Complete Amino Acid Sequence of Neutral Protease from *Bacillus subtilis* var. *amylosacchariticus*

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The neutral protease of *Bacillus subtilis* var. *amylosacchariticus* was cleaved chemically or digested with proteolytic enzymes, and the resultant peptides were separated and purified by high performance liquid chromatography. The sequence analyses of these peptides by the manual Edman procedure established the complete amino acid sequence of the enzyme. The neutral protease consisted of 300 amino acid residues with Ala and Leu as its amino- and carboxyl-termini, respectively, and the molecular weight was calculated to be 32,633. The sequence was found to be identical to that of *B. subtilis* 1A72 neutral protease, which was deduced from nucleotide sequencing. Comparison of the sequence with those of other *Bacillus* proteases revealed that the putative active site amino acid residues, Zn-binding ligands, and two Ca-binding sites were well conserved among them, as compared with those of thermolysin.

Several species of the genus *Bacillus* produce neutral and alkaline proteases (subtilisins) together with α -amylases, and their enzymatic properties have been reported.¹⁾ Among them, comprehensive studies have been done on protein chemical structures of subtilisins^{2~4)} and thermolysin, a thermostable neutral Zn-metallo protease.^{5~7)}

Bacillus subtilis var. *amylosacchariticus* (*B. amylosacchariticus*) was isolated from soil by Fukumoto *et al.*⁸⁾ as a producer of a saccharifying type α -amylase which was distinguishable from the liquefying type α -amylase produced by *Bacillus subtilis amyloliquefaciens* (*B. amyloliquefaciens*).⁹⁾ The former strain produces neutral protease (BANP) and subtilisin Amylosacchariticus (subtilisin AM) besides saccharifying type α -amylase. Some enzymatic and physicochemical properties of these two proteolytic enzymes have been characterized by Tsuru *et al.*^{1, 10~13)} and the amino acid sequence of subtilisin AM was determined from the protein sequencing by Kurihara *et al.*¹⁴⁾ and from nucleotide sequencing of the

gene by Yoshimoto *et al.*¹⁵⁾ On the other hand, *B. subtilis* neutral protease was characterized as a Zn-metalloenzyme with high specific activity,^{16, 17)} but the protein chemical properties of this enzyme remained to be clarified. Thus, we attempted to establish the amino acid sequence of the enzyme by protein sequencing. In this paper, we report the complete amino acid sequence of the neutral protease from *B. subtilis* var. *amylosacchariticus* and discuss its active site structure in comparison with that of thermolysin.

Materials and Methods

Materials. Cyanogen bromide (CNBr), trifluoroacetic acid (TFA), and other sequencing grade chemicals were obtained from Nakarai Chemicals, Kyoto. Lysyl endopeptidase from *Achromobacter lyticus* and phenylthiohydantoin (PTH) amino acids were from Wako Pure Chemicals, Osaka. *Staphylococcus aureus* V8 protease and endoproteinase Asp-N from a *Pseudomonas fragi* mutant were from Miles Laboratories, U.S.A. and Boehringer Mannheim, Germany, respectively. TPCK-treated trypsin, α -chymotrypsin, and thermolysin were from Sigma, U.S.A. Phenylisothiocyanate was from Pierce, U.S.A.

Abbreviations: BANP, neutral protease from *Bacillus subtilis* var. *amylosacchariticus*; HPLC, high performance liquid chromatography; subtilisin AM, subtilisin Amylosacchariticus; TFA, trifluoroacetic acid; TPCK, *N*²-tosyl phenylalanyl chloromethyl ketone; PTH-, phenylthiohydantoin; PTC, phenylthiocarbamyl.

HPLC grade acetonitrile and 2-propanol were from Kishida Chemicals, Osaka.

Purification of *Bacillus subtilis amylosacchariticus neutral protease*. The neutral protease of *Bacillus subtilis amylosacchariticus* (BANP) was purified by the method of Tsuru *et al.*¹⁰ with some modifications. Forty grams of crude powder of the enzyme (Uyeda Chemical Industries Co.) were suspended in 200 ml of 10 mM Tris-maleate buffer, pH 6.4, containing 5 mM Ca acetate, and the filtrate was put on a column (3 × 40 cm) of CM-Sephadex equilibrated with the same buffer. After the column was washed with 300 ml of the buffer, the enzyme was eluted with an increasing linear gradient of NaCl concentration (0 to 0.6 M). The active fractions eluted at 0.2 M NaCl were combined and concentrated by ultrafiltration, and then dialyzed against 10 mM Tris-HCl buffer, pH 8.0, with 2 mM Ca acetate. The dialyzate, 100 ml, was put onto a column (3 × 40 cm) of DEAE-Sephadex equilibrated with the same buffer, and the enzyme adsorbed was eluted with an increasing linear gradient of NaCl concentration.

Active fractions were combined, concentrated, and dialyzed against 5 mM Tris-HCl buffer, pH 7.2, containing 2 mM Ca acetate and then chromatographed on a column (3 × 20 cm) of DEAE-cellulose equilibrated with the buffer. The enzyme was found in break-through fractions, but the specific activity had increased significantly. The fractions which showed the specific activity higher than 9,000 were combined and dialyzed against 2 mM Ca acetate and lyophilized. The final preparation appeared homogeneous, as judged by amino terminal amino acid analysis but was further chromatographed by reverse phase HPLC using a TOSO system CCPM-8000, just before sequence analysis, to remove impurities which might come from its autolysis; the enzyme was denatured by dissolving in 70% formic acid and chromatographed on a Vydac C4 column (5 μ m, 300 Å, 4.6 × 250 mm; Separations Group, CA, U.S.A.) using a solvent system of 0.075% TFA in water vs. acetonitrile-2-propanol (3:1) with 0.06% TFA. The denatured protein was eluted by an increasing gradient of organic solvent concentration at a flow rate of 1.0 ml/min at room temperature.

