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Research paper

Molecular and structural characterization of a surfactant-stable high-alkaline protease AprB with a novel structural feature unique to subtilisin family

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ABSTRACT

High-alkaline proteases are of great importance because of their proteolytic activity and stability under high-alkaline condition. We have previously isolated a new protease (AprB) which has potential industrial applications based on its high-alkaline adaptation. However, the molecular and structural basis for alkaline adaptation of this enzyme has not been fully elucidated. In the present study, AprB gene was cloned and expressed in the *Bacillus subtilis* WB600. This gene codes for a protein of 375 amino acids comprised with a 28-residual signal peptide, a 78-residual pro-peptide, and a 269-residual mature protein. The deduced amino acid sequence has the highest homology of 63.2% with that of the high-alkaline proteases. Recombinant AprB was purified and determined to be monomeric with molecular mass of 26.755 kDa. The NH_2-terminal sequence of the purified AprB was A-Q-S-I-P-W-G-I-E-R. This enzyme exhibited high catalytic efficiencies (K_{cat}/K_m) towards natural, modified, and synthesis substrates with optimal activity at 60 °C and pH 10. AprB was stable over a wide range of pH 5 to 11 and various surfactants, and could be activated by Mg^{2+}, Ca^{2+} and Ba^{2+}. The structural properties of AprB, like a higher ratio of $R/(R+K)$, a larger area of hydrophobic surface, increased number of ion pairs formed by Arg residue, and the exposure of Asp active residue on the surface, might be responsible for its alkaline adaptation. In contrast with members of subtilisin family, such as M-protease and subtilisin BPN′, AprB harbored a high content of Glu and Asp residues, and a low content of Arg and Lys residues on the surface. Interestingly, these structural characters were similar with that of psychrophilic proteases, which suggested that these molecular factors were not restricted in the psychrophilic proteases, and therefore were not solely responsible for their cold-adaptation. Our results reveal a novel structural feature of AprB unique to subtilisin family and provide clues for its alkaline adaptation.

1. Introduction

Proteins of the subtilase superfamily have been identified in a diverse range of organisms including achaea, bacteria, fungi, yeasts, higher eukaryotes, and even viruses [1,2]. Subtilisins, one clan of the subtilase superfamily, play an important role in the environmental and agricultural fields, and have received extensive interest for their proteolytic properties, which make them useful in various industrial application [3–7]. Currently, based on the amino acid sequences, subtilisin family has been divided into six clans, i.e., true-subtilisins, high-alkaline proteases, intracellular proteases, oxidatively stable proteases, high-molecular-mass subtilisins and phylogenetically intermediate subtilisins [8].

High-alkaline proteases, the majority of which are extracellularly produced by alkaliphilic microorganisms, are one of the important members of subtilisin family. It is well known that the increase of protein function and stability is one of the major strategies for extremophiles to deal with extreme environments [9]. Therefore, enzymes from the alkaliphilic microorganisms have developed specific structural and catalyzing properties towards high-alkaline condition which can be used to adapt to harsh industrial desire, especially for the modern compact detergents [9,10]. Since the first discovery of protease no. 221, an increasing number of high-alkaline proteases have been successfully isolated and characterized [11–13]. For example, M-protease, one of the intensively studied high-alkaline protease from alkaliphilic *Bacillus clausii* KSM-K16, have been identified to be stable between pH 6–11

Abbreviation: AprB, alkaline protease from *Bacillus sp.* B001; PCR, polymerase chain reaction; ORF, open reading frame; SDS-PAGE, soduim dodecyl sulphate polyacrylamide gel electrophoresis; kDa, kilo Dalton; bp, base pairs; aa, amino acids; wt/vol, weight/volume; vol/vol, volume /volume; AAPF, *N*-succinyl-L-Ala-Ala-Pro-Phe-p-nitroanilide; R(or Arg), arginine; K (or Lys), lysine; E (or Glu), glutamic acid; D (or Asp), aspartic acid.

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with optimal pH at 12.3 and have been used as additives in compact heavy duty laundry detergents [8]. Although the sequences and structures of several members of the subtilase superfamily, such as M-protease (PDB code 1MPT), psychrophilic proteinase *Vibrio* (PDB code 1SH7), thermitase (PDB code 1THM), subtilisin BPN′ (PDB code 1UPS) and Carlsberg (PDB code 2SEC) have been determined and applied to analyze the mechanism of the adaptation of thermophilic and psychrophilic condition [10,11,14–16], structural analysis of high-alkaline proteases is still relatively limited. Determining the 3D structures is of particular importance to provide insights into the structural principles that control the variety of enzyme properties and further conduct the molecular direct evolution [17,18].

We previously obtained a new high-alkaline protease (AprB) from an alkaliphilic *Bacillus* sp. B001 [19]. AprB shows high similarity with high-alkaline proteases from *B. clausii*, *Bacillus alcalophilus*, and *Bacillus lentus* based on partial amino acid sequences. The fact that AprB has optimal catalytic temperature at 60 °C, optimal pH at 10.0, and stability towards surfactants and oxidants makes this enzyme potentially useful for various industrial applications. In the present study, the full nucleotide sequence of AprB was determined through degenerate and reverse PCR. This enzyme was then expressed in the *B. subtilis* and further purified by anion exchanger and gel filtration. The 3D structure of AprB was also modeled and compared with other reported subtilisins, which provide clues for the alkaline adaptation of AprB and reveal a novel structural feature unique to subtilisin family.

2. Materials and methods

2.1. Bacterial strain, plasmids and media

Bacillus sp. strain B001 was aerobically cultured in the medium (pH 10) containing (per liter) 10 g glucose, 5 g tryptone, 5 g yeast extract, 1 g KH_2PO_4, 0.2 g $MgSO_4$, and 10 g Na_2CO_3 as previously described [19]. *B. subtilis* WB600 (Δ *nprE*, Δ *aprA*, Δ *epr*, Δ *bpf*, Δ *mpr*, Δ *npr*B), a six extracellular protease deficient strain, was used as bacterial host for expression of alkaline protease AprB [20]. The plasmid pWB980 was used to express protein [21]. *Escherichia coli* DH5α (F$^-$ *supE44 Φ80 δlacZ ΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17* (r_K^-, m_K^+) *deoR thi-1* λ^- *gyrA96 relA1*) was used as clone strain, which cultured in Luria Bertani (LB) medium composed of (per liter) 10 g peptone, 5 g yeast extract, and 5 g NaCl. The medium used for expressing recombinant protein was 2YT (pH 7.0) containing (per liter) 10 g of yeast extract, 16 g of tryptone, and 5 g of NaCl.

