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Purification, Molecular Cloning, and Biochemical Characterization of Subtilisin JB1 from a Newly Isolated *Bacillus subtilis* JB1

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Abstract An extracellular gelatinolytic enzyme obtained from the newly isolated *Bacillus subtilis* JB1, a thermophilic microorganism relevant to the aerobic biodegradation process of fish-meal production, was purified via ammonium sulfate precipitation, Sephadex G-200 Gel filtration chromatography, and one-dimensional gel electrophoresis separation and subsequently identified via peptide mass fingerprinting and chemically assisted fragmentation matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The *subtilisin JB1* gene was sequenced and its recombinant protein prosubtilisin JB1 was expressed in *Escherichia coli*, and the purified prosubtilisin JB1 (62 kDa) protein was digested with gelatin, bovine serum albumin, azocasein, fibrinogen, and the fluorogenic peptide substrate Ala-Ala-Phe-7-amido-4-methylcoumarin hydrochloride, whereas the serine protease inhibitors phenylmethylsulfonyl fluoride and chymostatin completely inhibited its enzyme activity at an optimal pH of 7.5. Thus, our results show that subtilisin JB1 may serve as a potential source material for use in industrial applications of proteolytic enzymes and microorganisms for fishery waste degradation and fish by-product processing.

Keywords Skim milk agar plate · MALDI-TOF-MS · Peptide-mass fingerprinting (PMF) · Chemically assisted fragmentation (CAF) · Subtilisin · *Bacillus subtilis* JB1

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Introduction

In the fish processing industry, more than 50% of the total catch, including viscera, shells, scales, fins, and frame bones, is discarded as processing waste or by-products [1]. Moreover, fishery wastes are an important source of environmental contamination. Despite international attempts to decrease fishery wastes via various biological treatment technologies, the majority of the waste produced has been increasing annually [2]. The potential use of this fishery waste has been limited as the result of its notorious malodor [3]. Thus, there is an increasing need to find and develop ecologically acceptable alternatives by which this problem may be overcome.

Proteases are the most crucial groups of industrial enzymes and account for more than 60% of the worldwide enzyme market [4]. They have diverse applications in a broad variety of industries, including the production of detergent, food, agrochemical, leather, silk, and pharmaceuticals [5]. Microbes represent an excellent source of enzymes, including proteases, due to their vast range of physiological and biotechnological applications. Several proteolytic bacteria have been previously associated with fish processing wastes [6, 7]. Many serine proteases generated by *Bacillus* species have been characterized, and their encoding genes have been previously cloned and sequenced [8, 9]. Subtilisins are distributed widely in nature and are extracellularly secreted by a number of microorganisms, including *Bacillus* strains [10]. Subtilisins, including the subtilisin BPN′ from *Bacillus amyloliquefaciens* and the subtilisin Carlsberg from *Bacillus licheniformis*, have been the subject of several protein engineering studies [11].

In the current study, we have selected a bacterial extracellular serine protease, subtilisin JB1, from the thermophilic microorganism, *Bacillus subtilis* JB1, which was isolated from the aerobic biodegradation process involved in fish-meal production. This paper describes the separation and identification of the extracellular gelatinolytic enzyme from *B. subtilis* JB1 and provides some information regarding its biochemical and molecular characteristics.

Materials and Methods

Characterization of Bacteria

B. subtilis JB1, a thermophilic and aerobically fish-meal wastewater-degrading bacterium with the potential for use in the reutilization of the wastewater generated during the process of fish-meal production, was isolated and identified as previously described [7]. In order to observe protease production and for classification, the extracellular enzymes from *B. subtilis* JB1 were inoculated on agar plates (1% skim milk). The following known protease inhibitors were tested: cysteine protease inhibitors, *trans*-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), antipain, leupeptin (USB Co., USA); aspartic protease inhibitor, pepstatin A; serine protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), chymostatin, *N*-ethylmaleimide (NEM); and metalloproteinase inhibitors, ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(beta-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 1,10-phenanthroline. Unless indicated otherwise, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO, USA). The plates were incubated for 24 h at 37°C.

