

Molecular Cloning, Nucleotide Sequence, and Expression of the Structural Gene for Alkaline Serine Protease from Alkaliphilic *Bacillus* sp. 221

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The gene encoding an alkaline serine protease from alkaliphilic *Bacillus* sp. 221 was cloned in *Escherichia coli* and expressed in *Bacillus subtilis*. An open reading frame of 1,140 bases, identified as the protease gene was preceded by a putative Shine-Dalgarno sequence (AGGAGG) with a spacing of 7 bases. The deduced amino acid sequence had a pre-pro-peptide of 111 residues followed by the mature protease comprising 269 residues. The alkaline protease from alkaliphilic *Bacillus* sp. 221 had higher homology to the protease from alkaliphilic bacilli (82.1% and 99.6%) than to those from neutrophilic bacilli (60.6–61.7%). Also *Bacillus* sp. 221 protease and other protease from alkaliphilic bacilli shared common amino acid changes and 4 amino acid deletions that seemed to be related to characteristics of the enzyme of alkaliphilic bacilli when compared to the proteases from neutrophilic bacilli.

An alkaline protease from alkaliphilic *Bacillus* sp. 221 has been reported by Horikoshi¹⁾ as the first enzyme with an alkaline pH optimum produced by an alkaliphilic microorganism. This strain was later identified as *Bacillus alcalophilus* Vedder (ATCC 21522). The *Bacillus* strain 221 produces an alkaline protease that has a high optimum pH (pH 11.5–12.0), thermostability at highly alkaline pH, and stability to detergents. Since the first report on alkaline protease from alkaliphilic *Bacillus* sp. 221 was published, there have been extensive studies of the properties of alkaline proteases from other strains.²⁾ *Bacillus* species including alkaliphilic bacilli produce and secrete subtilisin-type alkaline serine proteases. Genes for alkaline serine proteases from neutrophilic bacilli such as *B. amyloliquefaciens* (subtilisin BPN'),³⁾ *B. licheniformis* (subtilisin Carlsberg),⁴⁾ *B. subtilis*,⁵⁾ and *B. subtilis* var. *amylosacchariticus*⁶⁾ have been cloned and sequenced. Recently, the genes encoding alkaline serine proteases from alkaliphilic *Bacillus* strains YaB⁷⁾ and PB92⁸⁾ have also been cloned and sequenced. Nucleotide and amino acid sequences of these subtilisin-type enzymes share significant homology although these enzymes are distinct from each other in their enzymatic and physicochemical properties. We isolated the gene encoding 221 alkaline protease, to allow structural and functional comparisons with genetic approach. In this report, we describe the cloning, sequencing, and expression of the gene for the 221 alkaline protease.

Materials and Methods

Bacterial strains, plasmids, and media. Alkaliphilic *Bacillus* sp. 221 was used as the chromosomal DNA source.²⁾ *Escherichia coli* MV1184 and XL1 blue were used as host strains for cloning. *Bacillus subtilis* DB-104 (*nprE18 nprR2 ΔaprE3 his-101*) carrying lesions in the structural genes for alkaline and neutral proteases⁹⁾ was also used as the host strain for

expression of the gene for 221 protease. The plasmids pUC119 and Bluescript II (KS and SK) were purchased from Takara Shuzo Co. (Kyoto, Japan) and Stratagene (CA, U.S.A.) respectively. The plasmid pHW1^{10,11)} was used for transformation of *B. subtilis*. A-II medium (pH 9.5) consisted of 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K₂HPO₄, 0.02% Mg₂SO₄·7H₂O, and 2% NaHCO₃.²⁾ N-II medium (pH 8.0) contained ingredients of A-II medium except for 2% NaHCO₃. *E. coli* was aerobically grown in LB-broth at 37°C. *Bacillus* sp. 221 and *B. subtilis* DB-104 were also aerobically grown at 37°C in A-II broth and in N-II broth, respectively.

DNA manipulations. Chromosomal DNA of *Bacillus* sp. 221 was prepared by phenol treatment.¹²⁾ Plasmid DNA was isolated from *E. coli* or *B. subtilis* by the alkaline extraction procedure.¹³⁾ *E. coli* was transformed by the CaCl₂ method.¹⁴⁾ *B. subtilis* was also transformed by Chang and Cohen's method.¹⁵⁾ Southern hybridization¹⁶⁾ was done using digoxigenin-labeled DNA probe and antidigoxigenin antibody coupled to alkaline phosphatase using a DNA labeling and detection kit (Boehringer-Mannheim, Germany).

