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

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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, subtilisins 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 wellknown 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

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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-Lleucyl-L-leucyl-L-valyl-L -tyrosyl-4-methyl-coumaryl-7amide (Suc-Leu-Leu-Val-Tyr-MCA) is preferable to succinyl-L-alanyl-L-alanyl-L-prolyl- L-phenylalanyl-4methyl-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.

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

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

| Frag- ments | Sequences | Sequence position | | |
|----------------|---------------------------------|----------------------|--|--|
| 1 | QTVPWGIPYIYSDVVHRQGYFGNGV | N-terminus | | |
| 2 | VAVLDTGVAPHPDLHIRGGVSFIS | 28-52ª | | |
| 3 | VLDRNGSGSHASIAQGIEWAMNNGM | 95-119 ^a | | |
| 4 | YASVMAVGAVDQNGNRANFSSYGSELEIMAP | 171-201ª | | |
| 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* DH5 α (*supE44*, Δ *lac*U169 (ϕ 80*lac*Z Δ M15), *hsd*R17, *rec*A1, *end*A1, *gyrA*96, *thi*-1, *rel*A1) was used for routine transformation. Plasmids pUC118 and pUC119 [20] were used for cloning into *E. coli* DH5 α cells. Alkalophilic *Bacillus* sp. NKS-21 was grown in 1% casein, 1% meat extract, 1% polypeptone, and 1% Na₂CO₃ 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 $(\phi 4.6 \times 75 \text{ mm})$ 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 CH₃CN 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[®] (\$4.6 × 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



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*).

performance liquid chromatography (HPLC) system attached to the sequencer.

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.

