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Molecular cloning, nucleotide sequence and expression of the structural gene for a thermostable alkaline protease from *Bacillus* sp. no. AH-101

Hideto Takami[1], Tetsuo Kobayashi[2], Rikizo Aono[1], and Koki Horikoshi[1,2]

[1] Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama, Kanagawa 227, Japan
[2] Microbiology Laboratory, The Institute of Physical and Chemical Research, 2-1, Hirosawa, Wako, Saitama 351-01, Japan

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Summary. Alkaliphilic *Bacillus* sp. no. AH-101 produces an extremely thermostable alkaline serine protease that has a high optimum pH (pH 12-13) and shows keratinolytic activity. The gene encoding this protease was cloned in *Escherichia coli* and expressed in *B. subtilis*. The cloned protease was identical to the AH-101 protease in its optimum pH and thermostability at high alkaline pH. An open reading frame of 1083 bases, identified as the protease gene, was preceded by a putative Shine-Dalgarno sequence (AAAGGAGG) with a spacing of 11 bases. The deduced amino acid sequence revealed a pre-pro-peptide of 93 residues followed by the mature protease comprising 268 residues. AH-101 protease showed slightly higher homology to alkaline proteases from alkaliphilic bacilli (61.2% and 65.3%) than to those from neutrophilic bacilli (54.9-56.7%). Also AH-101 protease and other proteases from alkaliphilic bacilli shared common amino acid changes and a four amino acid deletion when compared to the proteases from neutrophilic bacilli. AH-101 protease, however, was distinct among the proteases from alkaliphilic bacilli in showing the lowest homology to the others.

Introduction

There have been extensive studies on alkaline proteases from various strains of *Bacillus* (Horikoshi 1991). *Bacillus* species, including alkaliphilic bacilli, produce and secrete subtilisin-like alkaline serine proteases. The alkaline proteases from alkaliphilic bacilli show higher pH optima (pH 11-12) than those from neutrophilic bacilli (pH 10.5). *Bacillus* sp. no. AH-101 produces an extremely thermostable alkaline protease (Takami et al. 1989). This protease is characteristic in its highly alkaline pH optimum (pH 12-13), thermostability at high alkaline pH and keratinolytic activity (Takami et al. 1989, 1990).

Correspondence to: H. Takami

Genes for alkaline serine proteases from neutrophilic bacilli such as *B. amyloliquefaciens* (subtilisin BPN′), *B. licheniformis* (subtilisin Carlsberg), *B. subtilis* and *B. subtilis* var. *amylosacchariticus* (Wells et al. 1983; Jacobs et al. 1985; Stahl and Ferrari 1984; Yoshimoto et al. 1988) have been cloned and sequenced. Recently, the genes encoding alkaline serine proteases from alkaliphilic *Bacillus* strains YaB (Kaneko et al. 1989) and PB92 (Laan et al. 1991) have also been cloned and sequenced. Nucleotide and amino acid sequences of these subtilisin-like enzymes share significant homology although these enzymes are distinct from each other in their enzymatic and physicochemical properties. It is considered that subtilisin-like enzymes from *Bacillus* strains should be classified into a family along with alkaline proteases and that these enzymes have arisen from a common ancestor.

We isolated the gene encoding AH-101 thermostable alkaline protease, to enable structural and functional comparisons with genetic approaches. In this report, we describe cloning, sequencing, and expression of the gene for the AH-101 protease.

Materials and methods

Bacterial strains, plasmids and media. Alkaliphilic *Bacillus* sp. no. AH-101 was used as a DNA donor (Takami et al. 1989). *Escherichia coli* MV1184 and XL1 blue were used as host strains for cloning. *B. subtilis* DB-104 (*nprE18 nprR2 ΔaprE3 his-101*) carrying lesions in the structural genes for alkaline and neutral proteases (Kawamura and Doi 1984) was also used as a host strain for expression of the gene for AH-101 protease. Plasmids, pUC 119 and Bluescript II (KS and SK), were purchased from Takara Shuzo (Kyoto, Japan) and Stratagene (Calif., USA), respectively. Plasmid, pAHB1, was constructed by replacing the 635 bp *Hin*dIII/*Sac*I fragment of pHW1 (Horinouchi et al. 1982a, b) with the *Hin*dIII/*Sac*I fragment of the multi cloning site from pUC119.

