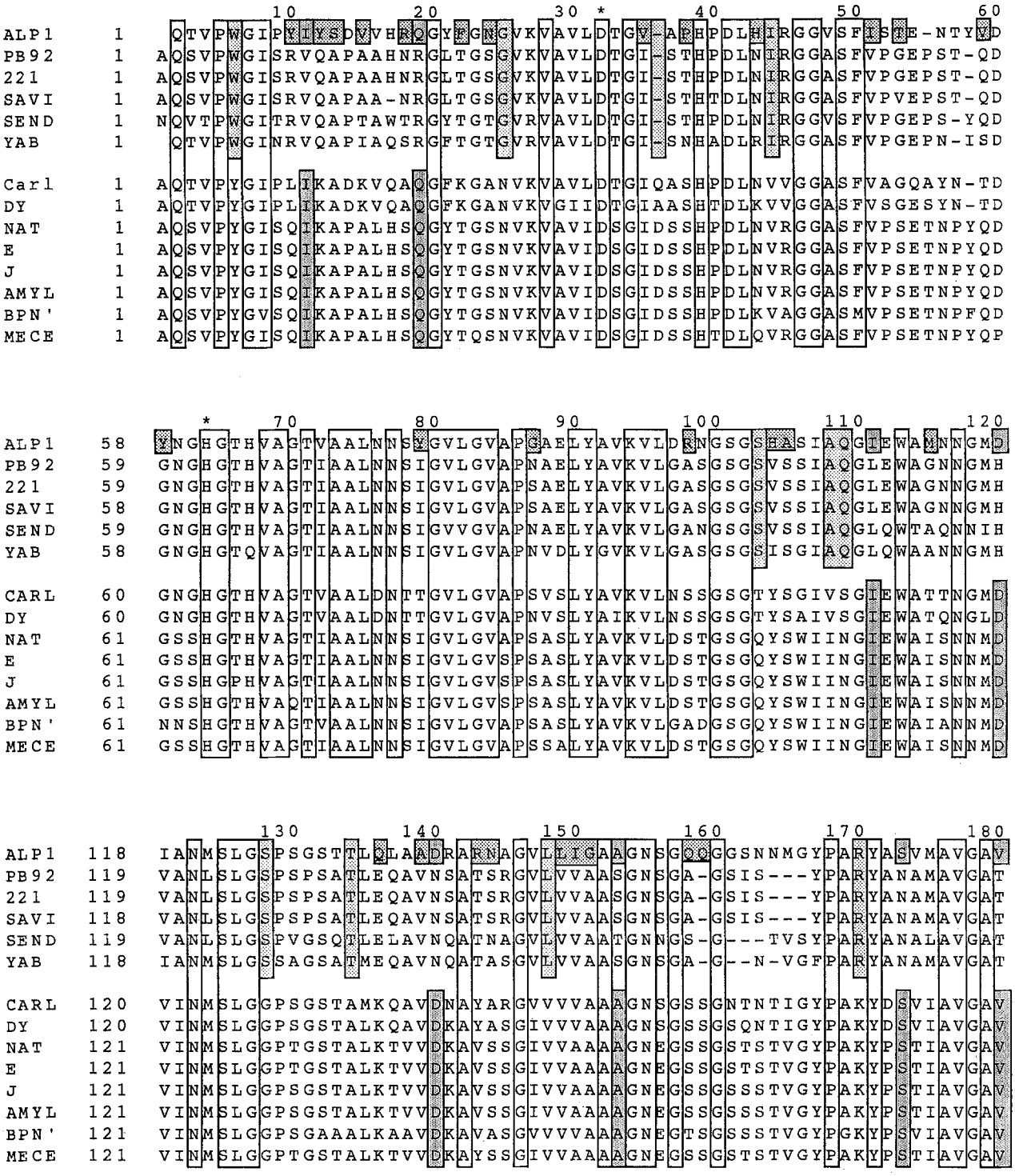
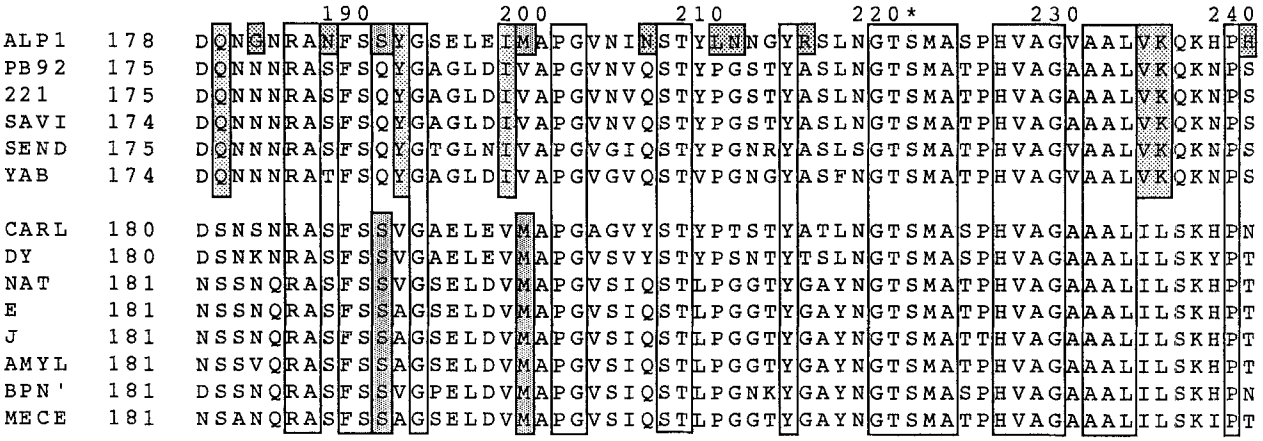