| -490 AATTAAAGTT | -480 Gaaaagctgct | -470 Atggagagga | -460 AAGGGTTAG | -450 ATTTAGATTT# | -440 ATTGATAACGO | -430 STCCAGGTAT | -420 TCCAAAAGATC | -410 GTCTTGCTC | -400 ATTTAGGAA | -390 CGCCTTTTTAT | -380 Acgacaaaag |
|----------------------------------|---------------------------------------|------------------------------|----------------------------|---------------------------------------|-----------------------------|------------------------------------|---------------------------------------|-----------------------------|----------------------------|--------------------------------------|------------------------------|
| -370 AAAAGGGGAT | – 360 CGGGCTTGGG1 | -350 TTAACAATCAG | -340 TCAAAAGAT | -330 TATCCATGAAC | - 320 ATAAGGGTG | -310 ATTTTAGGAT | -300 TCGCAGCGGAT | -290 ACAAAAGAGA | -280 SGACGTTGA | -270 FTGATATTATT | -260 TTGCCGCGCT |
| -250 Ataactaaat | -240 Atacaattaag | -230 GTATTTGAGA | -220 AGTTCCTCA | -210 AATGCCTTTTT | -200 TTTATGGGGA | -190 AATTCCACA1 | -180 CTTTCTATATI | -170 CTCAGCTTG | -160 ITTTTTGAA(| -150 CCTTTCATATA | -140 AAACTATTTA |
| -130 TATTACTATA | -120 TTTATTAAATO | -110 Stagcataata | -100 Gacgagaat | -90 CCTCCTATTAI | -80 TCTAGTAAA | -70 Faggttgtar | -60 ATAAQTTGTCAC -35 | -50 Agaaaataci | -40 ATACTTTTT | -30 IGAATITTGAT -10 | -20 Agattattca |
| -10 T <u>AGGAGGT</u> GF SD | +1 ATCGCCTATGAN M N 1 | 10 ATCTTCAAAAA L Q K | 20 ATACGCTCA I R S | 30 GCGTTGAAGG7 A L K V 10 | 40 TTAAGCAATCO K Q S | 50 GGCATTGGTC A L V | 60 CAGCAGTTTAAC S S L T 20 | 70 TATTTTGTT I L F | 80 ICTAATCATC L I M | 90 SCTAGTAGGTA L V G I 30 | 100 CGACTAGTGC T S A |
| 110 AAATGGTGCC N G A | 120 SAAGCAAGAGTI KQEY | 130 ACTTAATTGGT L I G | 140 TTCAACTCA F N S | 150 GACAAGGCAAJ D K A K | 160 AAGGACTTATY G L I | 170 CCAAAATGCA Q N A | 180 Aggtggagaaat g g e i | 190 TCATCATGAI H H E | 200 Atatacaga Y T E | 210 STTTCCAGTTA F P V I | 220 ATCTATGCAGA |
| 230 | 40 ignal peptidase 240 | cleavage site 250 | 260 | 50 270 | 280 | 290 | 60 300 | 310 | 320 TCCTTGGGG | 70 330 | 340 |
| L P E | A A V S 80 | G L X | N N P | H I D F 90 | I E E | N E E | V E I A 100 | | P W G | I P Y I 110 | Y S D |
| 350 TGTTGTTCA VVH | 360 ICGTCAAGGTTA R O G Y 120 | 370 ACTTTGGGAAC F G N | 380 GGAGTAAAA G V K | 390 GTAGCAGTAC V A V L 130 | 400 ITGATACAGG D T G | 410 AGTGGCTCCI V A P | 420 ICATCCTGATTI H P D L 140 | 430 CACATATTAG H I R | 440 AGGAGGAGTI G_G_V | 450 AAGCTTTATCI S F I 150 | 460 CTACAGAAAA T E N |
| 470 CACTTATGTO T Y V | 480 GGATTATAATGO DYNG 160 | 490 GTCACGGTACI H G T | 500 TCACGTAGCT H V A | 510 GGTACTGTAG G T V A 170 | 520 CTGCCCTAAA A L N | 530 CAATTCATAI NSY | 540 Iggcgtattggg g V L g 180 | 550 AGTGGCTCC V A P | 560 TGGAGCTGAJ G A E | 570 ACTATATGCTG L Y A V 190 | 580 TTAAAGTTCT / K V L |
| 590 TGATCGTAAC D R N | 600 CGGAAGCGGTTC G S G S 200 | 610 CGCATGCATCC H A S | 620 CATTGCTCAA | 630 GGAATTGAATC G_I_E_W 210 | 640 GGGCGATGAA A.M.N | 650 TAATGGGATC | 660 GGATATTGCCAF D I A N 220 | 670 Acatgagttti M S L | 680 AGGAAGTCC G S P | 690 ITCTGGGTCTA SGSI 230 | 700 ACAACCCTGCA TLQ |
| 710 ATTAGCAGCA L A A | 720 MGACCGCGCTAC D R A R | 730 Ggaatgcaggi N A G | 740 GTCTTATTA V L L | 750 ATTGGGGCGGG I G A A | 760 CTGGAAACTC G N S | 770 AGGACAACAA G Q Q | 780 AGGCGGCTCGAA G G S N | 790 ATAACATGGG4 N M G | 800 CTACCCAGCO Y P A | 810 GCGCTATGCAI R Y A S | 820 CTGTCATGGC V M A |
| 830 TGTTGGAGCO V G A | 840 BGTGGACCAAAJ | 850 ATGGAAATAGA G N R | 860 Agcgaacttt A N F | 250 870 TCAAGCTATG S.S.Y.G | 880 Gatcagaact S E L | 890 Igagattato E I M | 900 GCGCCTGGTGI | 910 CAATATTAA N I N | 920 CAGTACGTA S T Y | 270 930 Ittaaataacg L N N G | 940 Gatatcgcag 5 y r s |
| 950 TTTAAATGGI L N G | 960 960 TACGTCAATGGO T S M A | 970 CATCTCCACAI S P H | 980 GTTGCTGGG V A G | 990 GTAGCTGCAT V A A L | 1000 FAGTTAAACA V K Q | 1010 AAAACACCCT K <u>H P</u> | - 300 1020 FCACTTAACGGC | 1030 GGCACAAAT AQI | 1040 TCGTAATCG R N R | 1050 TATGAATCAAR | 1060 CAGCAATTCC |
| 1070 GCTTGGTAAC L G N | 1080 CAGCACGTATTI S T Y Y | 1090 Atggaaatggo g N g | 1100 TTTAGTGGAT | JJU 1110 GCTGAGTATGO A E Y A | 1120 CGGCTCAATA A Q * | 1130 ATCCCTAAGO | 1140 ATGTACTGGAT | 1150 GCAGAGAGA | 1160 GCTCTCTGC | 1170 1170 ATCCAACTTGC | 1180 CATTATGAGTA |

1190 1200 1210 1220 1230 1240 CATTTATATTTTAAAGAGATATACGAGTTATTCGCTTATTCAGGGGCTTTAATTAGTAAGAAAAT

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.