2.2. Cloning of the alkaline protease AprB gene

Genomic DNA from *Bacillus* sp. strain B001 was extracted as described previously [22]. A 467-bp gene fragment of AprB was amplified with a pair of degenerate primers as reported before [19]. To determine the complete nucleotide sequence of the gene for AprB protease, a pair of reverse primers WY311 and WY312 (Table 1) were designed according to the known DNA sequence. The genomic DNA of *Bacillus* sp. strain B001 was completely digested with *EcoR* I or *Sph* I, and the fragments were self-ligated at 16 °C overnight after purification. Aliquots of the ligation mixture were used as template DNA for reverse PCR which were optimized using a thermocycler (Eppendorf Mastercycler pro). The PCR was finally performed at 94 °C for 30 s, 30 cycles with denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 5 min, the last cycle was for 5 min at 72 °C. A 0.7-kbp and a 7.2-kbp fragment were amplified and purified, and then ligated into the pMD19T vector (TaKaRa, Japan). Subsequently, these DNA fragments were determined by an automated DNA sequencer (ABI 3730xl DNA analyzer, USA). The nucleotide sequence of protease gene had been deposited in the EMBL/GenBank/DDBJ database under accession no. GU136486. The deduced amino acid sequence was compared with those of close proteases obtained from the EMBL/GenBank/DDBJ databases.

Table 1
Primers used for cloning and expression of alkaline protease AprB.

Primers	Nucleotide sequences (5′ to 3′)	References
WY168	CAY GGN ACN CAY GTN GC	[19]
WY169	GC CAT NGA NGT NCC	[19]
WY311	GAAAGCACGTACCCAGGTGGAGGTTATG	This study
WY312	AACACCAGGAGCGACTAAGTCAAGACCC	This study
WY346	TCGCAAGCTTTTGCCGCAGAGGAGAAAAAATCTTATTTA	This study
WY347	CTAGATGTCGACCTATTGAACGGCATTTTCGGCATTCAATA	This study
WB102	TTGCTGAGGTGGCAGAGGGCAGGTT	This study
WB103	TCTTCATCGTTCATGTCTCCT	This study
WB104	AAGGAGACATGAACGATGAAGAAAAGATCAAACG	This study

2.3. Plasmid construction for expression of alkaline protease AprB

A set of primers WY346 and WY347 (Table 1) with *Hind* III and *Sal* I restriction sites was designed to amplify the pro- and mature protein of AprB gene. The amplified fragment digested with *Hind* III and *Sal* I was inserted into the plasmid pWB980 at the *Hind* III and *Sal* I sites, and introduced into the *B. subtilis* WB600 by a high osmolarity electroporation method [23]. A positive clone that contained recombinant plasmid (designated pSacBAprB) and exhibited hydrolysis halos on LB plates containing 1% casein was selected and confirmed by DNA sequencing. Then the signal peptide of AprB replaced the SacB signal peptide in the plasmid pSacBAprB by overlapping PCR using the primers WB102, WB103, WB104 and WY347 (Table 1). The overlapping PCR reaction was carried out in a final volume of 50 μl, using 0.2 μM each of the four dNTPs, 0.16 μM $MgCl_2$, 1.25 U *Taq* DNA polymerase (TaKaRa *Pyrobest*™ DNA Polymerase, Japan) with 1× *Taq* buffer, 0.4 μM each of the primer WB102 and WY347, and 1 μl of DNA templates. The P43 promoter located upstream of the SacB signal peptide in the plasmid pWB980 and the full open reading frame (ORF) of AprB gene in the chromosome DNA were amplified with the primers WB102/WB103 and WB104/WY347, respectively, and were further purified and used as the DNA templates. The PCR processes were operated under the following conditions: 5 min of initial denaturation at 94 °C, followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 58 °C, and 1.5 min at 72 °C, respectively. Final extension was carried out at 72 °C for 5 min. The resultant PCR product, which contained a P43 promoter in the NH_2-terminal sequence of AprB ORF, was digested with *Sac* I and *Sal* I, and then ligated into the pWB980 vector digested with the same restriction enzymes. The recombinant plasmid, named pFullAprB, was cloned into *B. subtilis* WB600 cells by using the high osmolarity electroporation method [23].

2.4. Protein purification

Overnight cultures of *B. subtilis* WB600 expressing AprB were inoculated in 2YT medium supplemented with 50 μg/ml kanamycine for 24 h at 37 °C. The supernatant from cultures was collected and precipitated by $(NH)_2$ SO_4 to 80% saturation, and the mixture was incubated overnight at 4 °C. The precipitate was collected by centrifugation, dissolved in the buffer containing 50 mM Tris–HCl, 50 mM NaCl, and 5% (wt/vol) glycerol (pH 7.9), and dialyzed overnight against the same buffer to remove the residual ammonium sulfate. The crude extract was purified to be homogeneous through a RESOURCE Q (6.4 × 30 mm diameter) and a Superdex 75 10/300 GL (6.4 × 30 mm diameter) high-performance columns using

an ÄKTA purifier system (Amersham Biosciences, Piscataway, NJ, UAS) as previously reported [19]. Protein concentration was determined according to the method of Bradford [24] using bovine serum albumin as a standard.

2.5. SDS-PAGE and zymogram analysis

SDS-PAGE was performed on a 3% stacking gel and a 10% running gel referred to the method of Laemmli [25] and protein bands were visualized by staining with Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany). Zymography was performed on SDS-PAGE as described before [19]. The molecular mass of the purified protein was determined by SDS-PAGE and confirmed by LC-Ion Trap MS spectrum (Finnigan LCQ Deca xp plus Ion Trap Mass Spectrometer, USA).