Preparation of DNA probe. The first 18 amino acid residues of the N-terminus of 221 protease were analyzed²⁾ and also the amino acid sequence of 221 protease corresponding to the region of the catalytic center (Asn218-Leu233) of subtilisins.¹⁷⁾ The amino acid sequence of N-terminal residues, Ala1-Gln-Ser-Val-Pro-Trp-Gly-Ile8, and the amino acid sequence around the catalytic center, Asn218-Gly-Thr-Ser-Met-Ala223, allowed us to design oligonucleotide primers to amplify a part of the gene for 221 protease by polymerase chain reaction (PCR).¹⁸⁾ The DNA primers (23- or 17-nucleotides) of 5'-GTNCARTCNGTNCNTG-GGGNAT-3' (N-terminus) and 5'-AAMGGNACNTCNATGGC-3' (C-terminus) were synthesized with an ABI DNA synthesizer Model 391 (Applied Biosystems Japan Co., Tokyo, Japan), in which equimolar mixtures of the following nucleotides were incorporated at N, R, and M: A, G, C, and T for N; A and G for R; and T and C for M. The intact chromosomal DNA of strain 221 was used as the template DNA for the PCR. A single 600-bp fragment amplified by PCR was purified by preparative agarose gel (1%) electrophoresis and labeled with digoxigenin-deoxy uridine triphosphate (dUTP) using a DNA labeling kit (Boehringer-Mannheim, Germany). The labeled PCR fragment was used as a DNA probe to detect transformants carrying the gene for 221 protease.

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Cloning of the gene for 221 alkaline protease. Chromosomal DNA of strain 221 was digested with an appropriate restriction enzyme and fractionated by preparative electrophoresis on a 1% agarose gel. DNA fragments of the fraction that hybridized to the probe were ligated with pUC119. The ligation mixture was used to transform competent *E. coli* MV 1184 and a subgenomic library was constructed. Transformants were grown overnight at 37°C on LB agar plates containing 150 µg/ml of ampicillin (LBA plate) and on nylon membranes Hybond-N (Amersham International plc., UK) overlaid on LBA plates. The nylon membranes carrying transformants grown were transferred to M9 plates containing casamino acid (0.4% w/v) and chloramphenicol (500 µg/ml) to amplify the plasmid. The nylon membranes were processed according to the supplier's recommendations for colony hybridization.

Preparation of cloned enzyme. *B. subtilis* DB-104 carrying pTHB2210,

which contained the whole gene for 221 protease, was aerobically grown at 37°C in 50 ml of N-II broth for 24 hr. The culture was centrifuged at 5000 × *g* for 15 min at 4°C. Protein in the supernatant was precipitated with cold acetone (−20°C) at a final concentration of 80% (v/v). The precipitate was collected by centrifugation at 5000 × *g* and washed with cold acetone. Residual acetone was evaporated *in vacuo* at 40°C. The dried powder was dissolved in 10 ml of 50 mM glycine–50 mM NaCl–NaOH buffer (pH 10.5). Caseinolytic activity was assayed by the method described previously.¹⁹⁾

DNA sequencing. Restriction fragments were cloned into appropriate restriction sites of pUC119 or Bluescript II plasmid vectors. Each fragment was sequenced by the dideoxy method.²⁰⁾ The sequences were analyzed by an ABI DNA sequencer Model 370A (Applied Biosystems Japan Co., Tokyo, Japan).