Fig. 5. Amino acid sequence of mature subtilisin ALP I and other subtilisins. The amino acid sequence enclosed in the open boxes (□) are common sequences among all subtilisins. The identical amino acids between subtilisin ALP I and all alkaline subtilisins are enclosed in the dark boxes (▩), and those between ALP I and neutral subtilisin are in darker boxes (▨). The amino acids enclosed in the darkest boxes (▩) are independent in subtilisin ALP I from all subtilisins. ALP I, subtilisin ALP I; PB92, serine protease from *Bacillus alcalophilus* PB92 [8]; 221, no. 221 protease from alkalophilic *Bacillus* sp. no. 221 [18]; SAVI, Savinase™ [2]; SEND, subtilisin Sendai from alkalophilic *Bacillus* sp. G-825-6 [23]; YAB, alkaline elastase Ya-B [7]; CARL, subtilisin Carlsberg [5]; DY, subtilisin DY [11]; NAT, subtilisin NAT [10]; E, subtilisin E [16]; J, subtilisin J [6]; AMYL, subtilisin amylosacchariticus [26]; BPN′, subtilisin BPN′ [21]; MECE, mecentericopeptidase [17].



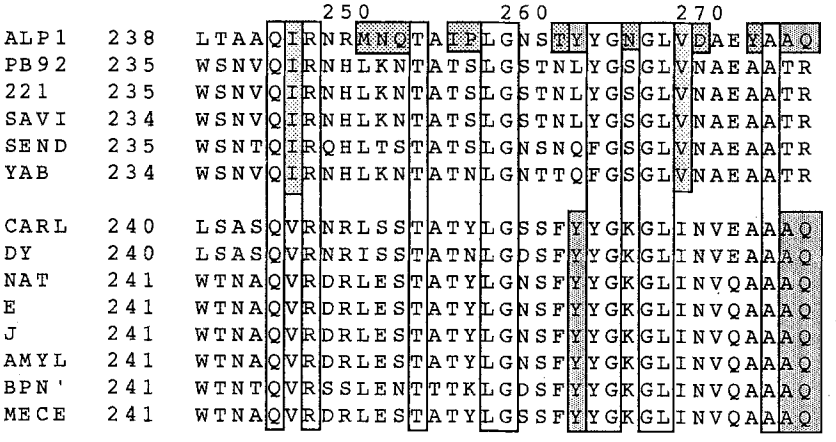


Fig. 5. Continued.

neutral subtilisins, and another subfamily of neutral ones contained subtilisin J [6], NAT [10], Amylosachariticus [26], E [16], BPN′ [21] and mecentericopeptidase [17]. The homologies of subtilisins belonging to each subclass were more than 80%. Subtilisin ALP I did not belong to any subfamily. It was thought to be in an independent subfamily between the alkaline group and the neutral one.

In comparison with the neutral subtilisins, the alkaline subtilisins had the deletion of amino acids in common (Fig. 5), so the total lengths of the mature enzymes of alkaline subtilisins were shorter than the neutral subtilisins by six or seven amino acids. The longest deletion was shown in the region around position 159 ~ 166. There was no deletion around position 159 ~ 166 in subtilisin ALP I. The amino acid sequence filled in this deletion did not resemble those of neutral subtilisins. The deleted amino acids around position 160 were thought to take part in the conformation of the P_1 pocket in subtilisin BPN′, and the deletion of these amino acids might reduce the structure of the P_1 pocket, and the P_1 preference of alkaline elastase Ya-B was for Ala [7].