Assays of proteolytic activity and protein concentration. The proteolytic activity was assayed by the method described previously,¹⁰ and the protein concentration was estimated spectrophotometrically by assuming that $E_{1\%, 1\text{ cm}}$ at 280 nm is 13.8.¹⁰ The specific activity was defined as the activity units per mg of protein.

Chemical or enzymatic digestion of BANP, separation of the resultant peptides and their amino acid sequence analysis CNBr-cleavage. BANP (10 mg) was incubated with CNBr (30 mg) in 4.0 ml of 70% formic acid for 24 hr at room temperature, diluted with water, and then lyophilized.

***S. aureus* V8 protease digestion.** Digestion with *S. aureus*

V8 protease was done at 37°C in 0.1 M ammonium acetate (pH 4.0) or in 0.1 M phosphate buffer (pH 7.9). BANP, 1 mg, was dissolved in 45 μ l of the above buffer and the V8 protease was added at the enzyme-substrate ratio of 1:50 (wt/wt). After 21 hr of incubation at 37°C, the precipitate was removed by centrifugation, and the supernatant was chromatographed by HPLC and the sequence of each purified fragment was analyzed.

Achromobacter lysyl endopeptidase digestion. BANP (1.5 mg) was dissolved in 200 μ l of 10 mM Tris-HCl buffer (pH 7.6) with 2 M urea and digested with lysyl endopeptidase for 5 hr at 30°C. The enzyme to substrate ratio was 1:300 (wt/wt). The digest was separated and purified by HPLC.

Pseudomonas fragi endoproteinase Asp-N digestion. BANP, 2.5 mg, was dissolved in 300 μ l of 0.1 M ammonium bicarbonate (pH 8.0) and digested with endoproteinase Asp-N for 4 hr at 37°C. The ratio of enzyme to substrate was 1:6000 (wt/wt). Urea, 72 mg, was added to the reaction mixture to dissolve some insoluble materials. Fragments were separated by HPLC.

Trypsin digestion. The intact enzyme or succinylated enzyme,¹⁸ 2.0 mg, was digested with TPCK-treated trypsin at an enzyme-substrate ratio of 1:50 (wt/wt) in 100 μ l of 0.1 M phosphate buffer, pH 7.9, at 37°C for 24 hr. The digest was separated by HPLC.

α -Chymotrypsin digestion. BANP (1.9 mg) was dissolved in 300 μ l of 0.1 M ammonium bicarbonate (pH 8.0) and digested with α -chymotrypsin (1 to 300, mol/mol) for 3 hr at 37°C, and then urea, 72 mg, was added to the reaction mixture to completely dissolve insoluble peptides. The digest was separated by HPLC and tryptophan-containing peptides were monitored by following the absorbance at 295 nm. TSK-gel ODS120T was used to purify peptides.

Separation and purification of peptides. Peptide mixtures obtained after chemical or enzymatic cleavage were separated by reverse phase HPLC using the system described above by monitoring absorbances at 214, 280, and/or 295 nm. A Vydac C18 column (5 μ m, 300 Å, 4.6 × 250 mm) proved to be most useful. Some fractions were further purified on the same column with a different gradient system (acetonitrile with 0.06% TFA) or on a Waters μ Bondasphere CN column (5 μ m, 300 Å, 3.9 × 150 mm; Waters, MA, U.S.A.) with the above solvent systems.

Amino acid sequence analysis. All of peptides and intact protein were sequenced by manual Edman degradation.¹⁹ Phenylthiohydantoin (PTH) amino acids were analyzed and quantified by HPLC as reported previously.¹⁹ The amino acid sequence of the carboxyl terminal region of the intact protein was analyzed by periodic monitoring of the amino acids liberated by carboxypeptidase A digestion as

described by Ikenaka.²⁰⁾

Amino acid analysis. The amino acid composition of the intact protein was determined as described previously,¹²⁾ except that the tryptophan content was analyzed by the method of Edelhoch.²¹⁾ The amino acid compositions of fragments resulted from chemical or enzymatic cleavages were analyzed by the phenylthiocarbamyl (PTC) amino acid method,¹⁸⁾ after hydrolysis with 6 N HCl containing a trace of phenol at 150°C for 1 hr.

Results

CNBr-cleavage of BANP, and amino terminal amino acid sequences of the fragments and intact protein

By CNBr-cleavage of BANP, three frag-

ments were obtained, after purification by gel filtration followed by HPLC, and their amino acid sequences were analyzed by manual Edman degradation. As shown in Table I, more than 15 residues from the amino termini of the intact protein and its fragments were clearly sequenced in pmol order. These fragments were deduced to be in the order M-1, M-2, and M-3 from the comparison with sequences of the intact protein and thermolysin. However, the sequences of the carboxyl terminal regions of these fragments remain to be clarified.