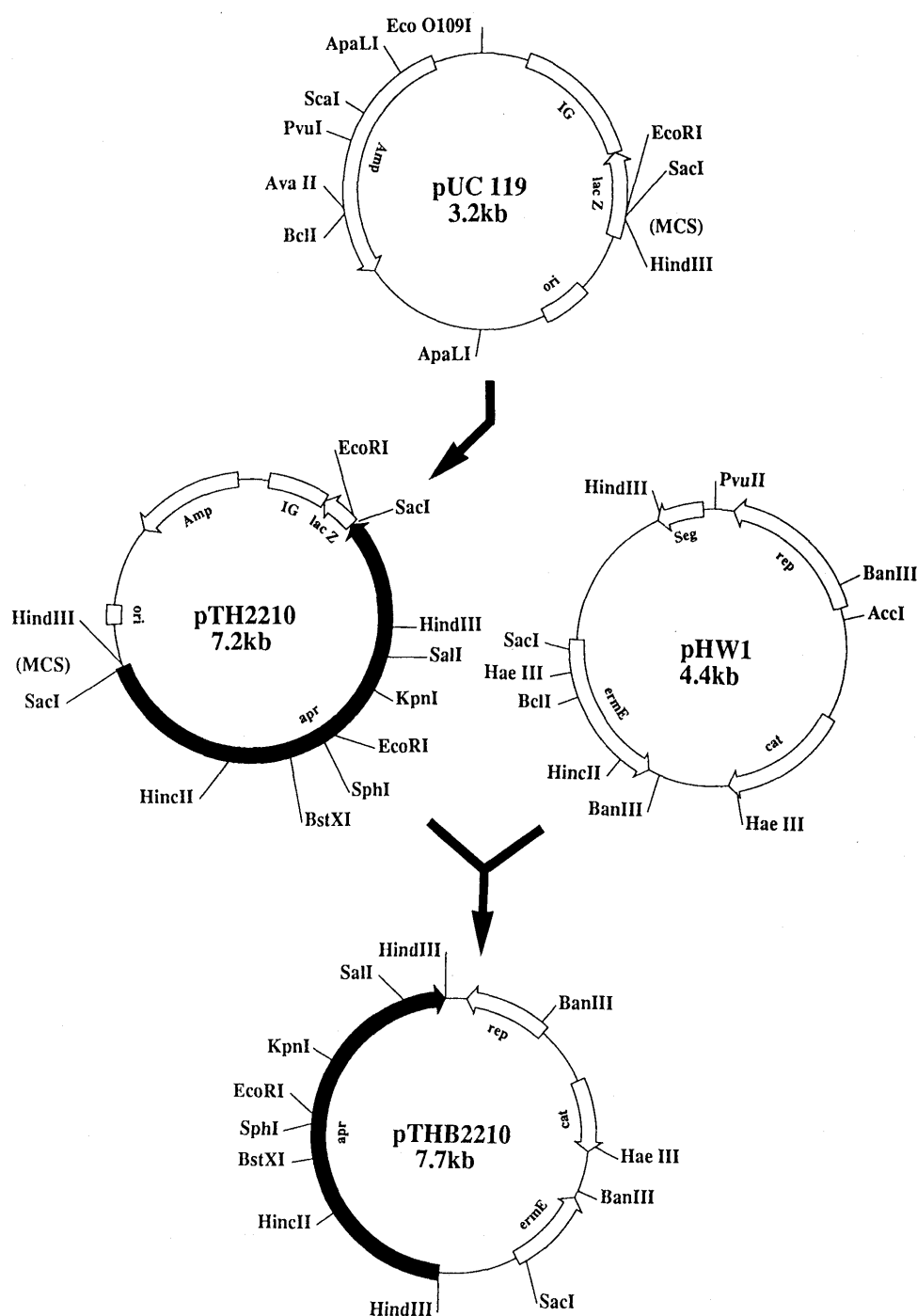


Fig. 1. Plasmid Constructions.

The insertions in vector pUC119 or pHW1 are indicated by black thick lines. The arrows within plasmids represent the direction of transcription of the structural gene. Amp, ampicillin resistance gene; IG, intergenic region; cat, chloramphenicol acetyl transferase gene; MCS, multi cloning site.

Results

Cloning and expression of the gene for 221 alkaline protease

A single 600-bp DNA fragment was amplified by PCR from the chromosomal DNA of strain 221 using the primers described in Materials and Methods, and used as a DNA probe. Southern blot analysis found that a 4 kb-*SacI* fragment of the strain 221 hybridized to this probe. Out of approximately 1500 colonies from a subgenomic library made of *SacI* digested, size-fractionated (<4 kb)

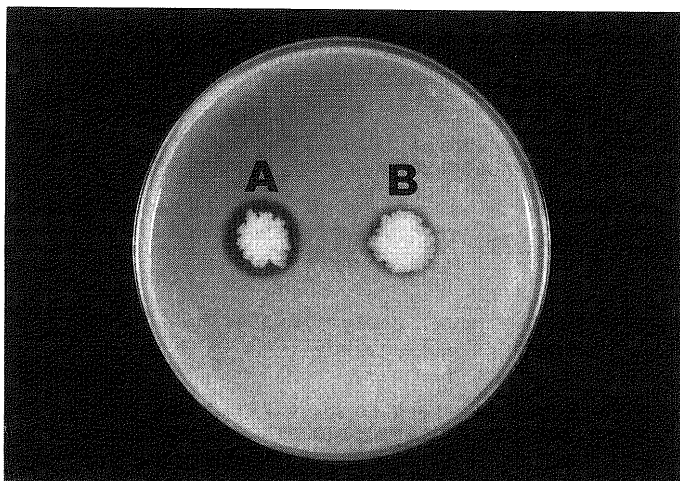


Fig. 2. Expression of the Gene for 221 Protease in *Bacillus subtilis* DB-104.