In our study, subtilisin Sendai [23], which showed more than 80% homology with PB 92 protease [8] or No. 221 protease [18], also deleted four amino acids in this region, but subtilisin Sendai did not show the P_1 preference for the small amino acid residues Ala and Gly. It preferred bulky and hydrophobic amino acids such as Leu, Tyr, or Phe in the P_1 position of the substrate. These observations showed that deletion around position 160 does not determine the only P_1 preference for small amino acid residue. There was no deletion in this region of subtilisin ALP I from

					YAB	56.0	54.9	53.3	54.4	53.3	54.0	54.7	53.3
				SEND	78.7	53.1	52.4	55.8	56.6	55.8	56.2	55.5	54.4
			SAVI	81.0	91.7	59.1	56.6	58.5	59.33	58.5	58.9	58.5	58.5
		221	98.9	81.7	82.1	59.9	56.6	59.3	60.0	59.5	59.6	59.3	58.5
	PB92	99.6	98.5	82.1	82.5	59.5	56.9	58.9	59.6	58.9	59.3	58.9	58.2
ALP I	62.3	62.3	61.9	61.4	59.9	61.3	60.8	57.7	57.5	57.5	57.1	56.4	55.1
						CARL	88.8	68.4	69.8	68.7	69.1	68.4	68.3
							DY	69.5	69.8	60.8	69.1	69.1	69.1
								J	92.8	98.5	98.5	85.8	95.3
									NAT	97.8	99.3	86.5	96.0
										AMYL	98.5	85.8	94.5
											E	85.8	96.0
												BPN'	83.3
													MECE

Fig. 6. Diagram of the homologies between subtilisins. The numbers represent homologies (%) between subtilisins. The thick lines separate the subtilisin family into subfamilies by their homologies. ALP I, subtilisin ALP I; PB92, serine protease from *Bacillus alcalophilus* PB92 [8]; 221, no. 221 protease from alkalophilic *Bacillus* sp. no. 221 [18]; SAVI, Savinase® [2]; SEND, subtilisin Sendai from alkalophilic *Bacillus* sp. G-825-6 [23]; YAB, alkaline elastase Ya-B [7]; CARL, subtilisin Carlsberg [5]; DY, subtilisin DY [11]; NAT, subtilisin NAT [10]; E, subtilisin E [16]; J, subtilisin J [6]; AMYL, subtilisin amylosaccharitícus [26]; BPN', subtilisin BPN' [21]; MECE, mecentericopeptidase [17].

alkalophilic *Bacillus*. It attacked well the oligomeric synthetic substrates, of which there were Phe, Tyr, or Leu in their P_1 site, but the substrate specificity of subtilisin ALP I against the oxidized B-chain of insulin was different from those of alkaline subtilisins. It was similar to those of neutral subtilisins. No neutral subtilisin deleted the amino acid in these region. If this deletion determined the substrate specificity of the enzyme, it might not be concerned only with the P_1 preference, but also with the recognition of steric conformation.

It can be concluded that subtilisin ALP I has a unique primary structure. The enzyme might be classified as intermediate between the alkaline and neutral groups of subtilisin.

ACKNOWLEDGMENT

We thank Mr. Yamamoto, Laboratory of Marine Biotechnology Institute Co., Ltd., Shimizu, for analysis of CD spectra measurement.