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

His-Arg-Gln-Tyr-Phe²⁰-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 ³²P-labeled oligonucleotides. The probe hybridized to an approximately 5-kb PstI and 4.5-kb HindIII. A fraction (5 kb) of genomic DNA from Bacillus sp. NKS-21 digested with PstI was isolated from the electrophoresed agarose gel with Prep-A-Gene. It was ligated at PstI site of plasmid vector pUC119. Escherichia coli DH5a 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 PstI fragment.

Nucleotide sequence of the subtilisin ALP I gene (aprQ) and its deduced amino acid sequence. Sequence strategy and a partial restriction map of *PstI* 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

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].

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).

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-

| | -80 . | -60 | • | -40 | • | -20 | • | -1 |
|----------------------|-------------------------|------------------------------------|---------------------|------------------------|--------------|------------------------|------------|-----------|
| No.221 protease | AEEAKEKYLIGFNEQE | AVSEFVEQVEANDE | VAILSEEEE | VEIELLHEFE | TPVLSVELSP | EDVDALELDP | AISYIEEDAE | VTTM |
| Elastase YaB | +-+ AEEAKEKYLIGFKEQI | EVMSQFVDQIDGDE | YSISSQAED | VEIDLLHEFT | FIPVLSVELDP | EDVDALELDP | ATAYIEEDAE | VTTM |
| Subtilisin ALP I | | + - AKQEYLIGFNS | -+ + SDKAKGLIQN | AGGEIHHEYTH | FPVIYAELPE | + AAVSGLKNNP | нфгратение | - VEIA |
| subtilisin BPN' | + _++ AGKSNGEKK | + YIVGF <mark>KQTMSTMS</mark> # | +++ AKKKDVISE | + + + + KGGKVQKQFKY | VDAASATI NE | + +- ++- KAVKELKKDP | SVAYVEEDHV | АНАҮ |
| subtilisin Carlsberg | + -· AQPAKNVE | ++ + KDYIVGFKSGVKT# | ++- +- SVKKDIIKE | + -+ + SGGKVDKQFR | + + -+ | - + + - EALKEVKNDP | DVAYVEEDHV | AHAL |
| subtilisin E | + _++ AGKSSTEKK | + YIVGEKQTMSAMSS | +++ SAKKKDVISE | + + + + KGGKVQKQFK | VVNAAAATI DE | + +- ++- KAVKELKKDP | SVAYVEEDHI | - AHEY |

Fig. 4. Pro-sequence of subtilisin ALP I and other subtilisins. The open boxes (\Box) show hydrophobic clusters. The dark box (\blacksquare) 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 proregion 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 -26of 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 Asp³², His⁶⁴, and Ser²²¹ of subtilisin BPN' [21] was well conserved in subtilisin ALP I as well as in other subtilisins. Only 18 amino acids of 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