B. subtilis DB-104 was transformed with (A) pTHB2210 or (B) pHW1. Each transformant was grown on N-II agar plate containing 1% (w/v) skim milk at 37°C.

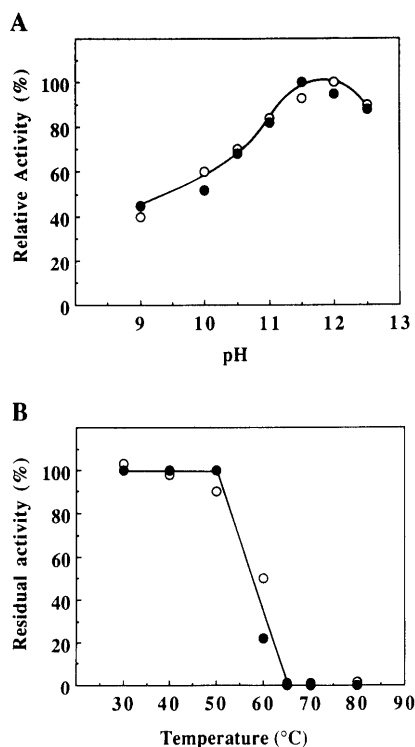


Fig. 3. Properties of the Cloned Protease.

The properties of cloned protease (○) were investigated and compared with 221 protease from the strain 221 (●) in their pH optima and thermostability at high alkaline pH (pH 11.0) by the methods described previously.²¹⁾ **A:** Effects of pH on enzyme activity. The substrate (casein) was dissolved at each pH and other conditions were the same as those of the standard method. **B:** Effects of temperature on enzyme stability. The enzyme was added to 50 mM glycine–50 mM NaCl–NaOH buffer (pH 11.0), incubated for 10 min, and the residual activities were measured.

chromosomal DNA, one colony showed hybridization to the probe. This transformant harbored a plasmid (pTH2210) containing the 4 kb-*SacI* fragment which hybridized to the probe described above (data not shown). *E. coli* carrying pTH2210 did not produce a detectable amount of alkaline protease. The 3.3-kb *HindIII* fragment which contains the structural gene for 221 protease was ligated with pHW1 digested with *HindIII* (pTHB2210, Fig. 1). *B. subtilis* DB-104 was transformed with a ligation mixture and grown on DM3¹⁵⁾ containing chloramphenicol (10 µg/ml). The transformant showed a halo on the N-II agar (pH 8.0) containing 1% (w/v) skim milk, while *B. subtilis* DB-104 carrying pHW1 formed very small halo around the colony (Fig. 2). The protease production by the transformant was about 3,000 units per 100 ml culture.

The *B. subtilis* DB-104-expressed alkaline protease was investigated for its optimum pH and thermostability at high alkaline pH, which were typical features of 221 protease. The enzyme was most active at a pH range of 11.5–12.0 and stable at 30–50°C at pH 11.0 (Fig. 3). These results indicated that the cloned protease in *B. subtilis* DB-104 was identical to that of *Bacillus* strain 221. In addition, the antiserum against the alkaline protease 221 cross-reacted with the cloned enzyme but did not cross-react with other subtilisin-like enzymes such as AH-101 alkaline protease,²¹⁾ subtilisin Carlsberg,⁴⁾ and subtilisin BPN⁷³⁾ (data not shown).

Nucleotide sequence of the gene for 221 protease

The nucleotides of the cloned gene and its flanking regions were sequenced (Fig. 4). There was an open reading frame between nucleotides 1 and 1140 that encoded a polypeptide of 380 amino acids. This open reading frame was preceded by a putative Shine-Dalgarno (SD) sequence²²⁾, 5'-AGGAGG-3', with a spacing of 7 bases and two putative sequences that are homologous to δ^{32} (P1) and δ^{29} (P2) promoters of *B. subtilis*, respectively (Fig. 4). The open reading frame was followed by two sequences which may form hairpin structures with clusters of T's, the structure resembling a rho-independent transcription terminator of *E. coli*.