2.6. NH_2-terminal sequencing

Protein bands of AprB on SDS-PAGE gel were blotted electrically onto a polyvinylidene difluoride membrane (Roche, USA). The membrane was stained with 0.1% (wt/vol) Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany) in 40% (vol/vol) methanol and 1% (vol/vol) acetic acid, and destained with 50% (vol/vol) methanol. NH_2-terminal sequences of the purified AprB expressed in two ways were determined by automated Edman protein degradation using a protein sequencer (ABI Procise™ 492cLc Protein Sequencing System, Applied Biosystems, USA).

2.7. Determination of proteolysis properties of AprB

Protease activity was determined in 50 mM Tris–HCl (pH 8.0) containing 3% azocasein (Sigma, USA) as reported before [19]. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1.0 mM of azodye per minute. The optimum temperature of the enzyme against azocasein was determined by incubating the reaction mixtures in 50 mM Tris–HCl (pH 8.0) at different temperatures (30–90 °C). Thermal stability assay was carried out by incubating 5 μl of enzyme solution at various temperatures (30–70 °C) for 30 min before the residual enzyme activities were determined. The effect of pH on enzyme activity against azocasein was measured at different pH values (5–13) at 60 °C. For pH stability, the enzyme was incubated with different pH buffers at 25 °C for 5 h before the residual enzyme activity was determined.

The effects of various metal ions on the enzyme activities of AprB were determined by incubating the reaction mixtures with $CaCl_2$, $MgCl_2$, $BaCl_2$, $MnCl_2$, $NiSO_4$, $CoCl_2$, $FeSO_4$, $FeCl_3$, and $CuSO_4$ at the concentration of 10 mM. To evaluate the effects of surfactants and oxidants, enzyme solutions were incubated with 1 M guanidine hydrochloride (GnHCl), 1 M urea, 5% (vol/vol) Tween 20, 40, 60, and 80, and 5% (vol/vol) Triton X-100 for 72 h, and with 1% (wt/vol) SDS, 1% (wt/vol) sarkosyl, and 1% (vol/vol) H_2O_2 for 1 h at 25 °C before the residual activity was measured. To evaluate the effects of inhibitors on the enzyme activity, AprB was pre-incubated with 10 mM dithio-bis-nitrobenzoic acid (DTNB), 1% (wt/vol) soybean tryptin inhibitor (SBTI), 10 mM dithiothreitol (DTT), 10 mM β-mercaptoethanol, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min at 25 °C before the residual activity was assayed. The activity of enzyme assayed in the absence of any chemical agents was set at 100%.

2.8. Kinetic measurements

Using natural protein (casein), modified protein (azocasein), and synthetic peptides (AAPF) as substrates, the kinetic parameters of purified protease AprB were determined by Lineweaver-Burk curves. Proteolytic activity was assayed at 60 °C for 5 min by mixing purified protease AprB and assay buffer (5 mM $CaCl_2$–50 mM glycine–NaOH buffer, pH 10.0) containing each substrate at a concentration of 0.06–5 mM. Each assay was carried out in triplicate. One unit of enzyme activity was defined as the amount of enzyme that released 1.0 mM of product (tyrosine, azodye or *p*-Na) per minute under experimental condition [26,27].

2.9. Molecular modeling and structural analysis

Based on the crystal structure of the alkaline protease BL from *B. lentus* (PDB code, 1ST3), the structure of AprB was modeled by Swiss-Model (http://swissmodel.expasy.org/) [28] and the quality of final model was analyzed with QMEANclust [29]. Hydrogen bonds and salt bridges defined as an ion pair with an interatomic distance within 4 Å between two oppositely charged residues (Asp, Glu, Arg, and Lys) were calculated through the Whatif web interface (http://swift.cmbi.ru.nl/servers/html/) [30]. The solvent accessible surfaces were calculated by the Getarea (http://curie.utmb.edu/) [31].

3. Results and discussion

3.1. Cloning and the deduced amino acid sequence of AprB

AprB gene was obtained from the genomic DNA of *Bacillus* sp. B001 through degenerate PCR and reverse PCR. The degenerate primers WY168 and WY169 [19] were synthesized on the basis of the two highly conserved sequences around catalytic amino acids His and Ser of known proteases (Fig. 1). A 467-bp DNA fragment was obtained and then used to design the reverse primers WY311 and WY312 (Fig. 1). The reverse PCR was carried out using the ligation mixture of the genomic DNA of *Bacillus* sp. B001 digested with *Eco*R I or *Sph* I as template. In this way, a complete gene sequence consist of 1128 bp was obtained to encode a new extracellular alkaline protease, starting with an ATG codon and ending with a TAG codon. A potential Shine-Dalgarno sequence (5′-AGGAGG-3′) was identified to be 9 bases upstream from the ATG initiation codon and a putative transcription terminator was located 10 nucleotides downstream from the stop codon TAG (Fig. 1). The deduced 375 amino acids sequence was composed of a 28-amino acid signal peptide, a 78-amino acid pro-peptide and a 269-amino acid mature protein with calculated molecular mass of 26.7 kDa and isoelectric point (pI) of 4.0. Similar molecular mass and prepropeptide region have been reported in other extracellular alkaline proteases from bacteria [18,32,33]. The catalytic triads of AprB were determined to be the Asp32, His62 and Ser215 included in the mature peptides, which is a common property in the serine proteases of subtilase superfamily [2].

3.2. Expression and purification of recombinant AprB

Protease activities with clear dissolving zones around colonies in the LB plates containing 1% casein were detected in the recombinant *B. subtilis* WB600 cells harboring pSacBAprB and pFullAprB, whereas no protease activity was observed around the WB600 cells harboring pWB980 (Fig. 2A). Similarly, the SDS-PAGE gel (Fig. 2B) also revealed that proteins around 29 kDa were obviously expressed in the recombinant strains *B. subtilis* WB600/pSacBAprB (band 7) and *B. subtilis* WB600/pFullAprB (band 4); while no corresponding protein was detected in the *B. subtilis* WB600/pWB980 cells (band 6). However, the expression levels of AprB in the cells *B. subtilis* WB600/pSacBAprB and *B. subtilis* WB600/pFullAprB were different, and represented 2×10^3 U/mL