Table I. SEQUENCE ANALYSIS OF INTACT PROTEIN AND CNBr FRAGMENTS

Edman cycle	Intact protein		M-1		M-2		M-3	
	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)
1	Ala	158	Ala	567	Ile	1226	Thr	445
2	Ala	122	Ala	949	Tyr	584	His	352
3	Ala	160	Ala	837	Gly	273	Gly	350
4	Thr	116	Thr	700	Asp	595	Val	903
5	Gly	88	Gly	459	Gly	320	Thr	156
6	Ser	51	Ser	23	Asp	383	Gln	490
7	Gly	64	Gly	523	Gly	123	Glu	461
8	Thr	58	Thr	56	Ser	25	Thr	178
9	Thr	70	Thr	233	Phe	87	Ala	372
10	Leu	39	Leu	258	Phe	109	Asn	323
11	Lys	9	Lys	149	Ser	13	Leu	348
12	Gly	34	Gly	267	Pro	21	Ile	232
13	Ala	39	Ala	140	Leu	23	Tyr	218
14	Thr	13	Thr	106	Ser	8	Glu	237
15	Val	42	Val	216	Gly	15	Asn	300
16	Pro	37	Pro	129	?	—	Gln	410
17	Leu	19	Leu	116	Leu	24	Pro	157
18	Asn	21	Asn	225	Asp	21	Gly	128
19			Ile	200			?	—
20			Ser	45			Leu	161
21			Tyr	36			Asn	128
22			Glu	129			Glu	292
23			Gly	58			?	—
24			Gly	37			Phe	44
25			Lys	23			Ser	28
26			Tyr	68			Asp	76
27			Val	27			Val	81
28			Leu	13				
29			Arg	12				
Yield of each peptide (%)			45		48		11	

Enzymatic digestion of BANP and sequence analyses of the resultant peptides

Figure 1 shows the HPLC profile of the peptides from lysyl endopeptidase digestion. Since BANP has been reported to contain 15 lysyl residues,¹²⁾ 16 peptides were expected to be produced by lysyl endopeptidase treatment. Unfortunately, however, only 10 peptides were highly purified by HPLC. Some of the peptides appeared insoluble and some were probably not to be eluted from the column under the conditions used. The amino acid sequence of each peptide was established and its location in protein was tentatively assumed to be in the order shown in Table II, from the comparison with sequences of thermolysin and of other *Bacillus* neutral proteases, most of which were deduced from nucleotide sequencing of the

genes.^{27~31)} About 70% of the whole sequence were established by these procedures.

Likewise, the intact protein was digested with *P. fragi* endoproteinase Asp-N which is active towards peptide bonds involving the amino groups of asparatyl residues (Fig. 2). Each peptide was purified to homogeneity, and the sequence and organization of each peptide were decided to be as shown in Table III. Surprisingly, the amino terminus of peptide ND-10 was found to be Leu, but the reason remains unknown.

To obtain overlapping peptides, the intact protein and CNBr-fragments were digested with trypsin and *S. aureus* V8 protease (Glu- and Asp-specific). Furthermore, the intact protein was succinylated to modify lysyl residues, and then digested with trypsin. By this pro-

Fig. 1. HPLC Profile of a Lysyl Endopeptidase Digest of BANP.

Lysyl endopeptidase digest was put on a Vydac C18 column as described in Materials and Methods. Fragments K7 and K8 were separated by rechromatography using the same column with a different gradient system. The numbering of peptides is based on the sequence order from the amino terminus of the protein.

Table II. SEQUENCE ANALYSIS OF PEPTIDES OBTAINED BY LYSYLENDOPEPTIDASE DIGESTION

Edman cycle	K-1		K-2		K-3		K-4		K-5	
	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)
1	Tyr	396	Pro	482	Tyr	511	Val	291	Tyr	864
2	Val	270	Thr	90	Phe	356	Tyr	798	Asn	658
3	Leu	177	Gly	347	Thr	322	Asp	934	Gln	697
4	Arg	201	Thr	117	Ser	214	Tyr	429	Pro	500
5	Asp	354	Gln	157	Ser	152	Phe	247	Asp	663
6	Leu	80	Ile	145	Ser	293	Tyr	312	Asn	635
7	Ser	117	Ile	226	Gln	144	Ser	113	Tyr	377
8	Lys	32	Thr	63	Arg	42	Asn	181	Ala	315
9			Tyr	197	Ala	153	Phe	63	Asn	91
10			Asp	176	Ala	126			Tyr	129
11			Leu	97	Val	162			Arg	173
12			Gln	49	Asp	184			Asn	132
13			Asn	51	Ala	126			Leu	42
14			Arg	18	His	59			Pro	57
15			Gln	20	Tyr	31			Asn	44
16					Asn	117				
Yield of each peptide (%)	25		48		20		21		47	

Table II. (continued).

Edman cycle	K-6		K-7		K-8		K-9		K-10	
	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)
1	Ala	196	Leu	100	Ser	248	Ala	5656	Val	810
2	Ala	70	Gly	125	Gln	433	Ala	3014	Glu	2342
3	Tyr	114	Val	41	Gln	510	Leu	4000	Ala	958
4	Asn	118	Ser	59	Ile	103	Ile	2890	Ala	558
5	Thr	68	Lys	—	Tyr	62	Gln	5008	Trp	195
6	Ile	26	Ser	51	Tyr	296	Ser	946	Asn	287
7	Thr	72	Gln	53	Arg	69	Ala	3237	Ala	112
8			Gln	61	Ala	242	Arg	5015	Val	59
9			Ile	26	Leu	71	Asp	5105	Gly	128
10			Tyr	18	Thr	117	Leu	1290	Leu	70
11					Thr	161	Tyr	1039		
12					Tyr	99	Gly	917		
13					Leu	52	Ser	507		
14					Thr	83	Thr	600		
15					Pro	161	Asp	1229		
16					Ser	51	Ala	279		
17					Ser	42	Ala	465		
18							Lys	100		
Yield of each peptide (%)	11		11		20		33		52

Fig. 2. HPLC Profile of an Endoproteinase Asp-N Digest of BANP.*

The digest was separated by a Vydac C18 column, and some peptides were further rechromatographed using the same column with a different gradient system before sequence analysis.