Literature Cited

1. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) Current protocols in molecular biology, vol 1. New York, Chichester, Brisbane, Toronto, Singapore: Greene Associates and Wiley-Interscience
2. Betzel C, Klupsch S, Papendorf G, Hastrup S, Branner S, Wilson KS (1992) Crystal structure of the alkaline proteinase Savinase® from *Bacillus lentus* at 1.4 Å resolution. J Mol Biol 223:427–445
3. Heijne GV (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 14:4683–4690
4. Hewick RM, Hunkapiller MW, Hood LE, Dreyer WJ (1981) A gas-liquid solid phase peptide and protein sequenator. J Biol Chem 256:7990–7997
5. Jacobs M, Eliasson M, Uhlen M, Flock J-I (1985) Cloning, sequencing and expression of subtilisin Carlsberg from *Bacillus licheniformis*. Nucleic Acids Res 13:8913–8926
6. Jang JS, Kang DO, Chun MJ, Byun SM (1992) Molecular cloning of a subtilisin J gene from *Bacillus stearothermophilus* and its expression in *Bacillus subtilis*. Biochem Biophys Res Commun 184:277–282
7. Kaneko R, Koyama N, Tsai Y-C, Juang R-Y, Yoda K, Yamazaki M (1989) Molecular cloning of the structural gene for alkaline elastase YaB, a new subtilisin produced by alkalophilic *Bacillus* strain. J Bacteriol 171:5232–5236
8. von der Laan JC, Gerritse G, Mulleners LJSM, von der Hoek RAC, Quax WJ (1991) Cloning, characterization, and multiple chromosomal integration of a *Bacillus* alkaline protease gene. Appl Environ Microbiol 57:901–909
9. Moran Jr CP, Lang N, LeGrice SFJ, Lee G, Stephens M, Sonenshein AL, Pero J, Losick R (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol Gen Genet 186:339–346
10. Nakamura T, Yamagata Y, Ichishima E (1992) Nucleotide sequence of the subtilisin NAT gene, *aprN*, of *Bacillus subtilis* (*natto*). Biosci Biotechnol Biochem 56:1869–1871
11. Nedkov P, Obserthuer W, Braunitzer G (1985) Determination of the complete amino acid sequence of subtilisin DY and its comparison with the primary structure of the subtilisin BPN', Carlsberg and amylosaccharitícus. Biol Chem Hoppe-Seyler 366:421–430
12. Saito H, Miura K (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim Biophys Acta 72:619–629
13. Shinde U, Li Y, Chatterjee S, Inouye M (1993) Folding pathway mediated by an intramolecular chaperone. Proc Natl Acad Sci USA 90:6924–6928

14. Shine J, Dalgarno L (1974) The 3'-terminal sequence of *Escherichia coli* 16 S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc Natl Acad Sci USA 71:1342–1346
15. Silen JL, Agard DA (1989) The α-lytic protease pro-region does not require a physical linkage to activate the proteae domain *in vivo*. Nature 341:462–464
16. Stahl ML, Ferrari E (1984) Replacement of the *Bacillus subtilis* subtilisin structural gene with an *in vitro* derived deletion mutation. J Bacteriol 158:411–418
17. Svendsen I, Genov N, Idakieva K (1986) Complete amino acid sequence of alkaline mecentericopeptidase. FEBS Lett 196:228–232
18. Takami H, Kobayashi T, Kobayashi M, Yamamoto M, Nakamura S, Aono R, Horikoshi K (1992) Molecular cloning, nucleotide sequence, and expression of the structural gene for alkaline serine protease from alkaliphilic *Bacillus* sp. 221. Biosci Biotechnol Biochem 56:1455–1460
19. Tsuchida O, Yamagata Y, Ishizuka T, Arai T, Yamada J, Takeuchi M, Ichishima E (1986) An alkaline proteinase of an alkalophilic *Bacillus* sp. Curr Microbiol 14:7–12
20. Vieira J, Messing J (1987) Production of single-stranded plasmid DNA. In: Wu R, Grossman L (ed) Methods Enzymol 153:3–11
21. Wells JA, Ferrari E, Henner DJ, Estell DA, Chen EY (1983) Cloning, sequencing, and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. Nucleic Acids Res 11:7911–7925
22. Yamagata Y, Ichishima E (1989) A new alkaline proteinase with pI 2.8 from alkalophilic *Bacillus* sp. Curr Microbiol 19:259–264
23. Yamagata Y, Isshiki K, Ichishima E (1993) Subtilisin Sendai from alkalophilic *Bacillus* sp.: Molecular and enzymatic properties of the enzyme and molecular cloning and characterization of the gene, *aprS*. Enz Microbial Technol (in press)
24. Yamaguchi M, Hanzawa S, Hirano K, Yamagata Y, Ichishima E (1993) Specificity and molecular properties of penicillolysin, a metalloproteinase from *Penicillium citrinum*. Phytochemistry 33:1317–1321
25. Yang JT, Wu C-SC, Martinez HM (1986) Calculation of protein conformation from circular dichroism. Methods Enzymol 130:208–269
26. Yoshimoto T, Oyama H, Honda T, Tone H, Takeshita T, Kamiyama T, Tsuru D (1988) Cloning and expression of subtilisin amylosacchariticus gene. J Biochem 103:1060–1065