Deduced amino acid sequence of 221 protease

The deduced amino acid sequence had a pre-pro-peptide of 111 residues followed by the mature protease comprising 269 residues (26.7 kDa). The amino acid sequence shared an overall homology to subtilisin-like enzymes of bacilli (Fig. 5), suggesting that the overall structure of 221 protease is similar to that of the other subtilisin-like enzymes. There were 54 residues out of 269 residues (enclosed in dark boxes) conserved only among 221 protease, YaB elastase,⁷⁾ and PB92 protease⁸⁾ (Fig. 5).

Discussion

The DNA sequence analysis showed that 5'-flanking sequence of 221 protease was almost identical to that of PB92 except for three bases at positions –105, –108, and –23 (Fig. 4). In the coding sequence of 221 protease, the third position of codons coding Val45, Ala178, Ala194, Gly255, and Ala284 and the second position of codon coding Ser196 differed from those of PB92 protease (Fig.

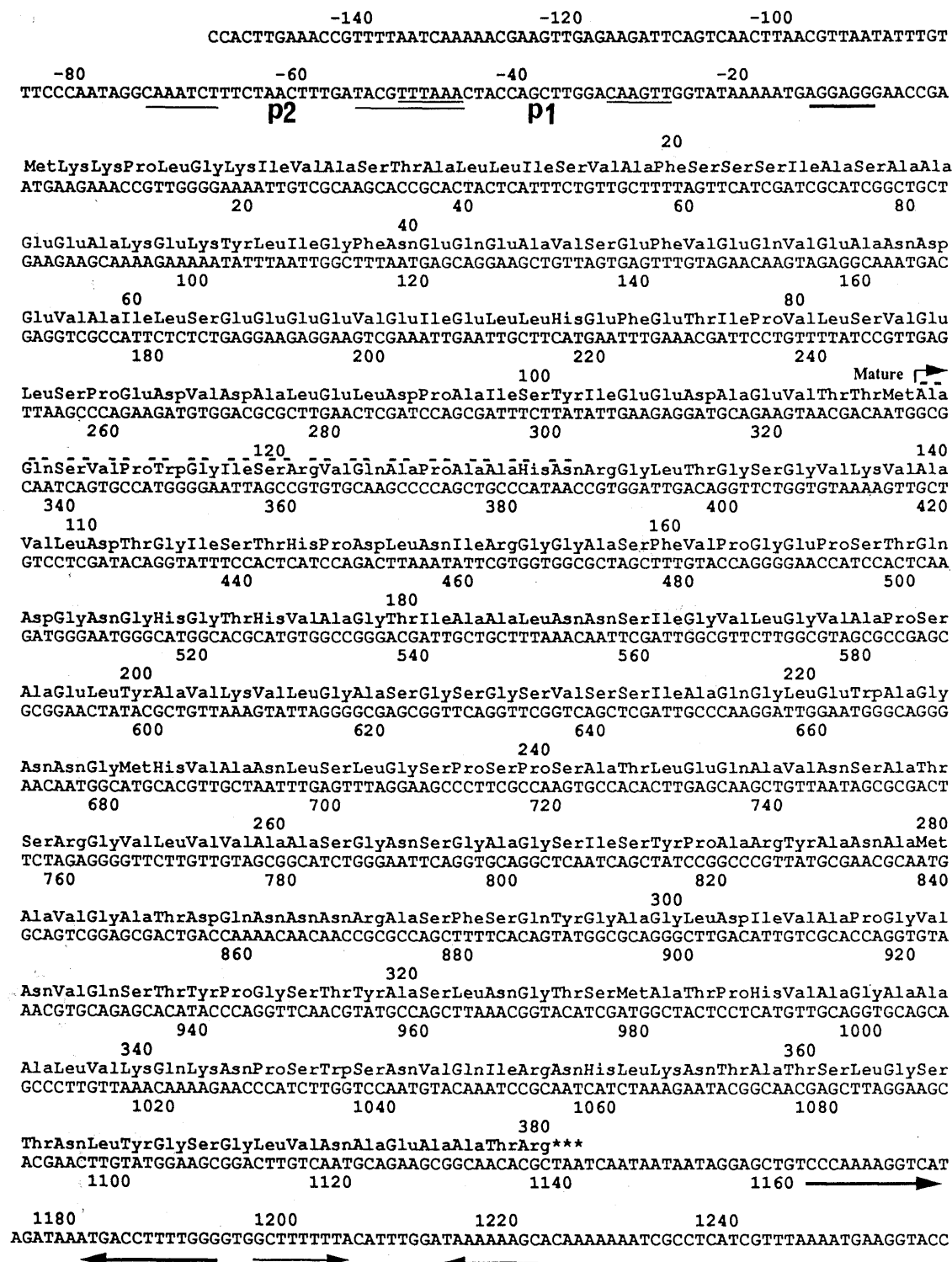


Fig. 4. Nucleotide Sequence of the Gene for 221 Protease and Deduced Amino Acid Sequence.

Both stands were sequenced from several independent overlapping fragments. The putative SD sequence and two promoters (P1 and P2) are underlined. The putative transcriptional terminator is shown by the arrows ($\rightarrow\leftarrow$). The N-terminal sequence of 18 residues of mature enzyme deduced from DNA sequence is identical to that of 221 protease from *Bacillus* sp. 221 (-----).²⁾

4). Consequently, the mature 221 protease was identical to PB92 protease except that Ser196 (Ser87 in the mature enzyme) was replaced by Asn in PB92 protease and it is interesting to consider an effect of this amino acid replacement on enzymatic properties of these two proteases. Laan *et al.* described how *Bacillus* strain PB92 produced a high-alkaline protease (PB92 protease) with unique pH optimum of 10.5—12⁸⁾ by quotation from the work of Zuidweg *et al.*²³⁾ However, we can not compare both

enzymes and strains since the properties of PB92 protease and the characteristics of *Bacillus* strain PB92 were not described at all in this paper.²³⁾ On the other hand, the DNA sequences in 3' flanking region entirely differ from those of PB92 and two characteristic sequences which may form hairpin structures with clusters of T's were observed, suggesting that these two strains should be distinct from each other. The 221 protease also showed very high homology (82.1%) with YaB elastase from alkaliphilic