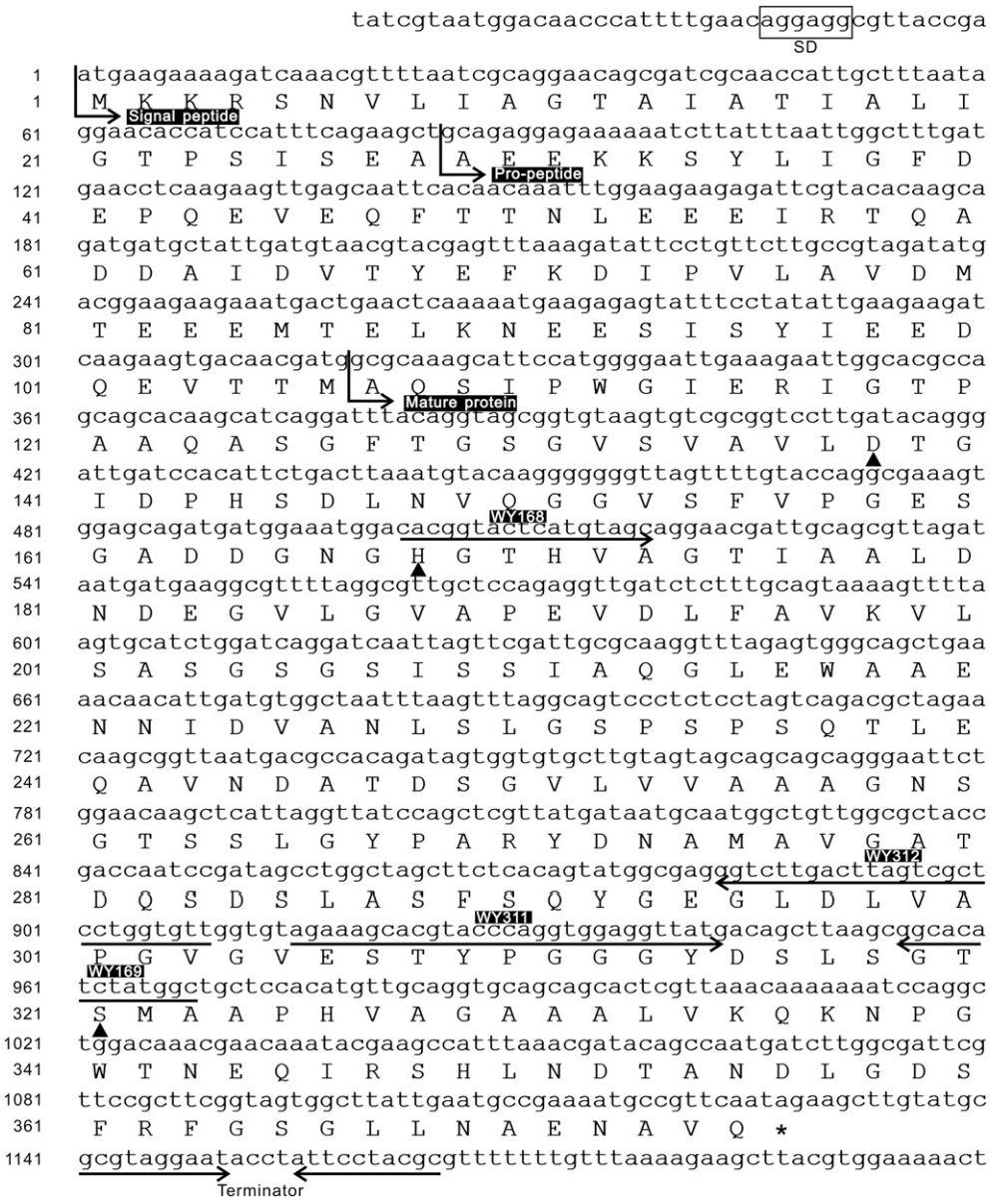

Fig. 1. Nucleotide and deduced amino acid sequences of AprB. "SD" indicates the putative ribosome biding site. The active sites and the stop codon are indicated as "▲" and "*", respectively. The start amino acids of signal peptide, pro-peptide and mature protein, the nucleotide sequences of primers, and potential terminator are indicated as arrows.

and 0.8×10^3 U/mL, respectively, after cultured in the same condition. Thus, compared with the SacB signal peptide from *B. subtilis*, the efficiency of recognition and transportation of protein in the recombinant cells by signal peptide from AprB was decreased by 2.5 times. These observations indicate that membrane transport proteins in *B. subtilis* can recognize the signal peptide of AprB and transport the protein into the media, although the similarity between the two signal peptides is relatively low (about 20.7%).

AprB from the cell-free supernatant of recombinant cells harboring pSacBAprB and pFullAprB were both purified to be homogeneous bands on SDS-PAGE gel (Fig. 2B, bands 2 and 9). They were monomeric with the same molecular size, which was further confirmed to be 26.755 kDa by LC-Ion Trap MS spectra (Fig. 2C). This value was in agreement with the deduced molecular mass (26.7 kDa).

The NH_2-terminal sequences of purified AprB from two recombinant strains *B. subtilis* WB600/pSacBAprB and *B. subtilis* WB600/pFullAprB also gave the same results, which were determined to be A-Q-S-I-P-W-G-I-E-R corresponding to the amino acids 107 to 117 of the putative protein sequence (Fig. 1). The NH_2-terminal sequences of two recombinant proteins confirmed the putative sequences of signal peptide, pro-peptide and mature peptide of AprB. All of the above findings suggest that AprB with different signal peptides has similar transportation, maturation and folding processes in the *B. subtilis*, but with different efficiencies.