A-II medium (pH 9.5) consisted of 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $Mg_2SO_4 \cdot 7H_2O$, and 2% $NaHCO_3$ (Horikoshi 1971). N-II medium (pH 8.0) contained the ingredients of A-II medium except 2% $NaHCO_3$. *E. coli* was aerobically grown in LB (Luria-Bertani) (Enquist and Sternberg 1979) broth at 37° C. *Bacillus* sp. no. AH-

101 and *B. subtilis* DB-104 were grown aerobically at 37° C in A-II broth and in N-II broth, respectively. Antibiotic selective medium contained ampicillin (150 µg/ml) or chloramphenicol (10 µg/ml).

DNA manipulations. Chromosomal DNA of *Bacillus* sp. no. AH-101 was prepared by phenol treatment (Saito and Miura 1963). Plasmid DNA was isolated from *E. coli* or *B. subtilis* by the alkaline extraction procedure (Birnboim and Doly 1979). *E. coli* was transformed by the $CaCl_2$ method (Mandel and Higa 1970). *B. subtilis* was transformed by the protoplast method (Chang and Cohen 1979). Southern hybridization (Southern 1975) was performed using a digoxigenin-labelled DNA probe and antidigoxigenin antibody coupled to alkaline phosphatase using a DNA labelling and detection kit (Boehringer, Mannheim, FRG).

Preparation of DNA probe. The first 20 amino acid residues of the N-terminal amino acid of 20 residues of AH-101 protease has been determined previously (Takami et al. 1990). The amino acid sequence around the catalytic centre of subtilisin BPN′ (Gly210-Ala223 and Phe225-Leu233) is conserved among all subtilisin-like enzymes so far reported (Wells et al. 1983; Stahl and Ferrari 1984; Jacobs et al. 1985; Yoshimoto et al. 1988; Kaneko et al. 1989; Laan et al. 1991). Since AH-101 protease can be classified in a family of alkaline proteases from *Bacillus* strains (Takami et al. 1990), it was considered possible that the region around the catalytic centre of AH-101 protease is also conserved. The amino acid sequence of N-terminal residues, Gln14-Gln-Ala-His-Asn-Arg-Gly-Ile21 of AH-101 protease, and the conserved amino acid sequence around the catalytic centre (His226-Val-Ala-Gly-Ala-Ala-Ala-Leu233, see Fig. 5), allowed us to design oligonucleotide primers to amplify a part of the gene for AH-101 protease by polymerase chain reaction (PCR) (Oste 1989). DNA primers (23 nucleotides) of 5′-CARCARGCNCAMAAMCGNGGNAT-3′ (N-terminus) and 5′-CAMGTNGCNGGNGCNGCNGCNMT-3′ (C-terminus) were synthesized with an ABI DNA synthesizer Model 391 (Applied Biosystems Japan, 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; T and C for M. The intact chromosomal DNA of strain AH-101 was used as template DNA for the PCR. The conditions for a cycle of PCR using *Taq* DNA polymerase were determined according to the supplier's recommendations (Perkin Elmer Cetus, Conn., USA). A single 600-bp fragment amplified by PCR was purified by preparative agarose gel (1%) electrophoresis and labelled with digoxigenin-deoxyuridine triphosphate (dUTP) using a DNA labelling kit (Boehringer). The labelled PCR fragment was used as a DNA probe to detect transformants carrying the gene for AH-101 protease.

Cloning of the gene for AH-101 thermostable alkaline protease. Chromosomal DNA of strain AH-101 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* MV1184 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, UK) overlaid on LBA plates. The nylon membranes carrying transformants were transferred onto M9 plates containing casamino acids (0.4% w/v) and chloramphenicol (500 µg/ml) to amplify plasmids. The nylon membranes were processed according to the supplier's recommendations for colony hybridization.

Preparation of cloned enzyme. B. subtilis DB-104 carrying pAH101B, which contained the whole gene for AH-101 protease, was aerobically grown at 37° C in 50 ml N-II broth for 24 h. 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).

Protease assay. Caseinolytic activity was assayed by the method described previously (Takami et al. 1989).

DNA sequencing. Restriction fragments were cloned into appropriate restriction enzyme sites of pUC119 or Bluescript II. Nucleotide sequence of each fragment was determined according to the dideoxy method (Sanger et al. 1977) using an ABI DNA sequencer Model 370A (Applied Biosystems Japan).