| 30 | 1 |
|------------|---|
| 2 U | D |

| | | | 1.0 | 20 | 30 * | 4.0 | 50 60 |
|-------------|-----|----------------------|--------------------|----------------|--------------|-------------------------|------------------|
| 1 מ ז ג | 1 | | Thereby | BOOVECNOV | | | VSFRSRR_NTV |
| ADF1 | 1 | | | | X VA VI DECT | | |
| PB92 | 1 | | TORVQAPAA | | | | |
| 221 | 1 | AUSVPWG | TORVQAPAA | HNRGLITGSGV | | | ASTVFGEFSI-QD |
| SAVI | 1 | AQSVPWG | ISRVQAPAA | - NRGLTGSGV | | S ТНТО ЦИТКСС | ASEVPVEPST-QD |
| SEND | 1 | NQVTPWG | ITRVQAPTA | WTRGYTGTGV | RVAVLDTGI | STHPDLNIRGO | VSFVPGEPS-YQD |
| YAB | 1 | QTVPWG | INRVQAPIA | QSRGFTGTGV | RVAVLDTGI | SNHADLRIRGO | ASFVPGEPN-ISD |
| | | | tean. | | | | |
| Carl | 1 | AQTVPYG | IPLIKADKV | QAQGFKGANV | KVAVLDTGIQ | ASHPDLNVVGO | ASFVAGQAYN-TD |
| DY | 1 | AQTVPYG | IPLIKADKV | QAQGFKGANV | KVGIIDTGIA | ASHTDLKVVGG | ASFVSGESYN-TD |
| NAT | 1 | AQSVPYG | ISQIKAPAL | HSQGYTGSNV | RVAVIDSGID | SSHPDLNVRGO | ASFVPSETNPYQD |
| Е | 1 | AOSVPYG | ISOIKAPAL | HSOGYTGSNV | KVAVIDSGID | SSHPDLNVRGO | ASFVPSETNPYQD |
| 3 | 1 | AOSVPYG | ISOIKAPAL | HSOGYTGSNV | KVAVIDSGID | SSHPDLNVRGO | ASFVPSETNPYOD |
| ΔΜΥΤ. | 1 | AOSVPVG | TSOTKAPAT | HSOGYTGSNV | KVAVTDSGTD | SSHPDINVRGO | ASEVPSETNPYOD |
| DDN! | 1 | AOSVIDVO | VSOTKADAT. | HEGOVICENV | KVAVTDSCTD | SSHPDTKVAGO | ASMVPSETNPFOD |
| BPN NTOT | 1 | | | | | C CHIND TO VDC C | ACRUDER NDVOD |
| MECE | T | AGEARIAG | LIS ARK VLAT | HSQGITQSNV | VAN TAPOT D | 2 2 PL D P O V C C | ABT PBEINPIQP |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | * | | | 90 | | |
| ALP1 | 58 | У ОН СТН | VAGTVAALN | NSEGVLGVAP | GAELYAVKVL | drngsgs <u>ha</u> s i | AQGIEWAMNNGMD |
| рв92 | 59 | G N G H G T H | VAGTIAALN | NSIGVLGVAP | NAELYAVKVL | GASGSGSVSSJ | AQGLEWAGNNGMH |
| 221 | 59 | GNGHGTH | IVAGTIAALN | NSIGVLGVAP | SAELYAVKVL | GASGSGSVSSJ | AQGLEWAGNNGMH |
| SAVI | 58 | GNGHGTH | IVAGTIAALN | NSIGVLGVAP | SAELYAVKVL | GASGSGSVSSJ | AOGLEWAGNNGMH |
| SEND | 59 | GNGHGTH | IVAGTIAALN | NSIGVVGVAP | NAELYAVKVL | GANGSGSVSSJ | AOGLOWTAONNIH |
| VAR | 58 | GNGHGTC | | NSTOVIGVAP | NVDTVGVKVT | GASGSGSTSGI | AOGTOWAANNGMH |
| IND | 50 | | | | | | |