3.3. Sequence similarity analysis of AprB

Compared with proteins in the EMBL/GenBank/DDBJ database, the amino acid sequence of AprB exhibited the highest similarities about 62.9–63.2% with that of M-protease, BP92, 221 and BL from *B. clausii* KSM-K16, *B. alcalophilus* PB92, *B. alcalophilus* 221, and *B. lentus*, respectively [13,16,34,35]. AprB also exhibited 60.4–61.0% similarities with protease AprN from *Bacillus* sp. B21-2 and protease

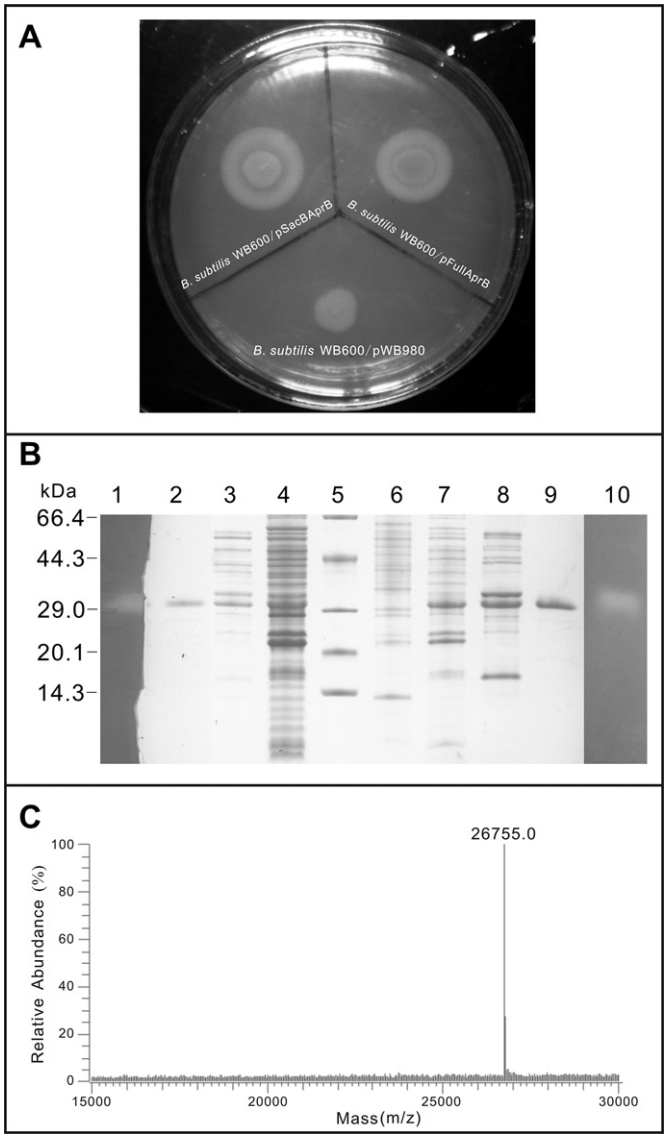

Fig. 2. The hydrolysis halos of recombinant cells *B. subtilis* WB600/pSacBAprB, *B. subtilis* WB600/pFullAprB and *B. subtilis* WB600/pWB980 on the LB plates contained 1% casein (A). SDS-PAGE and zymogram analysis of the crude and purified extracellular proteases AprB expressed in the *B. subtilis* WB600 (B). Lane 1: proteolytic band of purified AprB from lane 2; lane 2: AprB from lane 3 further purified by gel filtration; lane 3: AprB from lane 4 purified by ion exchange chromatography; lane 4: the extracellular proteins from recombinant cells *B. subtilis* WB600/pFullAprB; lane 5: molecular weight of marker standard; lane 6: extracellular proteins from recombinant cells *B. subtilis* WB600/pWB980; lane 7: extracellular proteins from recombinant cells *B. subtilis* WB600/pSacBAprB; lane 8: AprB from lane 7 purified by ion exchange chromatography; lane 9: AprB from lane 8 further purified by gel filtration; lane 10: proteolytic band of purified AprB from lane 9. LC-Ion Trap MS spectrum of purified recombinant AprB (C). The molecular mass of AprB was determined to be 26755.0 Da.

YaB from *Bacillus* sp. YaB [36,37]. However, AprB had less than 52% similarities with other proteases (Table 2). The homologies of amino acid sequences were obviously different among the signal peptide, pro-peptide and mature protein of AprB with that of other proteases. The mature protein of AprB had relatively higher homologies compared with that of other proteases, but the signal peptide and pro-peptide exhibited lower similarities. For example, the signal peptide of AprB only had the highest identity of 32.0% with that of ALP1 from *Bacillus* sp. NKS-21, but their mature proteins exhibited moderate similarity about 58.4%. Based on the sequence alignments of alkaline proteases, AprB exhibits the closest relationship with the high-alkaline proteases. Within this family, the mature amino acid sequence of AprB is most similar (about 72%) to that of M-protease, PB92, 221 and BL [13,16,34,35].

Table 2
Amino acid alignment of AprB and its closest members of subtilisin family.

Source of alkaline protease	Identities with AprB (%)			
	Signal peptide	Propeptide	Mature protein	Full ORF
High-alkaline proteases				
M-protease/PB92/221 /BL	19.2	43.6	72.1–72.5	62.9–63.2
AprN /YaB	23.1	43.6	69.8–69.0	60.4–61.0
AprM /AH101	5.3	36.2–40.6	57.5	50.7–51.5
ALP1	32.0	30.3	58.4	49.0
True-subtilisins				
Carlsberg	17.9	22.1	59.1	49.3
BPN′	25.9	23.5	58.0	49.1
E	23.1	25.0	57.2	48.9
Phylogenetically intermediate subtilisins				
ALTP	28.6	23.2	54.29	46.1
Oxidatively stable proteases				
KP-43	17.9	11.5	28.3	24.9

3.4. Proteolysis properties of recombinant AprB

The optimal temperature and pH of purified recombinant AprB were 60 °C and 10.0, respectively. The recombinant AprB was still stable at the temperature of lower than 50 °C and in a broad pH range from 5.0 to 11.0 (Fig. 3). It could retain about 70% residual activity even after incubating at 50 °C for 30 min; however, AprB lost 80% of its activity after incubating at 60 °C for 30 min (Fig. 3A). The effects of various metal ions, inhibitors, surfactants and oxidants on the AprB were summarized in Table 3. This enzyme could be activated to variant degrees by Ca^{2+}, Mg^{2+}, and Ba^{2+}, but was strongly inhibited by Fe^{3+}, Ni^{2+}, Cu^{2+}, and Co^{2+}. As shown from Table 3, recombinant AprB exhibited high stability with 1 M GnHCl, 1 M urea, 5% Tween 20, 40, 60 and 80, and 5% Triton X-100 after incubated for 72 h at 25 °C. After incubated with 1% sarkosyl, SDS and H_2O_2 for 1 h, AprB retained 80–98% activities. The fact that AprB was strongly inhibited by PMSF and was no or only slightly inhibited by DTNB, SBT1, DTT, β-mercaptoethanol and EDTA, suggested that AprB was a serine protease without any disulfide bond. Therefore, the recombinant AprB exhibited high activities and stability towards a wide range of temperatures, pH and various surfactants, which made this enzyme potential useful for various industrial applications [27,32,38].