Results

Cloning of the gene for thermostable alkaline protease from alkaliphilic Bacillus sp. no. AH-101

A single 600-bp DNA fragment was amplified by PCR from the chromosomal DNA of strain AH-101 using the primers described in Materials and methods, and used as a DNA probe. Out of approximately 1500 colonies from a subgenomic library made of *Sph*I digested, size-fractionated (<3.8 kb) chromosomal DNA of strain AH-101, only one colony showed hybridization to the probe. This transformant harboured a plasmid (pAH101N) containing the 3.8-kb *Sph*I fragment, which hybridized to the probe described above (data not shown). A partial DNA sequence of pAH101N indicated that the 3.8 kb *Sph*I fragment contained a part of the structural gene for AH-101 protease. We could find the N-terminal amino acids of 20 residues of AH-101 protease and the conserved amino acids around the catalytic centre region among subtilisin-like enzymes within the sequenced region. However the 3.8-kb *Sph*I fragment did not contain a stop codon for the structural gene.

A similar approach was used to clone the 3′-terminal end of the structural gene. The chromosomal DNA was digested with *Hin*dIII and *Bcl*I. DNA fragments around 1.0 kb, the size of which showed hybridization to the digoxigenin-labelled *Sph*I fragment of pAH101N, were purified, and ligated with *Hin*dIII-*Bam*HI digested pUC119. The pAH101N probe was used as a probe to detect a transformant carrying a plasmid (designated pAH101C, Fig. 1) containing the *Hin*dIII-*Bcl*I fragment on which the C-terminal region of the enzyme was encoded. The inserts in plasmid pAH101C and pAH101N overlapped by 340 bp. The complete structural gene for AH-101 protease was constructed by recombining the N-terminal and C-terminal regions at a common *Ban*III site. The resulting plasmid, pAH101NC contained the complete sequence for AH-101 protease (28.8 kDa) (Fig. 1).

Expression of the gene for thermostable alkaline protease AH-101 in B. subtilis DB-104

E. coli carrying pAH101NC did not produce a detectable amount of alkaline protease. The *Hin*dIII-*Sac*I fragment from pAH101NC was ligated with *Hin*dIII-*Sac*I-

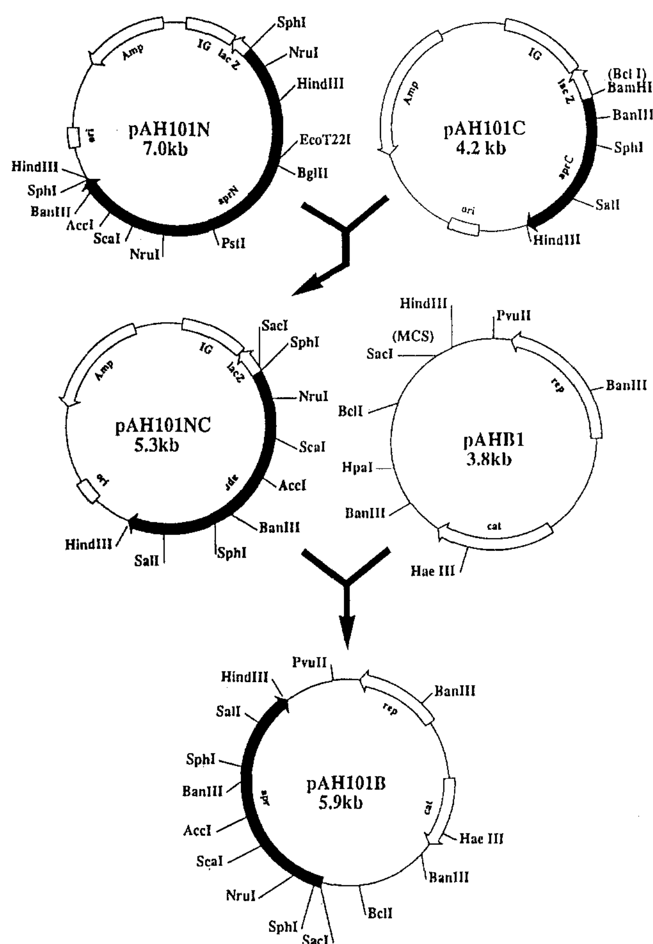

Fig. 1. Plasmid constructions. The insertions in vector pUC119 or pAHB1 are indicated by *thick black lines*. The *arrows* within plasmids represent the direction of transcription of the structural gene. The whole structural gene for AH-101 protease was constructed by recombining pAH101N and pAH101C by using the *Ban*III site on both inserts: Amp, ampicillin resistance gene; IG, intergenic region; cat, chloramphenicol acetyl transferase gene; MCS, multicloning site