*The sequences of peaks designated by small letters (a to i) were not shown in Table III, since they were found to be the peptides having the sequences already shown in Tables I and II, after several cycles of manual Edman degradation.

cedure, only the peptide bonds involving arginyl residues became susceptible to trypsin. α -Chymotrypsin was also used to get overlapping peptide fragments.

The resultant peptides were separated and purified by HPLC and their amino acid sequences were analyzed. Through these experiments, fragments of unidentified regions and overlapping peptides were obtained (Fig. 3), except for the overlapping peptide linked between the Lys-269 and Asp-270 region. The amino acid sequence of the carboxyl terminus of the intact protein was -Asn-Ala-Val-Gly-LeuOH. From these experimental results, the entire amino acid sequence of BANP was deduced to be as shown in Fig. 3.

BANP consists of 300 amino acid residues with a molecular weight of 32,633. The amino

acid composition calculated from sequence analysis and that found by amino acid analysis agreed well, except for serine and isoleucine residues (Table IV).

In Fig. 4, the amino acid sequences of *Bacillus* neutral proteases hitherto reported are compared with that of BANP. BANP was found to be identical to the neutral protease from *B. subtilis* IA72,²⁴⁾ which was derived from *Bacillus subtilis* 168.

Discussion

There are several reports on the sequence analyses of *B. subtilis* neutral proteases, but the entire amino acid sequence was all deduced only from nucleotide sequencing, i.e., from gene analyses of *B. amyloliquefaciens*,^{22, 23)} *B.*

Table III. SEQUENCE ANALYSIS OF PEPTIDES OBTAINED BY ENDOPROTEINASE ASP-N DIGESTION

Edman cycle	ND-1		ND-2		ND-3		ND-4		ND-5		ND-6		ND-7	
	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)
1	Asp	131	Asp	570	Asp	455	Asp	1307	Asp	222	Asp	74	Asp	1600
2	Leu	13	Ala	502	Tyr	1325	Asn	461	Gln	187	Gly	197	Val	284
3	Gln	87	His	72	Phe	457	Lys	30	Met	158	Asp	152	Thr	60
4	Asn	50	Tyr	460	Tyr	909	Gly	244	Ile	48	Gly	80	Ala	65
5	Arg	14	Asn	273	Ser	144	Ser	51	Tyr	187	Ser	62	His	267
6	Gln	36	Leu	165	Asn	239	Lys	23	Gly	124	Phe	16	Glu	146
7	Ser	2	Gly	80	Phe	76	Ile	10			Phe	16	Met	10
8	Arg	9	Lys	10	Lys	15	Val	90			Ser	17	Thr	20
9	?	—	Val	130	Arg	42	Ser	32			Pro	14	His	108
10	Pro	18	Tyr	148	Asn	35	Ser	4			Leu	10	Gly	40
11	Gly	6			Ser	6	Val	27			Ser	14	Val	5
12	Thr	3			Tyr	62	His	66			Gly	8	Thr	24
13							Tyr	26			Ser	14	Gln	29
14											Leu	3	Glu	10
15													?	—
16													Ala	13
17													Asn	29
18													Leu	13
19													Ile	10
20													Tyr	26
21													Glu	16
22													Asn	36
Yield of each peptide (%)		16		28		20		50		15		24		36

Primary Structure of *B. subtilis* Neutral Protease

Table III. (continued).

Edman cycle	ND-8		ND-9		ND-10		ND-11			ND-12		ND-13	
	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)	Yield ^a (pmol)	Residue	Yield (pmol)	Residue	Yield (pmol)
1	Asp	65	Asp	1624	Leu	1677	Asp	1088	507	Asp	1544	Asp	813
2	Val	503	Ile	206	Ser	1431	Tyr	390	738	Ala	1591	Ala	1779
3	Phe	184	Gly	731	Asn	3385	Gly	304	821	Lys	1073	Ala	1721
4	Gly	267	Glu	1031	Pro	4571	Gly	—	949	Ala	767	Lys	138
5	Tyr	47	Asp	811	Thr	444	Val	22	432	Ala	1926	Val	470
6	Phe	54	Ile	361	Lys	423	His	149	120	Leu	1277	Glu	275
7	Asn	26	Thr	483	Tyr	1486	Thr	28	250	Ile	1161	Ala	851
8			Val	300			Asn	146	335	Gln	1158	Ala	1165
9			Ser	211			Ser	12	158	Ser	389		
10			Gln	634			Gly	37	160	Ala	530		
11			Pro	830			Ile	10	194	Arg	45		
12			Ala	52			Pro	36	129				
13			Leu	7			Asn	75	54				
14			Arg	24			Lys	6	38				
15							Ala	23	60				
16							Ala	14					
17							Tyr	9					
18							Asn	30					
19							Thr	8					
20							Ile	7					
21							Thr	5					
22							Lys	5					
23							Leu	7					
24							Gly	6					
25							Val	7					
26							?	—					
27							Lys	4					
							?	—					
28							Gln	8					
Yield of each peptide (%)		30		56		12		40			56		58

^a Reexamined to confirm glycine residue.