Partial Purification of *B. subtilis* JB1 Extracellular Proteases

The 16-h culture broth (1,000 ml) of *B. subtilis* JB1 was centrifuged (6,000×*g*, 20 min, and 4°C), and the supernatant was filtered through a 0.45-μm pore size filter. The resultant

supernatant was then saturated to 40–80% with ammonium sulfate and incubated overnight at 4°C with stirring. The precipitate was subsequently collected by centrifugation (8,000×*g*, 20 min, 4°C), dissolved in 100 mM Tris-HCl/pH8.0, and dialyzed overnight against the same buffer at 4°C. The concentrated samples (2 ml) were subjected to Sephadex G-200 Gel filtration chromatography (high-performance liquid chromatography, HP 1100 series, Hewlett Packard Co., USA) on a column (21.5 mm×15 cm, TOSOHAS Co., Japan) equilibrated previously with 100 mM Tris-HCl/pH7.5 buffer. Proteins were eluted from the column with a linear NaCl gradient of 0.01 to 1.0 M. Protein concentrations were determined with a Bio-Rad protein assay solution (Bio-Rad, USA) on the basis of the Bradford method, using bovine serum albumin (BSA) as a standard. The proteolytic activity in each fraction was assessed using the fluorogenic substrate Ala-Ala-Phe-7-amido-4-methylcoumarin hydrochloride (AAF-AMC) and gelatin zymography.

Enzyme Activity Assay

The serine protease activity was assessed via the previously described method [12], with slight modifications, using the peptidyl fluorogenic substrate, AAF-AMC. In brief, 10 μl of purified enzyme was preincubated for 2 h at 37°C with 85 μl of 100 mM Tris buffer/pH7.5 and 2 mM dithiothreitol (DTT). The enzyme reaction was initiated via the addition of 5 μl of 1 mM AAF-AMC at 37°C for 10 min. The AMC was assessed using a Microplate Fluorometer (Packard Co., USA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

SDS-PAGE and Zymogram

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as previously described by Laemmli [13]. All samples were denatured in buffer containing 60 mM Tris/pH6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue, boiled for 5 min, and separated via 12% SDS-PAGE (Bio-Rad, USA). Stained molecular weight markers (Amersham, USA) were run as standards on each of the gels. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and the other via silver staining [14] with slight modification. After electrophoresis, the gel was fixed for 30 min in a 50% methanol solution. The fixed gel was washed several times with deionized water, sensitized for 2 min with 0.02% sodium thiosulfate, and again washed three times for 20 s with deionized water. The gel was then incubated for 30 min with 0.2% $AgNO_3$ at room temperature. After discarding the silver nitrate solution, the gel was rinsed quickly with water twice for 30 s. The gel was developed with developing solution (0.04% formaldehyde in 2% sodium carbonate) followed by replacement with 1% acetic acid.

Substrate zymography was conducted via a modified method using gels with gelatin (Sigma), azocasein (Sigma), fibrinogen (Sigma), and BSA (Sigma; 0.1%, *w*/*v*) as described previously [15]. In order to prepare the zymogram, a 30-μl protease sample was mixed with 10 μl of 4× SDS sample buffer (0.5 M Tris/pH6.8, 10% SDS, 20% glycerol, and 0.02% bromophenol blue) without reducing agent and without boiling. The sample was then applied to the gel and electrophoresed using a Bio-Rad Mini-Protean system (Bio-Rad, USA) with a constant applied current of 12 mA per gel at 4°C. After electrophoresis, the gels were immersed in 100 ml of 2.5% (*v*/*v*) Triton X-100 for 1 h to remove the SDS and were then washed once in incubation buffer (100 mM Tris-HCl/pH7.5, 2 mM DTT), then immersed for 18 h in incubation buffer at 37°C. The gels were subsequently washed with water and stained in 5% methanol/10% acetic acid/water containing 0.1% Coomassie brilliant blue R-250. The protease bands appeared as clear zones on a blue background.

MALDI-TOF-MS, PMF Analysis, and CAF-MALDI Sequencing

Electrophoretic bands from SDS-PAGE were excised from the Coomassie blue-stained gel, and mass spectra were acquired via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Ettan™ MALDI-TOF, Amersham Biosciences) by Genomine, Inc. (Korea). The assigned peak values of the peptide masses were searched against the peptide mass fingerprinting search program proFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe).