| CADT | 60 | CNCHCMP | | NINIT CVT CVAD | GVGT VAURT | Neecechveci | VS CHEWA THAC MO |
| CARD | 00 | | | | | | |
| DY | 60 | GNGHGIH | | NTTGVLGVAP | | NSSGSGTISAI | |
| NAT | 61 | GSSHGTH | VAGTIAALN | NSIGVLGVAP | SASLYAVKVL | DSTGSGQYSWJ | LINGLEWAISNNMD |
| E | 61 | ссснотн | IVAGTIAALN | NGIGVLGVSP | SASLYAVKVL | DSTGSGQYSWJ | INGTEWAISNNMD |
| J | 61 | GSSHGPH | VAGTIAALN | NSIGVLGVSP | SASLYAVKVL | DSTGSGQYSWJ | INGLEWAISNNMD |
| AMYL | 61 | GSSHGTH | IVADTIAALN | NSIGVLGVSP | SASLYAVKVL | DSTGSGQYSWI | INGIEWAISNNMD |
| BPN' | 61 | NNSHGTH | IVAGTVAALN | NSIGVLGVAP | SASLYAVKVL | GADGSGQYSWI | IINGIEWAIANNMD |
| MECE | 61 | GSSHGTH | VAGTIAALN | NSIGVLGVAP | SSALYAVKVL | DSTGSGQYSWI | INGIEWAISNNMD |
| | | k | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | 130 | 140 | 150 | 160 | 170 180 |
| מד. דס 1 | 118 | TANKSTO | le s c s m m h . B | | WTTTGAAGNS | GOOGGSNNMG | |
| | 110 | TANT ET C | | OAVNEAUSPC | | CA CETE | |
| PB92 | 119 | VANUSIC | | QAVNSATSRG | | GA-GSISS | IPARIANAMA VGAT |
| 221 | 119 | VAMUSLO | SPSPSPSATLE | QAVNSATSRG | | GA-GSISI | PARIANAMA VGAT |
| SAVI | 118 | VANLSLO | SPSPSPSATLE | QAVNSATSRG | | GA-GSIS) | |
| SEND | 119 | VANLELO | SPVGSQTLE | LAVNQATNAG | VLVVAATGNN | GS - G TVSY | PARYANALAVGAT |
| YAB | 118 | IANMSLO | SSAGSATME | QAVNQATASG | VLVVAASGNS | GA - G - - N - V G I | PARYANAMAVGAT |
| | | | | 1000 | | | |
| CARL | 120 | лімырго | GPSGSTAMK | QAVDNAYARG | VVVAAAGNS | GSSGNTNTIGS | PAKYDSVIAVGAV |
| DY | 120 | VINMBLO | GPSGSTALK | QAVDKAYASG | IVVVAAAGNS | GSSGSQNTIGY | PARYDSVIAVGAV |
| NAT | 121 | VINMSLO | GPTGSTALK | TVVDKAVSSG | IVVAAAAGNE | GSSGSTSTVG | PAKYPSTIAVGAV |
| Е | 121 | VINMBLO | GPTGSTALK | TVVDKAVSSG | IVVAAAAGNE | GSSGSTSTVG | PARYPSTIAVGAV |
| J | 121 | VINMBIC | GPSGSTALK | TVVDKAVSSG | IVVAAAAGNE | GSSGSSSTVG | PAKYPSTIAVGAN |
| AMYT. | 121 | VTNMSTO | GPSGSTALK | TVVDKAVSSG | TVVAAAAGNE | GSSGSSSTVG | PAKYPSTTAVGAN |
| יזרניני | 121 | VTNMET | GDGGAAATV | AAVINKAVACO | VVVVAAAACME | CT SCS S S T V C Y | DGKVDSWTAVCAW |
| DEN Nege | 101 | | | | | | |
| MECE | ΤΖΤ | <u>атымыто</u> | JGPTGSTALK | TVVDKAYSSG | TVVAAAAAGNE | ызысытытке | (FWVLTBLTTWAGWA |