3.5. Kinetic parameters of recombinant AprB towards different substrates

Kinetic parameters of purified AprB indicated that it had maximum catalytic velocities (V_{max}) towards casein, azocasein, and AAPF of 12.54, 102.54, and 242.09×10^3 U/mg, respectively (Table 4). Moreover, the apparent K_m values for casein, azocasein, and AAPF revealed that the recombinant AprB protease had a substrate order preference of azocasin > casein > AAPF. The deduced K_{cat}/K_m values of AprB for casein, azocasein and AAPF were found to be 57.42, 906.31, and 151.01×10^3 min^{-1} mM^{-1}, respectively. These high catalytic efficiencies of AprB strongly indicates that it is a potential candidate used in the laundry additive and other commerce applications [7,9,32].

3.6. Homology modeling and secondary structural analysis of AprB

The overall molecular structure of AprB in Fig. 4A comprised nine α helices, one β sheets comprised of seven parallel strands, and

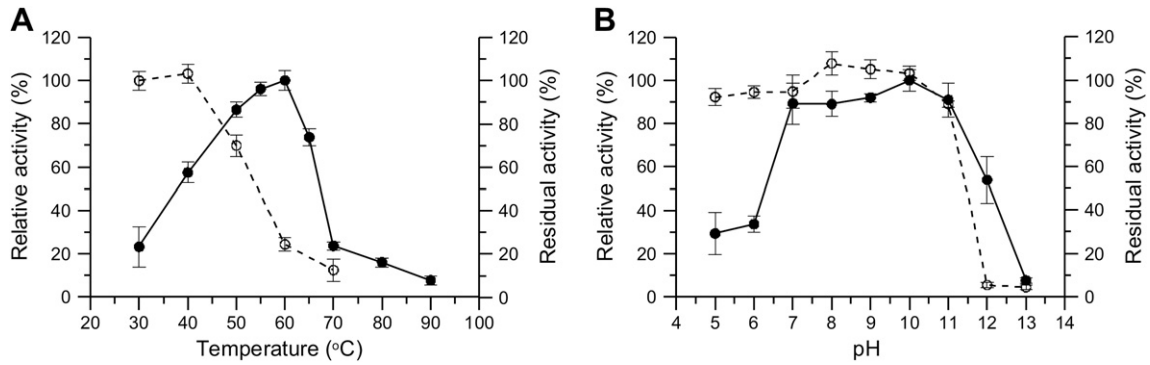

Fig. 3. Effects of temperature (A) and pH (B) on activity (solid lines) and stability (dashed lines) of recombinant AprB. The data are arithmetic means of triplicate independent experiments and the error bars indicate standard deviations.

one β sheets made of two anti-parallel stands. The catalytic triad of Asp32, His62 and Ser215, located closely in 3D structure but distantly in primary structure, made the catalytic triad of AprB. Based on the secondary structural alignment of AprB with its most related high-alkaline proteases M-protease (PDB code 1MPT) [39] and true-subtilisins BPN′ (PDB code 1SUP) [40], structural homology of these three enzymes was observed. All of AprB, M-protease, and BPN′ contained nine α helices and nine β sheets, and the third and sixth helices contained active residuals His62 and Ser215 were highly conserved among these proteases (Fig. 4b). However, several distinct parts were also observed: (i) BPN′ contained more alpha and beta turns between β1/β2, β2/α2 and β5/β6, compared to AprB and M-protease, owing to the multiple residues insertions. (ii) Although the second α helix, the sixth β sheet and the ninth α helix on the surface of AprB were identical with that of M-protease, they were different in comparison with that of BPN′. (iii) The fifth α-helix of AprB surface loops was shorter than the corresponding helix of M-protease and BPN′. (iv) The seventh β sheet of AprB was identical with that of BPN′, but was shorter than that of M-protease. All together, the high-alkaline proteases harbored more loops on the surface relative to the subtilisin counterpart. The structural differences of these three enzymes probably resulted from multiple amino acids insertions, deletions and replacements which might contribute to their different hydrolytic properties [41].

3.7. Structural features of AprB

The numbers of ion pairs and the contents of proline, glycine, and aromatic residues were similar between AprB and M-protease, and exhibited no significant distinct with that of BPN′ (Table 5). However, AprB contained less numbers of hydrogen bonds than the M-protease and BPN′. Besides, AprB exhibited an increased $R/(R+K)$ ratio and a higher content of Asp and Glu residues compared with that of BPN′. The total area of accessible surfaces was similar in the AprB, M-protease, and BPN′, but their composition was different. AprB and M-protease contained more hydrophobic residues on their surface compared to BPN′. Furthermore, smaller positively charged and larger positively charged surfaces of AprB were obviously different from that of M-protease and BPN′.

The comparison of structural features of AprB with those of M-protease and BPN′ provides some clues for the adaptation of AprB to alkaline condition. A frequently observed difference between the high-alkaline proteases and the less alkaline relatives is the ratio of $R/(R+K)$. As previously noted in the high-alkaline protease PB92, YaB, and M-protease, the ratio of $R/(R+K)$ was increased relative to the less alkaline homologues [37,39]. The relatively high $R/(R+K)$ was observed for AprB as well (Table 5), which might reveal the alkaline adaptation of AprB by retaining a positive charge under high-alkaline condition [39]. In addition, the number of ion pairs is not significantly distinct among the AprB, M-protease, and BPN′; however, the numbers of ion pairs formed by Arg in AprB and M-protease are more than non-alkaliphilic BPN′. The increased Arg ion pairs may be one of important components of alkaline adaptation as suggested by Shirai et al. [39,42]. It has been suggested that the hydrophobic effect plays important role in maintaining the stability of protein [10]. Thus the increased hydrophobic surfaces of high-alkaline proteases AprB and M-protease would be expected to contribute to their stability under high-alkaline condition [43]. Moreover, a notable difference in the surface of high-alkaline

Table 3
Effects of various reagents on the activity of the alkaline protease AprB.