	1	50	100
	AAATGSOTTLKGATVPLNISYEGGKYVLRDLRSKPTGTQIIITYDLQNRQSRLPGTLVSSSTTKTFTSSSQRAAVDAHYNLGKVYDYFYSNFKRNSYDNKGSK		
Intact	AAATGSOTTLKGATVPLN		
Peptides obtained after cleavage with			
CNBr	AAATGSOTTLKGATVPLNISYEGGKYVLR		
V8 protease pH 4.0	GGKYVLRDLRSKPTGTQII-YDLQNRQSR		
Lysylendopeptidase	YVLRDLRSK		
	PTGTQIIITYDLQNRQ		
Endoproteinase Asp-N	DLQNRQSR-PGTL		
	DAHYNLGKVY		
	DYFYSNFKRNSY		
V8 protease pH 7.9	LPGTLVSSSTTK		
Trypsin	LPGTLVSSSTT-TFTSSSQ		
Trypsin (succ)			
	101	150	200
	IVSSVHYGTQYNNAAWTGDQMIYGDGDSFFSPLSGSLDVTAHEMTHGVTQETANLIYENQPGALNESPSDFVGYFNDDTDWDIGEDITVSQPALRSLN		
CNBr	IYGDGDSFFSPLSG-LD		
V8 protease pH 4.0	THGVTQETANLIYENQPG-LNE-FSDV		
	TANLIYEN-PG		
	NQPAL		
Endoproteinase Asp-N	IVSSVHY	DQMIYG	DVTAHEMTHGVTQE-ANLIYEN
		DGDGDSFFSPLSGSL	DVFGYFN
			DIGEDITVSQPALR
V8 protease pH 7.9			LSN
			SFSDV-GYF
			DWDIGE
			DITVSQPALRSLN
Trypsin	IV--VHYGTQYNNAAWTGDQMIYGD		
α -Chymotrypsin	GYFNDDTDWDIGE		
	201	250	300
	PTKYNQPDNYANYRNLPNTDEGDYGGVHTNSGIPNKAAYNTITKLGVSKSQIYYRALTTYLTPSSTPKDAKAALIQSARDLYGSTDAAKVEAAWNAVGL		
V8 protease pH 4.0	YNQPDNYANYRNLPN		
Lysylendopeptidase	LGVS-SQIY		
	AAYNTIT		
Endoproteinase Asp-N	PTKY	DYGGVHTNSGIPNKAAYNTITKLGV-K-Q	AAWNAVGL
V8 protease pH 7.9	PT-YN		VEAAWNAVGL
Trypsin		NLPNTDEGDYGGVH	DAKAALIQSAR
		AA-NTITK	DAAKVEAA
Carboxypeptidase A		ALTTYLTPSSTPK	(W)NAVGL

Fig. 3. Overlap Strategy of Peptides and the Entire Amino Acid Sequence of *Bacillus subtilis* amylosacchariticus Neutral Protease. The top line in boldface represents the entire sequence of BANP. The one letter codes of amino acids below the entire sequence indicate residues found by manual sequence analysis of peptides obtained by chemical or enzymatic cleavages.