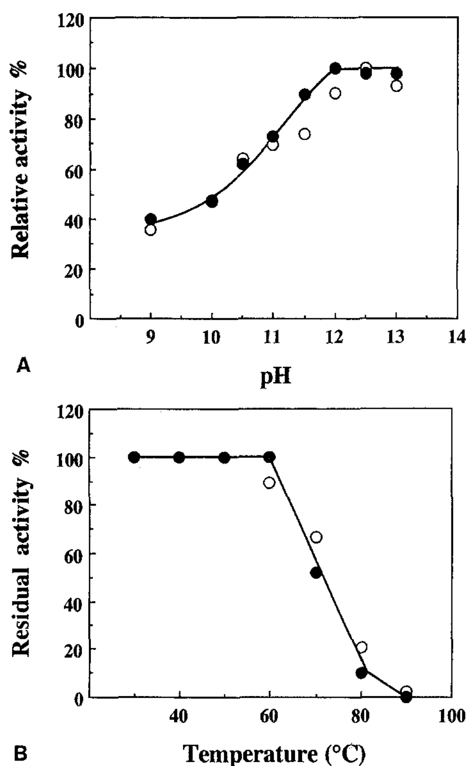

Fig. 2A, B. Properties of the cloned protease. The properties of cloned protease (○) were investigated and compared with AH-101 protease from *Bacillus* sp. strain AH-101 (●) in their pH optima and thermostability under high alkaline pH (pH 11.0) according to methods described previously (Takami et al. 1989). **A** Effect 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** Effect of temperature on enzyme stability. The enzyme was added to 50 mM glycine-50 mM NaCl-NaOH buffer (pH 11), incubated for 10 min, and the residual activities were measured

digested pAHB1. *B. subtilis* DB-104 was transformed with the ligation mixture and grown at 37° C for 2 days on DM3 (Chang and Cohen 1979) containing chloramphenicol (10 μg/ml). The transformant, carrying a plasmid, containing the structural gene for AH-101 protease (designated pAH101B), exhibited a large halo on an N-II agar plate (pH 8.0) containing 1% (w/v) skim milk (data not shown). The protease production by the transformant was about 3000 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. The enzyme was most active at pH 12–13 and stable at 30–70° C at pH 11 (Fig. 2). These results indicated that the enzyme produced by *B. subtilis* DB-104 was identical to that of strain AH-101. In addition, the antiserum against AH-101 protease cross-reacted with the enzyme produced by *B. subtilis* DB-104 (pAH101B) but did not cross-react with other subtilisin-like enzymes such as YaB elastase, 221 protease, subtilisin Carlsberg, and subtilisin BPN′ (data not shown).

Nucleotide sequence of the gene for AH-101 thermostable alkaline protease

The nucleotide sequence of the cloned gene and its flanking regions was determined (Fig. 3). There was an open reading frame between nucleotides 1 and 1083 that encoded a polypeptide of 361 amino acids. This open reading frame was preceded by a putative Shine-Dalgarno (SD) sequence (Shine and Dalgarno 1974), 5′-AAAGGAGG-3′, with a spacing of 11 bases (Fig. 3). There was no *B. subtilis* consensus promoter sequence upstream of the SD sequence. The open reading frame was followed by a sequence which may form a hairpin structure with a cluster of T's, the structure resembling the rho-independent transcription terminator of *E. coli*. Free energy calculated by the method of Zuker and Stiegler (1981) for the hairpin structure was −30.6 kcal/mol.