Fig. 5. Amino acid sequence of mature subtilisin ALP I and other subtilisins. The amino acid sequence enclosed in the open boxes (\Box) are common sequences among all subtilisins. The identical amino acids between subtilisin ALP I and all alkaline subtilisins are enclosed in the dark boxes (\blacksquare), and those between ALP I and neutral subtilisin are in darker boxes (\blacksquare). The amino acids enclosed in the darkest boxes (\blacksquare) 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].

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| | | | | | | | • • • | |
|--------|-----|---------|----------|-----------|-----------------------|--------------|------------|---------|
| | | | 190 | 200 | 210 | 220* | 230 | 240 |
| ALP1 | 178 | DONGNRA | NFSSYGSI | ELEIMAPG | 7 N I N S T Y L N N G | YRSLNGTSMAS | PHVAGVAALV | 7КQКНРН |
| PB 9 2 | 175 | DONNNRA | SFSQYGA | GLDIVAPG | NVQSTYPGST | YASLNGTSMAT | PHVAGAAALV | KQKNPS |
| 221 | 175 | DONNNRA | SFSQYGA | GLDIVAPG | NVQSTYPGST | YASLNGTSMAT | PHVAGAAAL | KQKNPS |
| SAVI | 174 | DONNNRA | SFSQYGA | GLDIVAPG | 7 N V Q S T Y P G S T | YASLNGTSMAT | PHVAGAAAL | KQKNPS |
| SEND | 175 | DONNNRA | SFSQYGT | GLNIVAPG | / G I Q S T Y P G N R | YASLSGTSMAT | PHVAGVAAL | KQKNPS |
| YAB | 174 | DONNNRA | TFSQYGA | GLDIVAPG | 7 G V Q S T V P G N G | YASFNGTSMAT | PHVAGVAALV | KQKNPS |
| | | 16665 | | | | | | |
| CARL | 180 | DSNSNRA | SFSSVGAI | ELEVMAPGZ | AGVYSTYPTST | YATLNGTSMAS | PHVAGAAALI | LSKHPN |
| DY | 180 | DSNKNRA | SFSSVGAI | ELEVMAPG | /SVYSTYPSNT | YTSLNGTSMAS | PHVAGAAALI | LSKYPT |
| NAT | 181 | NSSNQRA | SFSSVGSI | ELDVMAPG | /SIQSTLPGGT | YGAYNGTSMAT | PHVAGAAALI | LSKHPT |
| E | 181 | NSSNQRA | SFSSAGS | ELDVMAPG | /SIQSTLPGGT | YGAYNGTSMAT | PHVAGAAALJ | LSKHPT |
| J | 181 | NSSNQRA | SFSSAGSI | ELDVMAPG | /SIQSTLPGGT | YGAYNGTSMAT | THVAGAAALI | LSKHPT |
| AMYL | 181 | NSSVQRA | SFSSAGSI | ELDVMAPG | 7 SIQSTLPGGT | YGAYNGTSMAT: | PHVAGAAALI | LSKHPT |
| BPN' | 181 | DSSNQRA | SFSSVGPI | ELDVMAPG | 7 SIQSTLPGNK | YGAYNGTSMAS | PHVAGAAALI | LSKHPN |
| MECE | 181 | NSANQRA | SFSSAGS | ELDVMAPG | /SIQ <u>ST</u> LPGGT | YGAYNGTSMAT | PHVAGAAALI | LSKIPT |

| | | | 250 | 260 | 270 |
|--------|-----|----------|--------|----------------|--------------|
| ALP1 | 238 | LTAAQIR | NRMNQT | AIPLGNSTYY | GNGLVDAEYAAQ |
| PB 9 2 | 235 | WSNVQIR | NHLKNT | ATSLGSTNLY | GSGLVNAEAATR |
| 221 | 235 | WSNVQIR | NHLKNT | ATSLGSTNLY | GSGLVNAEAATR |
| SAVI | 234 | WSNVQIRI | NHLKNT | ATSLGSTNLY | GSGLVNAEAATR |
| SEND | 235 | WSNTQIR | QHLTST | ATSLGNSNQF | GSGLVNAEAATR |
| YAB | 234 | WSNVQIRI | NHLKNT | ATNLGNTTQF | GSGLVNAEAATR |
| | | | | | |
| CARL | 240 | LSASQVR | NRLSST | ATYLGSSFYY | GKGLINVEAAAQ |
| DY | 240 | LSASQVRI | NRISST | ATNLGDSFYY | GKGLINVEAAAQ |
| NAT | 241 | WTNAQVRI | DRLEST | ATYLGNSFYY | GKGLINVQAAAQ |
| Е | 241 | WTNAQVRI | DRLEST | ATYLGNSFYY | GKGLINVQAAAQ |
| J | 241 | WTNAQVRI | DRLEST | ATYLGNSFYY | GKGLINVQAAAQ |
| AMYL | 241 | WTNAQVRI | DRLEST | ATYLGNSFYY | GKGLINVQAAAQ |
| BPN ' | 241 | WINTQUR | SSLENT | TTKLGDSFYY | GKGLINVQAAAQ |
| MECE | 241 | WTNAOVRI | DRLEST | ATT VIL CRSEVV | CKGTTWVOAAA |

Fig. 5. Continued.