		1		10		20
B. amylosacchariticus	Ala-Ala-Ala-Thr-Gly-Ser-Gly-Thr-Thr-Leu-Lys-Gly-Ala-Thr-Val-Pro-Leu-Asn-Ile-Ser-Tyr-Glu-					
B. amylioliquefaciens P	-Thr-	-Thr-		-Lys-	-Ser-	-Ser-
B. amylioliquefaciens F	-Thr-	-Thr-		-Lys-	-Ser-	-Ser-
B. thermoproteolyticus	-----Ile-	-Thr-Ser-	-Val-Gly-Val-	-Arg-Gly-	-Leu-Gly-Asp-Gln-Lys-Asn-Ile-	
B. stearothermophilus	-----Val-Ala-	-Ala-Ser-	-Val-Gly-Val-	-Arg-Gly-	-Leu-Gly-Asn-Gln-Lys-	-Ile-
B. cereus	-----Val-	-Thr-Asn-Lys-Val-Gly-Thr-		-Lys-Gly-	-Leu-Gly-Asp-Thr-Lys-Ser-Leu-	
				30		40
B. amylosacchariticus	Gly-Gly-Lys-Tyr-Val-----Leu-Arg-Asp-Leu-Ser-Lys-Pro-Thr-----Gly-Thr-Gln-Ile-Ile-					
B. amylioliquefaciens P	Ser-	-----			-----	
B. amylioliquefaciens F	Ser-	-----				-Asn-Asn-
B. thermoproteolyticus	Asn-Thr-Thr-	-Ser-----	-Thr-Tyr-Tyr-Tyr-	-Gln-Asn-Asn-	-Arg-	-Asp-Gly-
B. stearothermophilus	Asn-Thr-Thr-	-Ser-Ser-Tyr-Tyr-Gly-Tyr-Tyr-		-Gln-Asp-Asn-	-Arg-	-Ser-Gly-
B. cereus	Asn-Thr-Thr-Leu-Ser-----	-Gly-Ser-Ser-Tyr-Tyr-		-Gln-Asp-Asn-	-Arg-	-Ala-Thr-
				50		60
B. amylosacchariticus	Thr-Tyr-Asp-Leu-Gln-Asn-Arg-Gln-Ser-Arg-Leu-Pro-Gly-Thr-Leu-Val-Ser-Ser-Thr-Thr-Lys-					
B. amylioliquefaciens P	Gln-		-Glu-Tyr-Asn-			-Asn-
B. amylioliquefaciens F			-Glu-Tyr-Asn-			-Asn-
B. thermoproteolyticus		-Ala-Lys-Tyr-	-----Thr-Thr-	-Ser-	-Trp-Ala-Asp-Ala-Asp-Asn-	
B. stearothermophilus		-Gly-Arg-	-----Thr-Val-	-Ser-	-Trp-Thr-Asp-Gly-Asp-Asn-	
B. cereus		-Ala-Lys-	-----Thr-		-Trp-Ala-Asp-Ala-Asp-Asn-	
					Ca3	Ca3
				70		80
B. amylosacchariticus	Thr-Phe-Thr-Ser-Ser-Ser-Gln-Arg-Ala-Ala-Val-Asp-Ala-His-Tyr-Asn-Leu-Gly-Lys-Val-					
B. amylioliquefaciens P	Gln-	-Thr-				
B. amylioliquefaciens F	Gln-	-Thr-			-Asn-	
B. thermoproteolyticus	Gln-	-Phe-Ala-	-Tyr-Asp-Ala-Pro-		-Tyr-Ala-	-Val-Thr-
B. stearothermophilus	Gln-	-Ala-	-Tyr-Asp-Ala-		-Tyr-Ala-	-Val-
B. cereus	Val-	-Asn-Ala-Ala-Tyr-Asp-Ala-			-Tyr-Ala-	-Thr-
				90		100
B. amylosacchariticus	Tyr-Asp-Tyr-Phe-Tyr-Ser-Asn-Phe-Lys-Arg-Asn-Ser-Tyr-Asp-Asn-Lys-Gly-Ser-Lys-Ile-					
B. amylioliquefaciens P		-Gln-Lys-	-Asn-			-Gly-
B. amylioliquefaciens F	-Glu-	-Gln-Lys-Tyr-Asn-				-Gly-
B. thermoproteolyticus		-Tyr-Lys-Asn-Val-His-Asn-	-Leu-		-Gly-Asn-Asn-Ala-Ala-	
B. stearothermophilus		-Tyr-Lys-Asn-Val-His-Gly-	-Leu-		-Gly-Ser-Asn-Ala-Ala-	
B. cereus		-Tyr-Lys-Ala-Thr-	-Asn-		-Ile-Asn-Asp-Ala-	-Ala-Pro-Leu-
				110	S S S	120
B. amylosacchariticus	Val-Ser-Ser-Val-His-Tyr-Gly-Thr-Gln-Tyr-Asn-Asn-Ala-Ala-Trp-Thr-Gly-Asp-Gln-Met-					
B. amylioliquefaciens P		-Gln-	-Ser-Arg-		-Ile-	
B. amylioliquefaciens F		-Gln-	-Ser-Arg-		-Ile-	
B. thermoproteolyticus	Arg-		-Ser-Gln-Gly-		-Phe-	-Ser-Glu-
B. stearothermophilus	Arg-	-Thr-	-Arg-Gly-		-Phe-	-Asn-
B. cereus	Lys-	-Thr-	-Ser-Asn-		-Phe-	-Asn-
				130		140
B. amylosacchariticus	Ile-Tyr-Gly-Asp-Gly-Asp-Gly-Ser-Phe-Phe-Ser-Pro-Leu-Ser-Gly-Ser-Leu-Asp-Val-Thr-				Ca1	
B. amylioliquefaciens P					-Met-	
B. amylioliquefaciens F					-Met-	
B. thermoproteolyticus	Val-		-Gln-Thr-	-Ile-	-Gly-Ile-	-Val-
B. stearothermophilus	Val-		-Gln-Thr-	-Leu-	-Gly-Ile-	-Val-
B. cereus	Val-		-Val-Thr-	-Thr-Ser-	-Gly-Ile-	-Ile-
		Zn C	Zn	150	C	160
B. amylosacchariticus	Ala-His-Glu-Met-Thr-His-Gly-Val-Thr-Gln-Glu-Thr-Ala-Asn-Leu-Ile-Tyr-Glu-Asn-Gln-					
B. amylioliquefaciens P					-Asn-	
B. amylioliquefaciens F					-Asn-	
B. thermoproteolyticus		-Leu-	-Ala-	-Asp-Tyr-	-Gly-	-Gln-
B. stearothermophilus	Gly-	-Leu-	-Ala-	-Asp-Tyr-	-Gly-	-Val-
B. cereus	Gly-	-Leu-	-Ala-	-Glu-Asn-Ser-Ser-		-Gln-
			Zn	170	Ca1,2	180
B. amylosacchariticus	Pro-Gly-Ala-Leu-Asn-Glu-Ser-Phe-Ser-Asp-Val-Phe-Gly-Tyr-Phe-Asn-Asp-Thr-Glu-----					
B. amylioliquefaciens P						
B. amylioliquefaciens F						
B. thermoproteolyticus	Ser-	-Ile-	-Ala-Ile-	-Ile-	-Thr-Leu-Val-Glu-Phe-Tyr-Ala-	
B. stearothermophilus	Ser-	-Ile-	-Ala-Met-	-Ile-	-Thr-Leu-Val-Glu-Phe-Tyr-Ala-	
B. cereus	Ser-		-Ala-Ile-	-Ile-	-Thr-Leu-Val-Glu-Phe-Tyr-Asp-	
		Ca1,2	Ca1,2	190		200
B. amylosacchariticus	-----Asp-Trp-Asp-Ile-Gly-Glu-Asp-Ile-Thr-Val-Ser-Gln-Pro-----					
B. amylioliquefaciens P	-----					
B. amylioliquefaciens F	-----					
B. thermoproteolyticus	Asn-Lys-Asn-Pro-	-Glu-		-Val-Tyr-Thr-Pro-Gly-Ile-Ser-Gly-Asp-		
B. stearothermophilus	Asn-Arg-Asn-Pro-	-Glu-		-Tyr-Thr-Pro-Gly-Val-Ala-Gly-Asp-		
B. cereus	Asn-Arg-Asn-Pro-	-Glu-		-Tyr-Thr-Pro-Gly-Lys-Ala-Gly-Asp-	Ca4 Ca4	Ca4
		S		210		220
B. amylosacchariticus	Ala-Leu-Arg-Ser-Leu-Ser-Asn-Pro-Thr-Lys-Tyr-Asn-Gln-Pro-Asp-Asn-Tyr-Ala-Asn-Tyr-Arg-					
B. amylioliquefaciens P				-Gly-	-Phe-Lys-	-Lys-
B. amylioliquefaciens F				-Gly-	-Phe-Lys-	-Lys-
B. thermoproteolyticus	Ser-	-Met-	-Asp-	-Ala-	-Gly-Asp-	-His-
B. stearothermophilus		-Met-	-Asp-	-Ala-	-Gly-Asp-	-His-
B. cereus		-Met-	-Asp-		-Gly-Asp-	-His-
			C	230	C	240
B. amylosacchariticus	Asn-Leu-Pro-Asn-Thr-Asp-Glu-Gly-Asp-Tyr-Gly-Gly-Val-His-Thr-Asn-Ser-Gly-Ile-Pro-Asn-Lys-Ala-					
B. amylioliquefaciens P			-Ala-			
B. amylioliquefaciens F			-Ala-			
B. thermoproteolyticus	Tyr-Thr-Gly-Thr-Gln-				-Ile-	-Ile-
B. stearothermophilus	Tyr-Thr-Gly-Thr-Gln-					-Ile-
B. cereus	Tyr-Thr-Gly-Ser-Ser-					-Ile-