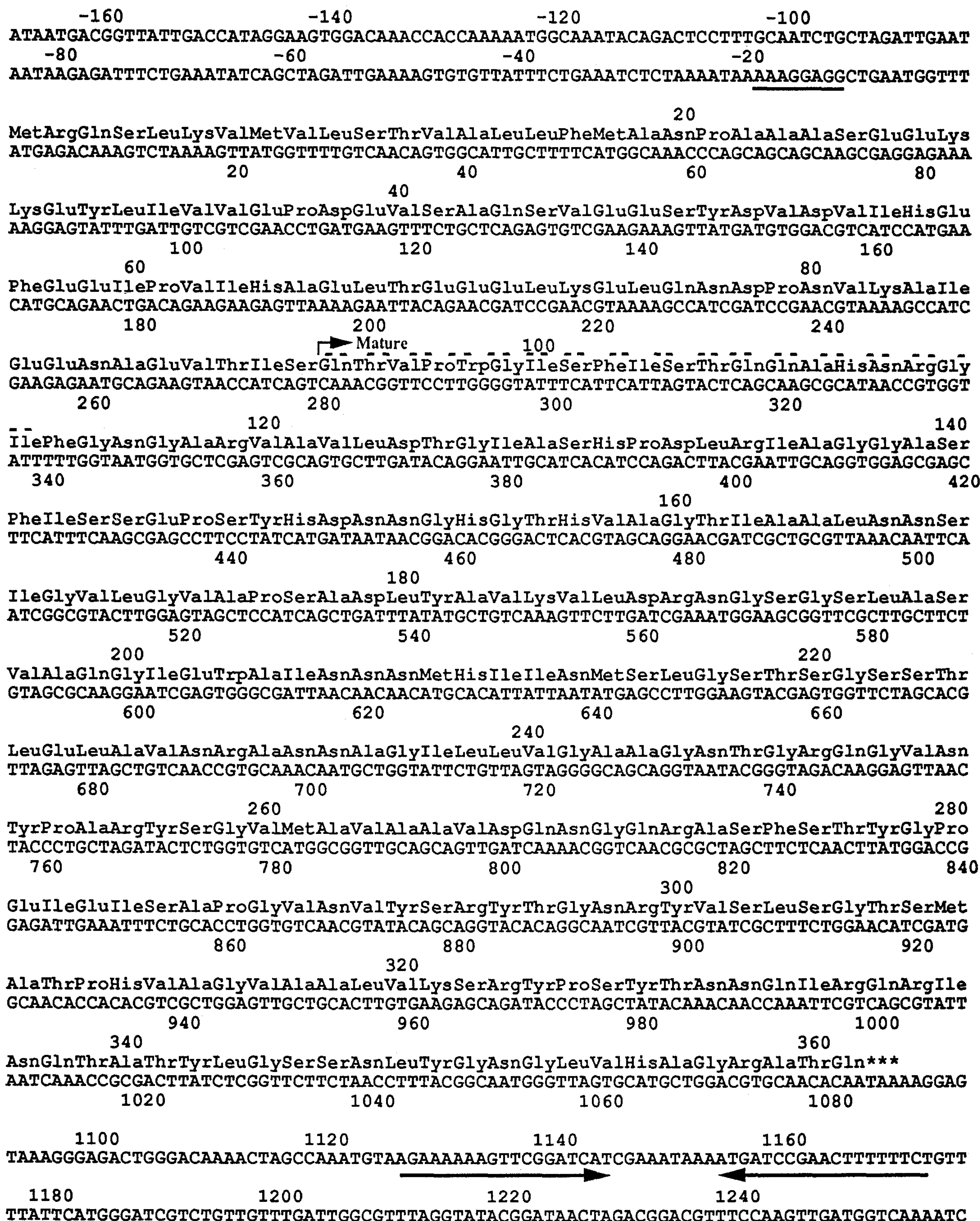

Fig. 3. Nucleotide sequence of the gene for AH-101 protease and deduced amino acid sequence. Both strands were sequenced from several independent overlapping fragments. The putative Shine-Dalgarno (SD) sequence (—) is observed at 12 bases upstream from the translation start site (ATG). The putative transcriptional terminator is shown by the *arrows* (→←). The N-terminal 20 sequence of mature enzyme deduced from the DNA sequence is identical to that of the AH-101 protease from *Bacillus* sp. no. AH-101 (-----) (Takami et al. 1990)