Agents	Concentration	% Activity of origin	Agents	Concentration	% Activity of origin
Metal ions			Tween 40	5%[b]	95 ± 5.0
Ca^{2+}	10 mM	214 ± 6.2	Tween 60	5%[b]	105 ± 8.6
Mg^{2+}	10 mM	216 ± 1.5	Tween 80	5%[b]	107 ± 5.6
Ba^{2+}	10 mM	124 ± 5.2	Triton X-100	5%[b]	109 ± 5.2
Mn^{2+}	10 mM	128 ± 15.9	Sarkosyl	1%[a]	98 ± 36.0
Ni^{2+}	10 mM	4 ± 2.9	SDS	1%[a]	81 ± 5.6
Cu^{2+}	10 mM	13.4 ± 0.8	H_2O_2	1%[b]	97 ± 2.4
Co^{2+}	10 mM	11.4 ± 12.0	Inhibitors		
Fe^{3+}	10 mM	5.8 ± 3.7	DTNB	10 mM	109 ± 3.9
Fe^{2+}	10 mM	78 ± 9.0	SBT1	1%[a]	92 ± 2.9
Surfactants and oxidants			EDTA	10 mM	112 ± 4.6
GnHCl	1 M	112 ± 1.7	β-mercaptoethanol	10 mM	113 ± 1.3
Urea	1 M	117 ± 1.4	DTT	10 mM	99 ± 5.4
Tween 20	5%[b]	86 ± 5.5	PMSF	10 mM	2 ± 1.9

[a] wt/vol.
[b] vol/vol.

Table 4
Kinetic parameters of purified alkaline protease AprB.

Substrate	Apparent K_m (mM)	V_{max} ($\times 10^3$ U mg^{-1})	Apparent K_{cat} ($\times 10^3$ min^{-1})	K_{cat}/K_m ($\times 10^3$ min^{-1} mM^{-1})
Casein	0.44 ± 0.020	12.54 ± 0.374	250.86	57.42
azocasein	0.23 ± 0.003	102.57 ± 0.460	205.13	906.31
AAPF	3.21 ± 0.251	242.09 ± 19.764	484.18	151.01

proteases AprB and M-protease and non-alkaliphilic BPN′ is the active site Asp. As shown in Fig. 5, parts of the catalytic residue Asp-32 were found to be exposed on the surface strands of high-alkaline proteases AprB and M-protease, whereas the corresponding catalytic residue was hidden in the entry of BPN′. As a structure- and sequence-conserved basic residue in all subtilase families, the catalytic residue Asp plays a key role in delocalization of charged His by forming a very stable hydrogen bond [44]. Thus the exposure of catalytic Asp residue could enhance electrostatic interactions in high-alkaline condition and thus might contribute to the alkaline adaptation of AprB as well.

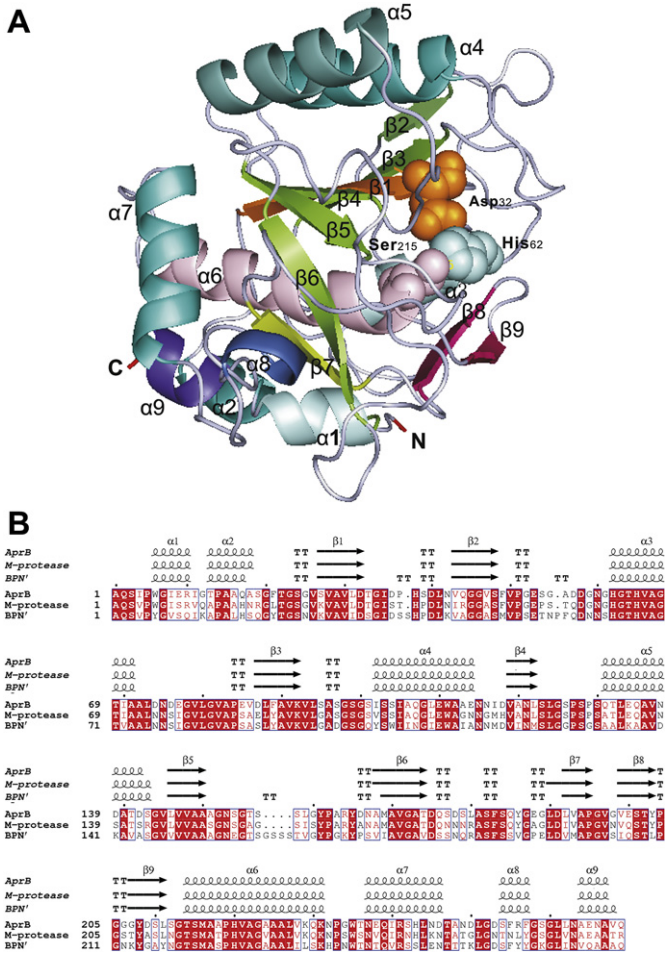

Fig. 4. Model of the overall 3-D structure of AprB (A) and secondary structural alignment of AprB, M-protease and BPN′ (B). (A) The letters "N" and "C" indicate the N and C terminus in red coils, respectively. α-helices and β-strands expressed as α1–9 and β1–9 are drawn as coils and flat arrows, respectively. The catalytic triad of Asp32, His62, and Ser215 are expressed as the spheres in orange, cyan, and pink, respectively. This image was prepared with the PYMOL program. (B) M-protease (PDB code 1MPT) and BPN′ (PDB code 1SUP) were isolated from *B. clausii* KSM-K16 and *B. amyloliquefaciens*, respectively. The α-helices, β-strands, alpha and beta turns expressed in helices, arrows, TTT and TT letters, respectively, indicate secondary structure elements above the corresponding amino acid sequences.

Table 5
Structural properties of AprB and its closely related alkaline proteases.