		250		260
<i>B. amylosacchariticus</i>	Ala-Tyr-Asn-Thr-Ile-Thr-Lys-Leu-Gly	-----Val-Ser-----		
<i>B. amyloliquefaciens</i> P		-Ile-----	-Asn-----	
<i>B. amyloliquefaciens</i> F		-Ile-----	-Asn-----	
<i>B. thermoproteolyticus</i>	-----Leu- -Ser-Gln-Gly-	-Thr-His-Tyr-Gly-	-Val-Val-Gly-Ile-Gly-Arg-	
<i>B. stearothermophilus</i>	-----Leu-Leu-Ser-Gln-Gly-	-Val-His-Tyr-Gly-	-Val-Asn-Gly-Ile-Gly-Arg-	
<i>B. cereus</i>	-----Leu-Leu-Ala-Asn-Gly-	-Thr-His-Tyr-Gly-	-Thr-Val-Thr-Gly-Ile-Gly-Lys-	
		270		280
<i>B. amylosacchariticus</i>	---Lys-Ser-Gln-Gln-Ile-Tyr-Tyr-Arg-Ala-Leu-Thr-Thr-Tyr-Leu-Thr-Pro-Ser-Ser-Thr-			
<i>B. amyloliquefaciens</i> P	-----Ala-Glu-	-Val-		
<i>B. amyloliquefaciens</i> F	-----Ala-Glu-	-Val-		
<i>B. thermoproteolyticus</i>	Asp- -Leu-Gly-Lys- -Phe-	-Gln-	-Thr-	-Asn-
<i>B. stearothermophilus</i>	Asp- -Met-Gly-Lys- -Phe-	-Val-Tyr-	-Thr-	-Asn-
<i>B. cereus</i>	Asp- -Leu-Gly-Ala-	-Asn- -Gln- -Phe- -Gln- -Thr-		
		290		300
<i>B. amylosacchariticus</i>	Phe-Lys-Asp-Ala-Lys-Ala-Ala-Leu-Ile-Gln-Ser-Ala-Arg-Asp-Leu-Tyr-Gly-Ser-Thr----			
<i>B. amyloliquefaciens</i> P			-Gln----	
<i>B. amyloliquefaciens</i> F			-Gln----	
<i>B. thermoproteolyticus</i>	-Ser-Gln-Leu-Arg- -Ala-Val- -Thr-		-Ser-	
<i>B. stearothermophilus</i>	-Ser-Gln-Leu-Arg- -Cys-Val- -Ala- -Ala-		-Ser-	
<i>B. cereus</i>	-Ser-Gln- -Arg- -Gly-Ala-Val- -Ala- -Ala-		-Ala-Asn-Ser-	
		310		
<i>B. amylosacchariticus</i>	---Asp-Ala-Ala-Lys-Val-Glu-Ala-Ala-Trp-Asn-Ala-Val-Gly-Leu----			
<i>B. amyloliquefaciens</i> P	-----Ser-			
<i>B. amyloliquefaciens</i> F	-----Arg-Leu-			
<i>B. thermoproteolyticus</i>	Gln-Glu-Val- -Ser- -Lys-Gln- -Phe-Asp-	-Val-Lys		
<i>B. stearothermophilus</i>	Gln-Glu-Val-Asn-Ser- -Lys-Gln- -Phe-	-Val-Tyr		
<i>B. cereus</i>	Ala-Glu-Val- -Ala- -Lys-Gln-Ser-Phe-Ser-	-Val-Asn		

Fig. 4. Sequence Homology Found in *Bacillus* Neutral Proteases.