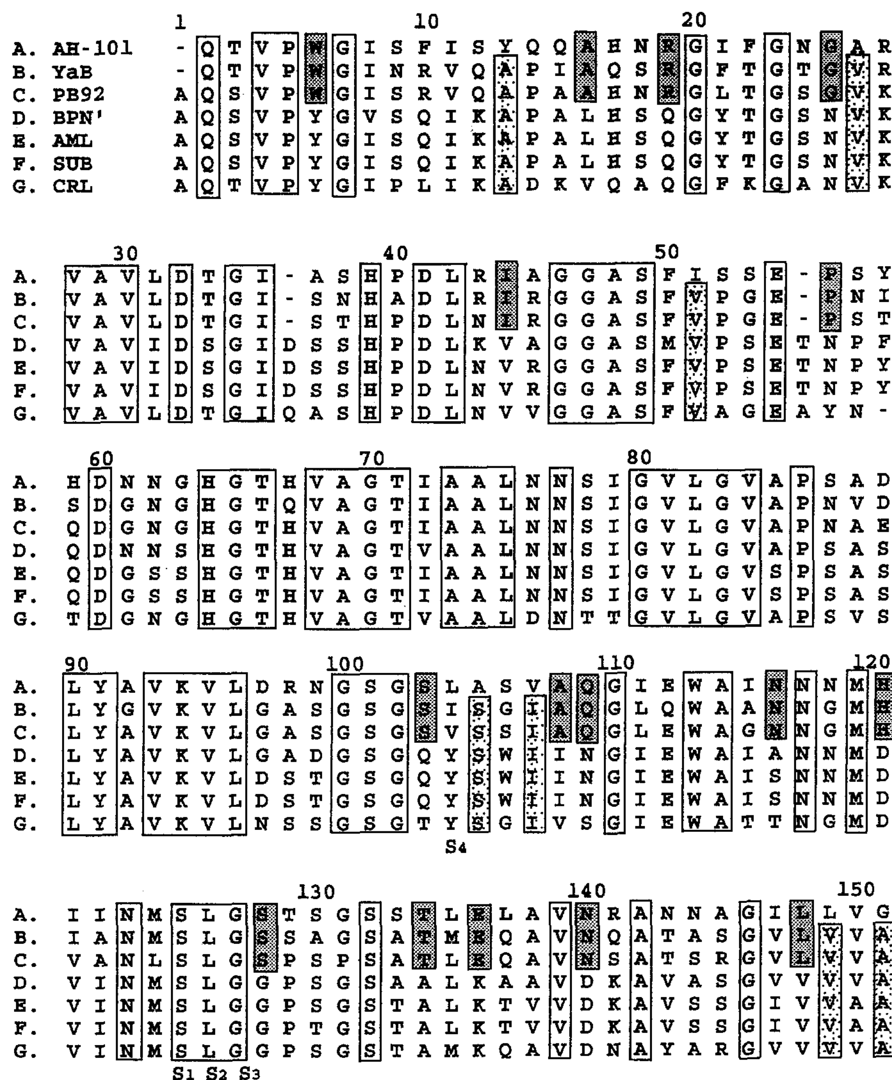

Fig. 4. Amino acid sequences of AH-101 mature enzyme and other subtilisin-like enzymes. The amino acid sequences enclosed in the *white boxes* (□) and in the *dark boxes* (▩) are common sequences among all of the subtilisin-like enzymes and among the enzymes from alkaliphilic bacilli, respectively. Those enclosed in the *stipped boxes* (▨) are common sequences among subtilisin-like enzymes except the alkaline protease AH-101: AH-101 protease, YaB elastase (YaB) (Kaneko et al. 1989); PB92 protease (PB92) (Laan et al. 1991); subtilisin BPN′ (BPN′) (Wells et al. 1983). *B. subtilis* var. *amylosacchariticus* (AML) subtilisin (Yoshimoto et al. 1988); *B. subtilis* subtilisin (SUB) (Stahl and Ferrari 1984); subtilisin Carlsberg (CRL) (Jacobs et al. 1985)

Table 4 continuated on page 106

Deduced amino acid sequence of AH-101 thermostable alkaline protease

The deduced amino acid sequence revealed a pre-propeptide of 93 residues followed by the mature protease comprising 268 residues (27.8 kDa). The amino acid sequence showed an overall homology to those of subtilisin-like enzymes of bacilli (Fig. 4), suggesting that the overall structure of AH-101 protease is similar to that of other subtilisin-like enzymes. There were 29 residues out of 268 residues (enclosed in dark boxes) conserved only among AH-101 protease, YaB elastase, and PB92 protease (Fig. 4). An additional common feature among these three enzymes was deletions of amino acid residues at positions 36, 55 and 161–164 when compared to subtilisins from neutrophilic *Bacillus* strains (Fig. 4). Several amino acids (16 out of 268), conserved among all the other enzymes, were unique in the AH-101 protease.

Discussion

Secretory proteins are generally synthesized with a signal peptide for translocation across a cell membrane (Watson 1984). In the case of the subtilisin-like enzymes of bacilli, the signal peptide is cleaved off within the conserved sequence, Ala-Ser/Gln-Ala-*Ala (where the asterisk represents the cleavage site) (Fig. 5). The conserved sequence is followed by glutamic acid in both YaB elastase and PB92 protease from alkaliphilic bacilli. The conserved sequence was not observed in the 93 residues preceding AH-101 mature protease; however, we were able to deduce the cleavage site as Ala22-Ala-Ala-*Ser-Glu by considering Ser as an equivalent to Ala. The putative signal peptide of 24 residues of AH-101 protease showed homologies of approximately 30% with YaB elastase and PB92 protease, and homologies of below 20% with the subtilisins from neutrophilic bacilli.