neutral subtilisins, and another subfamily of neutral ones contained subtilisin J [6], NAT [10], Amylosacchariticus [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 $159 \sim 166$. There was no deletion around position $159 \sim 166$ 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 P₁ pocket in subtilisin BPN', and the deletion of these amino acids might reduce the structure of the P₁ pocket, and the P₁ 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 |
| 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 |
| | | | | | CAF | RL 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 |
| | | | | | | | <u>.</u> | 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 |
| | P B92 62.3 | 221 PB92 99.6 62.3 62.3 | 221 98.9 PB92 99.6 98.5 62.3 62.3 61.9 | SEND SAVI 81.0 221 98.9 81.7 PB92 99.6 98.5 82.1 62.3 62.3 61.9 61.4 | YAB SEND 78.7 SAVI 81.0 91.7 221 98.9 81.7 82.1 PB92 99.6 98.5 82.1 82.5 62.3 62.3 61.9 61.4 59.9 | YAB 56.0 SEND 78.7 59.1 221 98.9 81.0 91.7 59.9 PB92 99.6 98.5 82.1 82.5 59.5 62.3 62.3 61.9 61.4 59.9 61.3 | YAB 56.0 54.9 SEND 78.7 53.1 52.4 SAVI 81.0 91.7 59.1 56.6 221 98.9 81.7 82.1 59.9 56.6 PB92 99.6 98.5 82.1 82.5 59.5 56.9 62.3 62.3 61.9 61.4 59.9 61.3 60.8 CARL 98.8 DY | YAB 56.0 54.9 53.3 SEND 78.7 53.1 52.4 55.8 221 98.9 81.0 91.7 59.1 56.6 59.3 PB92 99.6 98.5 82.1 82.5 59.9 56.6 59.3 62.3 62.3 61.9 61.4 59.9 61.3 60.8 57.7 CARL 88.8 68.4 DY 69.5 J | YAB 56.0 54.9 53.3 54.4 SEND 78.7 53.1 52.4 55.8 56.6 SAVI 81.0 91.7 59.9 56.6 59.3 60.0 PB92 99.6 98.5 82.1 82.5 59.5 56.9 58.9 59.6 62.3 62.3 61.9 61.4 59.9 61.3 60.8 57.7 57.5 CARL 88.8 68.4 69.8 DY 69.5 69.8 J 92.8 J 92.8 J 92.8 J 92.8 | YAB 56.0 54.9 53.3 54.4 53.3 SAVI 81.0 91.7 59.1 56.6 58.5 59.33 58.5 PB92 99.6 98.5 82.1 82.5 59.5 56.9 58.9 59.5 62.3 62.3 61.9 61.4 59.9 61.3 60.8 57.7 57.5 57.5 CARL 88.8 68.4 69.8 68.7 DY 69.5 69.8 60.8 NAT 97.8 | YAB SEND 78.7 SAVI 81.0 91.7 221 98.9 81.7 82.1 99.6 98.5 82.1 82.5 62.3 62.3 61.9 61.4 59.9 62.3 62.3 61.9 61.4 59.9 62.3 62.3 61.9 61.4 59.9 62.3 62.3 61.9 61.4 59.9 62.3 62.3 61.9 61.4 59.9 62.3 62.3 61.9 61.4 59.9 61.3 60.8 57.7 57.5 57.5 59.9 59.6 59.8 59.9 59.6 91.7 59.9 50.8 59.9 59.6 92.8 93.6 59.7 57.5 57.1 92.8 93.5 93.5 93.5 94.5 92.8 93.5 93.5 94.5 94.5 92.8 93.5 94.5 94.5 14.4< | YAB 56.0 54.9 53.3 54.4 63.3 54.0 54.7 SEND 78.7 53.1 52.4 55.8 56.6 55.8 56.2 55.5 59.1 56.6 58.5 59.33 58.5 58.9 58.5 PB92 99.6 98.5 82.1 82.5 59.5 56.6 59.3 60.0 59.5 59.3 58.9 58.4 59.3 58.9 58.4 59.3 58.9 58.4 59.3 58.4 59.3 58.4 |

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⁽¹⁾ [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].

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.

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