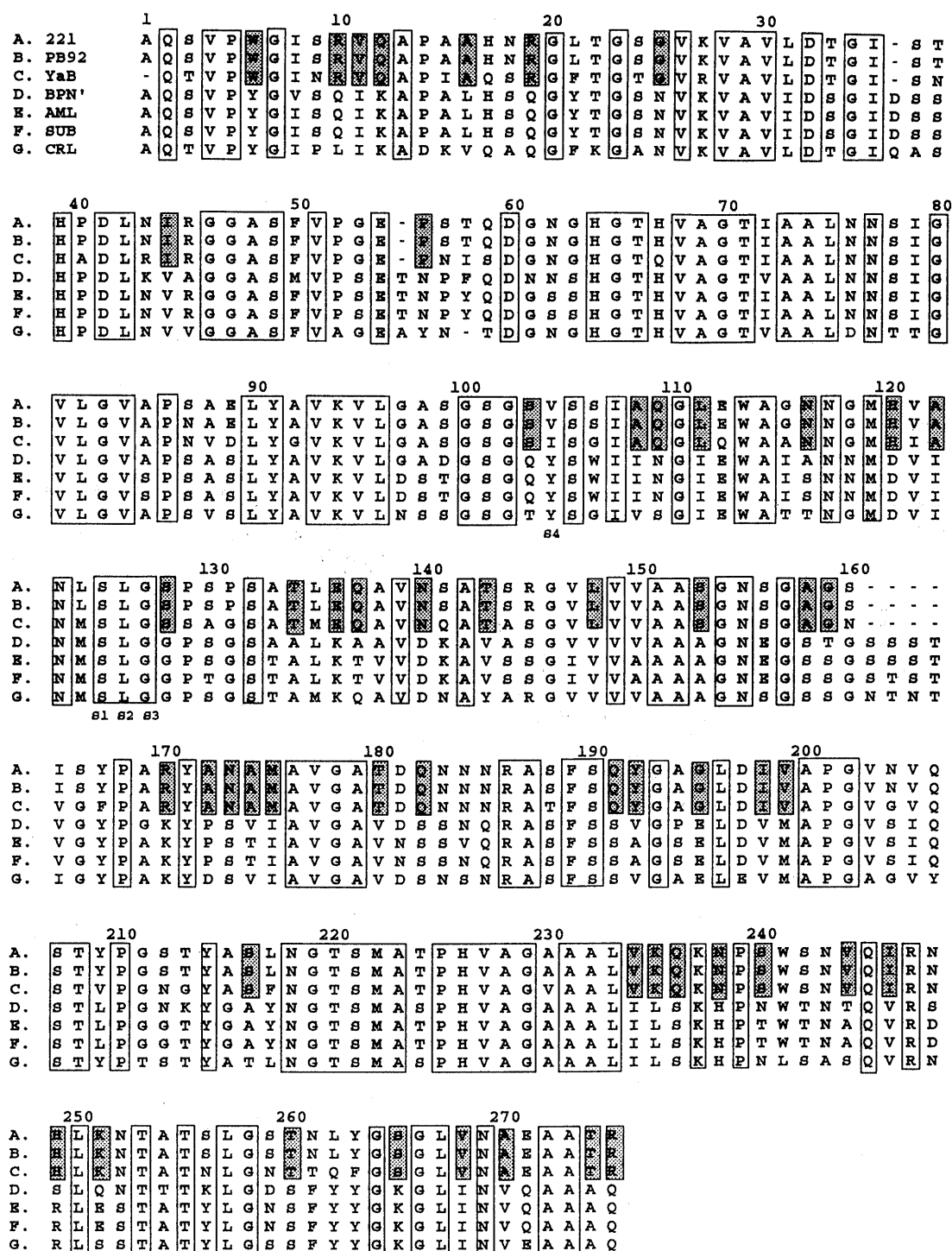


Fig. 5. Amino Acid Sequences of 221 Mature Enzyme and Other Subtilisin-like Enzymes.

The amino acid sequences enclosed in the boxes (□) and in the dark boxes (■) are common sequences among all of subtilisin-type enzymes and among the enzymes from alkaliphilic bacilli respectively. 221, 221 protease; YaB, YaB elastase⁷; PB92, PB92 protease⁸; BPN', subtilisin BPN'³; AML, *B. subtilis* var. *amylosacchariticus* subtilisin⁶; SUB, *B. subtilis* subtilisin⁵; CRL, subtilisin Carlsberg.⁴

Bacillus strain, which had very similar enzymatic and physicochemical properties. The evolutionary distance from a common ancestor among these alkaline protease from alkaliphilic bacilli seemed to be close to each other.

The deduced amino acid sequence of 221 mature protease shares moderate homology with those of subtilisins from neutrophilic bacilli. The 221 protease showed 61.7% identity with subtilisin Carlsberg,⁴ 61.3% with subtilisin amylosacchariticus,⁶ 61.0% with *B. subtilis* subtilisin,⁵ and 60.6% with subtilisin BPN'.³ A three-dimensional structural model has been proposed for subtilisin BPN'.²⁴

The regions containing the catalytic triad composed of Asp32, His64, and Ser221 of subtilisin BPN'²⁵ were well conserved in 221 protease as well as other subtilisin-type enzymes (Fig. 5). The sequences including subsites S₁, S₂, and S₃ (Ser125-Leu126-Gly127) of subtilisin BPN' were also conserved in 221 protease (Fig. 5).

The subtilisin-like enzymes from alkaliphilic bacilli were distinguished from subtilisins from neutrophilic bacilli by common amino acid sequences among those from alkaliphiles. These were deletions at positions 36, 55, and 161–164 (Fig. 5). It was pointed out that Gly166 formed

the bottom of P₁ pocket of subtilisin BPN'.²⁷⁾ On the basis of this finding, Kaneko *et al.*⁷⁾ suggested that the deletion of four amino acids at the region between 161 and 164 might change the conformation of P₁ pocket and deduced that this distortion correlated with the P₁ preference of YaB elastase for Ala, in contrast to that of subtilisin BPN' for Tyr. Since 221 protease, which shared the same deletion, also had high elastolytic activity^{28,29)} and similar cleavage pattern on oxidized insulin B-chain,³⁰⁾ this conformational change of the P₁ pocket might also happen in this protease. Furthermore, 54 amino acid residues in the enzymes from alkaliphiles are commonly different from those in the enzymes from neutrophiles. It is especially interesting that 11 amino acids are different in charge; ex., Arg19 (alkaliphile)→Gln19 (neutrophile) (Fig. 5). It seems likely that such diversity is related to characteristic properties of alkaline proteases from alkaliphiles.

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