The deduced amino acid sequence of AH-101 mature protease was overall homologous to the subtilisin-like enzymes of bacilli. The homologies among the mature forms of subtilisin-like enzymes are shown in Fig. 6. AH-101 protease showed 56.3% identity with subtilisin

160 170

A. AH-101 A A G N T G R Q G - - - - V N Y P A R Y S G V M A V A
B. YaB A S G N S G A G N - - - - V G F P A R Y A N A M A V G
C. PB92 A S G N S G A G S - - - - I S Y P A R Y A N A M A V S
D. BPN' A A G N E G S T G S S S T V G Y P G K Y P S V I A V G
E. AML A A G N E G S S G S S S T V G Y P A K Y P S T I A V G
F. SUB A A G N E G S S G S T S T V G Y P A K Y P S T I A V G
G. CRL A A G N S G S S G N T N T I G Y P A K Y D S V I A V G

180 190 200

A. A V D Q N G Q R A S F S T Y G P E I E I S A P G V N V Y S R
B. A T D Q N N N R A T F S Q Y G A G L D I V A P G V G V Q S T
C. A T D Q N N N R A S F S Q Y G A G L D I V A P G V N V Q S T
D. A V D S S N Q R A S F S S V G P E L D V M A P G V S I Q S T
E. A V N S S N Q R A S F S S A G S E L D V M A P G V S I Q S T
F. A V N S S N Q R A S F S S A G S E L D V M A P G V S I Q S T
G. A V D S N S N R A S F S S V G A E L E V M A P G A G V Y S T

210 220 230

A. Y T G N R Y V S L S G T S M A T P H V A G V A A L V K S R Y P
B. V P G N G Y A S F N G T S M A T P H V A G V A A L V K Q K N P
C. Y P G S T Y A S L N G T S M A T P H V A G A A A L V K Q K N P
D. L P G N K Y G A Y N G T S M A S P H V A G A A A L I L S K H P
E. L P G G T Y G A Y N G T S M A T P H V A G A A A L I L S K H P
F. L P G G T Y G A Y N G T S M A T P H V A G A A A L I L S K H P
G. Y P T S T Y A T L N G T S M A S P H V A G A A A L I L S K H P

240 250 260 270

A. S Y T N N Q I R Q R I N Q T A T Y L G S S N L Y G N G L V H A
B. S W S N V Q I R N H L K N T A T N L G N T T Q F G S G L V N A
C. S W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A
D. N W T N T Q V R S S L Q N T T T K L G D S F Y Y G K G L I N V
E. T W T N A Q V R D R L E S T A T Y L G N S F Y Y G K G L I N V
F. T W T N A Q V R D R L E S T A T Y L G N S F Y Y G K G L I N V
G. N L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V

A. G R A T Q
B. E A A T R
C. E A A T R
D. Q A A A Q
E. Q A A A Q
F. Q A A A Q
G. E A A A Q

Fig. 4. (continued)

1 10 20

A. AH-101 Met Arg Gln Ser Leu Lys Val Met Val Leu Ser Thr Val Ala Leu Leu Phe Met Ala Asn Pro Ala
B. PB92 Met Lys Lys Pro Leu Gly Lys Ile Val Ala Ser Thr Ala Leu Leu Ile Ser Val Ala Phe Ser Ser
C. YaB Met Asn Lys Lys Met Gly Lys Ile Val Ala Gly Thr Ala Leu Ile Ile Ser Val Ala Phe Ser Ser
D. BPN' Met Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe
E. AML Met Arg Ser Lys Lys Leu Trp Ile Ser Leu Leu Phe Ala Leu Thr Leu Ile Phe Thr Met Ala Phe
F. SUB Met Arg Ser Lys Lys Leu Trp Ile Ser Leu Leu Phe Ala Leu Thr Leu Ile Phe Thr Met Ala Phe
G. CRL Met Met Arg Lys Lys Ser Phe Trp Leu Gly Met Leu Thr Ala Phe Met Leu Val Phe Thr Met Ala