Features	Source of extracellular alkaline proteases		
	AprB	M-protease	Subtilisin BPN′
Optimal pH/temperature	10.0/60 °C	12.3/55 °C [11]	9.0/48 °C [27]
$R/(R+K)$ ratio	0.57	0.62	0.17
$E+D$	30	10	15
$(R+K)/(E+D)$ ratio	0.23	1.30	0.86
Proline content (%)	4.5	4.8	5.1
Glycine content (%)	14.1	13.4	12.0
Aromatic residues (%)	5.20	4.46	5.81
Total residues	269	269	275
No. ion pairs[a]			
Total[a]	9	11	12
Formed by Arg residue [a]	8	10	5
No. hydrogen bonds	150	207	345
Accessible surface (Å^2)			
Total	9689	9261	9668
Hydrophobic[b] (%)	18.85	24.08	15.29
Positively charged[c] (%)	4.99	12.72	11.57
Negatively charged[d] (%)	21.24	3.36	5.08

[a] An interaction is assigned to two oppositely charged residues (Asp, Glu, Arg and Lys) within a distance of 4 Å.
[b] The hydrophobic residues were Ala, Ile, Leu, Pro, Trp, and Val.
[c] The positively charged residues were Arg, Lys and Met.
[d] The negatively charged residues were Glu and Asp.

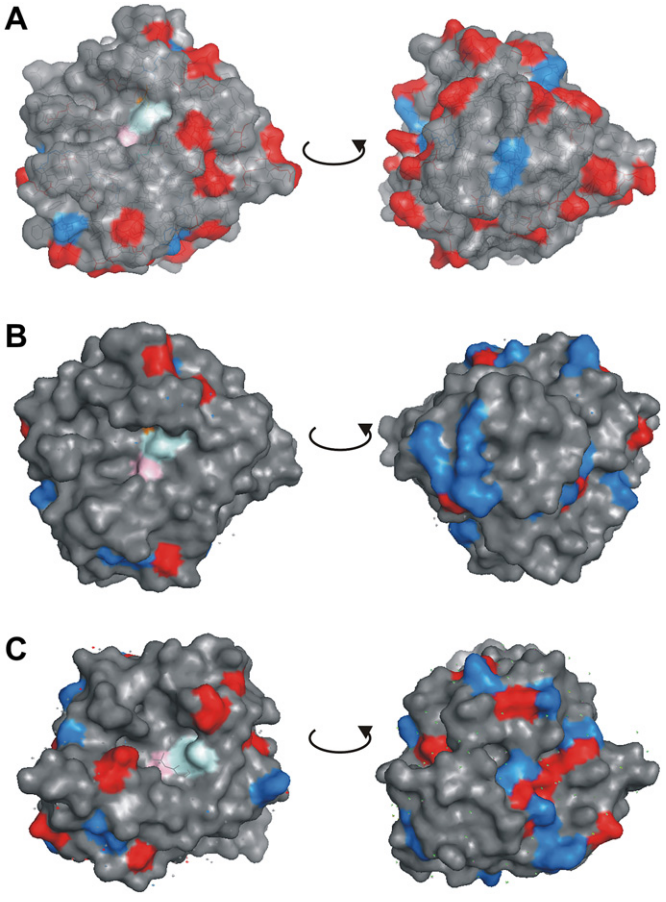

Fig. 5. Comparison of the molecular surfaces in gray of AprB (A), M-protease (B) and subtilisin BPN′ (C) in two views related by rotation along the y axis. The negatively charged residues (Asp/Glu) and the positively charges residues (Lys/Arg) are highlighted in red and blue, respectively. The catalytic active sites of Asp, His and Ser residues on the molecular surface are shown in orange, cyan, and pink, respectively. This image was prepared with the PYMOL program.

The dominant deviations were the low content of alkaline residues and high content of acid residues of AprB compared with the M-protease and BPN′. These three proteins contained 4, 8 and 2 Arg residues, 3, 5 and 11 Lys residues, 11, 5 and 5 Glu residues, and 19, 5 and 10 Asp residues, respectively. As shown in Fig. 5, Glu and Asp residues were mainly on the surface of AprB, whereas on the surfaces of M-protease and BPN′, the main residues were Arg residues and Lys residues, respectively. Thus, the charged surface of AprB was notably different with that of M-protease and BPN′. AprB exhibited 4.99% positively charged surface and 21.24% negatively charged surface, whereas M-protease and BPN′ exhibited 12.72% and 11.57% positively charged surface, respectively, and 3.36% and 5.08% negatively charged surface, respectively (Table 5). However, none of known structures of members of subtilisin family have been reported to have the relatively high negatively charged surface [8,14,45]. Previous research on psychrophilic proteases belonging to the subtilase superfamily found that some cold-adapted enzymes had a higher content of negatively charged residues and a reduced $(R+K)/(E+D)$ ratio [46–48]. For example, the psychrophilic subtilisin-like proteases APR and S41 have been reported to harbor a higher content of Glu and Asp residues and the $(R+K)/(E+D)$ ratios to be 0.58 and 0.61 [10,49]. The fact that AprB had low content of alkaline residues and high content of acid residues is similar to these psychrophilic subtilisin-like proteases [15]. Therefore, it is reasonable to assume that the negatively charged residues might not be the main molecular features responsible for the adaptation to low temperatures.

4. Conclusion

In this study, we successfully cloned and expressed a novel alkaline protease gene (aprB) in the *B. subtilis* WB600. Protease AprB was then purified by anion exchanger and gel filtration. NH_2-terminal sequence of AprB was determined to be A-Q-S-I-P-W-G-I-E-R, and the molecular mass of AprB was about 26.755 kDa. AprB had high catalytic efficiency (K_{cat}/K_m) towards casein, azo-casein and AAPF with optimal activity at 60 °C and pH 10. This protease was stable over a wide range of pH values (5 to 11) and towards various surfactants, and could be activated by Mg^{2+}, Ca^{2+} and Ba^{2+}. These characters make AprB potentially useful for various industrial purposes. In addition, based on the determined amino acid sequence, the structure of AprB was modeled and compared with other alkaline proteases from subtilisin family. AprB exhibits some structural features responsible for its alkaline adaptation, like increased quantity of $R/(R+K)$ ratio, ion pairs formed by Arg, hydrophobic surface area, and the exposure area of Asp active residue on the surface. However, AprB had a high content of Glu and Asp residues, and a low content of Arg and Lys residues on the surface, which were similar to that of cold-adaptation proteases but different with that of members of subtilisin family. These results suggest that AprB has a novel structural feature unique to subtilisin family and provide clues for its alkaline adaptation.

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