The numbering of amino acid residues is based on that for thermolysin. C, residues involved in catalytic reaction; S, residues related to interaction with substrate; Zn, zinc-binding ligands; Ca, residues participating in binding of Ca-1, 2, 3 and 4 in thermolysin.

subtilis GSY264* (*B. subtilis* IA72),²⁴⁾ and of *B. amylosacchariticus* (Yoshimoto *et al.*, unpublished). On the other hand, protein sequencing was attempted for the neutral protease A of *B. amyloliquefaciens*, but the results were incomplete.²⁵⁾

This paper is the first report on the complete amino acid sequence of a neutral protease from *Bacillus subtilis*, found from protein sequencing. The amino acid sequence of BANP was identical to that of *B. subtilis* IA72, which was deduced from nucleotide sequencing.²⁴⁾ The enzyme was 89% homologous to the neutral protease of *B. amyloliquefaciens* P (ATCC 23844),²²⁾ 87% homologous to *B. amyloliquefaciens* F (ATCC 23350),²³⁾ 47 and 48% homologous to thermolysin⁶⁾ and the thermostable neutral protease from *B. stearothermophilus* CU21,²⁶⁾ and 45% homologous to *B. cereus* enzyme.²⁷⁾ The putative active site amino acid residues (Glu-143, Tyr-157, Asp-226 and His-231), which seem to be involved in catalytic reaction, Zn-binding ligands (His-142, His-146, and Glu-166) and residues related to interaction with the substrate (Asn-112, Ala-113, Trp-115, and Arg-

203) were well conserved among them, as deduced from comparison with thermolysin.^{7, 28, 29)}

These results indicate that the reaction mechanisms of *Bacillus* neutral proteases for peptide bond hydrolysis are quite similar. Of four amino acid residues involved in binding of Ca-1 and Ca-2, Asp-138 and Asp-185 were conserved in *Bacillus* neutral proteases, but the other two, Glu-177 and Glu-187, in thermolysin were replaced by Asp in *B. subtilis* neutral proteases. On the other hand, Ca-binding ligands for Ca-3 (Asp-57 and Asp-59) and Ca-4 (Tyr-193, Thr-194 and Asp-200) in thermolysin were not conserved; Asp-57 and Asp-59 for Ca-3 were replaced by Ser and Thr, respectively, and those for Ca-4 were by Thr-193 and Val-194, Asp-200 being deleted in *B. subtilis* neutral proteases. These discrepancies seem to be related to the instability of *B. subtilis* neutral proteases towards autolysis and/or heat treatment, though BANP was supposed to contain 3 or 4 gram atoms of Ca per mol.^{10, 30)}

In the genus *Bacillus*, cases where an identical protease is produced by different strains are

* *Bacillus subtilis* GSY264,²⁴⁾ which was derived from *B. subtilis* 168, is now registered as *B. subtilis* IA72 at the *Bacillus* Genetic Stock Center, Ohio State University (comment from Dr. Donald H. Dean).

Table IV. AMINO ACID COMPOSITION OF
B. amylosacchariticus NEUTRAL
PROTEASE

	Sequencing	Amino acid analysis	
		Calcd.	Integral
Lysine	15	14.8	15
Histidine	5	5.1	5
Arginine	9	9.0	9
Aspartic acid	20	41.8	42
Asparagine	22		
Threonine	30	29.8 ^a	30
Serine	30	28.8 ^a	29
Glutamic acid	9	22.4	22
Glutamine	13		
Proline	11	10.5	11
Glycine	26	25.8	26
Alanine	26	26.1	26
1/2 Cystine	0	0	0
Valine	15	15.4 ^b	15
Methionine	2	2.0 ^b	2
Isoleucine	12	10.6 ^b	11
Leucine	21	21.0	21
Tyrosine	22	22.4	22
Phenylalanine	9	8.7	9
Tryptophan	3	2.6 ^c	3
Total	300		298

^a Values extrapolated to zero hydrolysis time.

^b Maximal values were adopted.

^c Determined by the method of Edelhoch.²¹⁾

very rare; for example, even in the neutral proteases produced by two strains of the same *Bacillus* species, *B. amyloliquefaciens* strains P²²⁾ and F,²³⁾ there were observed 11 alternation in nucleotides of the genes encoding mature enzyme protein, resulting in 8 amino acid changes out of 314 residues of the mature protein. A quite similar relationship was also observed between subtilisin AM¹⁵⁾ produced by *B. amylosacchariticus* and subtilisin E³¹⁾ purified from *B. subtilis* 168, the parent strain of *B. subtilis* IA72; comparison of nucleotide sequences of both subtilisins indicated that there were 10 discrepancies in nucleotides encoding mature enzymes, which resulted in alternation of two amino acid residues between two subtilisins.

Nevertheless, *B. subtilis* IA72 and *amylosacchariticus* were found to produce an identical

neutral protease. This was also confirmed by nucleotide sequencing of the gene encoding BANP (Yoshimoto *et al.*, unpublished) and comparison with that encoding *B. subtilis* IA72 neutral protease.²⁴⁾ *B. subtilis* neutral proteases are commonly unstable, as compared with thermolysin and subtilisins. Taking into account these facts, it might be possible to conclude that *B. subtilis amylosacchariticus* and IA72 are taxonomically very close and presumably be derived from a single ancestor, *B. subtilis* 168, and that a minor alteration in the amino acid sequence of the neutral protease make the enzyme more unstable, leading the mutated proteases to be excluded from nature.

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