30

A. AH-101 Ala Ala ↓ - - - - - -
B. PB92 Ser Ile Ala Ser Ala - - -
C. YaB Ser Ile Ala Gln Ala - - -
D. BPN' Gly Ser Thr Ser Ser Ala Gln Ala
E. AML Ser Asn Met Ser Ala Gln Ala -
F. SUB Ser Asn Met Ser Ala Gln Ala -
G. CRL Phe Ser Asp Ser Ala Ser Ala -

Fig. 5. Comparison of the putative signal sequence of AH-101 protease with those of other subtilisin-like enzymes (for abbreviations, see Fig. 4). The amino acid sequences enclosed in the *boxes* (□) are identical with those of AH-101 protease. The *arrow* indicates where the deduced signal peptide deduced is cleaved

BPN′. A three-dimensional structural model has been proposed for subtilisin BPN′ (Wright et al. 1969). The charge relay system of the catalytic triad of subtilisin BPN′ was found to be composed of Asp32, His64, and Ser221 and the deprotonation of His64 was closely related to pH-dependence of the catalytic activity (Carter and Wells 1988). These residues and amino acid sequences around these residues were found to be well conserved in the AH-101 protease, as is the case for other subtilisin-like enzymes (Fig. 4). The sequences including subsites S_1, S_2 and S_3 (Ser125–Leu126–Gly127) of subtilisin BPN′, and the sequences (Ala152–Ala153–Gly154) forming the wall of the active-site crevice of subtilisin BPN′ (Robertus et al. 1972) were also conserved in AH-101 protease (Fig. 4). From these results, it is reasonable to consider that the three-dimensional structure of AH-101 protease is overall similar to that of subtilisin BPN′.

The subtilisin-like enzymes from alkaliphilic bacilli were distinguished from subtilisins from neutrophilic bacilli by the amino acid sequences that were common among those of alkaliphiles. These were deletions at positions 36, 55 and 161–164 (Fig. 4). It was pointed out that Gly166 formed the bottom of the P_1 pocket of subtilisin BPN′ (Estell et al. 1986). This Gly166 was replaced with asparagine in the AH-101 protease. On the basis of this finding, Kaneko et al. (1989) suggested that the deletion of four amino acids in the region between 161 and 164 may change conformation of the P_1 pocket and deduced that this distortion correlated with the P_1 preference of YaB elastase for Ala, in contrast to that of subtilisin BPN′ for Tyr. Since AH-101 protease, which shared the same deletion, also exhibited high elastolytic activity and preferentially hydrolysed ester bonds of alanine (Takami et al. 1992), this conformational change of the P_1 pocket may also occur in this protease. Furthermore, 29 amino acid residues in the enzymes from akaliphiles were commonly different from those in the enzymes from neutrophiles. Interestingly, five of these amino acids showed charges of: Arg (alkaliphile)→Glu19 (neutrophile), His→Asp120, Glu→Lys136, Asn→Asp140, and Lys→Leu235 (Fig. 4). Although AH-101 protease shared common features among subtilisin-like enzymes from alkaliphiles, unique amino acid replacements in AH-101 protease were observed in 16 residues of 268 residues, ex., Arg (AH-101)→Thr208. These replacements may be related to the characteristic features of AH-101, the extreme thermostability at high alkaline pH and keratinolytic activity.

The subtilisin-like enzymes including AH-101 protease, showed high homologies of over 54% with each other (Fig. 6), suggesting that they should be classified in a family of alkaline proteases and may arise from a common ancestor. AH-101 protease was relatively more homologous to YaB elastase and PB92 protease from alkaliphilic bacilli than to the proteases from neutrophilic bacilli. Among the proteases from alkaliphiles, AH-101 protease showed low homologies of below 66% to the others compared to the very high homology of 82.5% observed between YaB elastase and PB92 protease. These results may suggest that AH-101 protease is evolutionarily distant from the other proteases of alkaliphiles.

	AH-101	YaB	PB92	CRL	BPN′	SUB
YaB	61.2					
PB92	65.3	82.5				
CRL	54.9	58.6	61.3			
BPN′	56.3	56.3	60.2	69.3		
SUB	56.3	55.2	60.6	69.7	86.2	
AML	56.7	55.2	61.0	69.7	86.9	99.3

Fig. 6. Similarity matrix based on the homologies among seven subtilisin-like enzymes from the genus of *Bacillus*. For calculations of homology, only those amino acids that could be aligned in all seven enzymes were considered (i.e., gaps were not counted)

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