In order to further improve the identification of *B. subtilis* JB1 extracellular proteases, chemically assisted fragmentation (CAF)-MALDI sequencing was utilized on proteins that had not been successfully identified by PMF. Peptide sequences were acquired using CAF-MALDI sequencing in conjunction with MALDI-TOF-MS by Genomine, Inc. (Korea). The sequences and positions were deduced from the CAF-derivatized peptides using Ettan MALDI-TOF software or the PepFrag search engine (http://prowl.rockefeller.edu/prowl/pepfrag.html).

Subtilisin-Specific Primers Design and Cloning of *Subtilisin JB1*

The methods utilized for the extraction and purification of genomic DNA have been described previously [7]. The genomic DNA of *B. subtilis* JB1 was extracted from cells grown in the given medium with an AccuPrep Genomic DNA extraction kit (Bioneer, Korea). The homologous subtilisin sequences identified by BLASTX and BLASTP searches of the GenBank™ database were then aligned manually using the BioEdit Sequence Alignment Editor, version 5.0.9. According to the sequences of the subtilisin genes (accession nos. K02496, AY627764, AY720895, AF093112, X00165) from *Bacillus* sp. in the GenBank (National Center for Biotechnology Information) nucleotide sequence database, two oligonucleotide primers were designed and synthesized to allow for the polymerase chain reaction (PCR) amplification of the entire subtilisin gene from the genomic DNA of *B. subtilis* JB1. The nucleotide sequences of these primers were as follows: The "sense primer" was subtilisin-F1 (5′-ATGAGAGGCAAAAARGTATGGATCAGTTTG-3′) and the "anti-sense primer" was subtilisin-R1 (5′-MTGWGCTGCYGCYTGTACGTTGATCAG-3′).

The PCR mixture contained 1 μl of genomic DNA template, 5 μl of PCR buffer, 3 μl of deoxynucleotide triphosphates (10 mM), 2 μl of subtilisin-F1 and subtilisin-R1 primers, 1 μl of *Taq* DNA polymerase (1 U per μl), and 36 μl of sterilized water. The PCR program was conducted, in brief, as follows: initial denaturation at 96 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min 30 s using a thermal cycler DICE model TP600 (Takara, Japan). The PCR products were then recovered with Wizard® SV gel and the PCR Clean-up system (Promega). The purified products were ligated into pGEM T-Easy vector (Promega) and transformed into *Escherichia coli* DH5αMCR competent cells in accordance with the manufacturer's instructions. The *E. coli* clones harboring the recombinants were overlaid with 100 μg/ml of ampicillin, 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG), and 40 μg/ml 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside was added to agar gel in Luria–Bertani (LB) broth. White colonies were randomly selected, cultivated, and stored in freezing medium at −80 °C. Plasmid DNA was prepared from *E. coli* using an AccuPrep® Plasmid DNA Extraction Kit (Bioneer). DNA sequencing was conducted using the T7 promoter and SP6 primers by COSMO Co, Ltd. (Seoul, South Korea). The nucleotide and predicted peptide sequences were analyzed using DNAsis for Windows version 2.5 (Hitachi software engineering), BioEdit Sequence Alignment Editor, and BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The signal

sequence and putative cleavage site of *subtilisin JB1* were identified using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP).

Expression and Purification of Recombinant Prosubtilisin JB1 in *E. coli*

The glutathione S-transferase (GST) fusion plasmid with promature *subtilisin JB1* (*prosubtilisin JB1*) was constructed via PCR with the primers prosubtilisin-BamHI-F (5′-CCGCGGATCCATGAGAGGCAAAAAGGTATGG-3′) and prosubtilisin-XhoI-R (5′-CCGCTCGAGCTGTGCTGCTGCTTGTACGTTG-3′). Two primers were modified to harbor *Bam*HI and *Xho*I recognition sites (underlined) in order to facilitate cloning into the GST fusion protein expression vector, pGEX-4T-1 (GE Healthcare, formerly Amersham Biosciences, USA). The resultant plasmid (*prosubtilisin JB1*/*pGEX*) was transformed into *E. coli* DH5αMCR. The transformed cells were then grown in LB broth (100 ml) containing 100 μg/ml of ampicillin at 37°C for approximately 16 h, diluted to 1/100 with the same medium, and grown to an A_{600} of 0.6. Then, IPTG was added to a final concentration of 0.4 mM, and the incubation was continued for 3 h. The cells were collected via centrifugation, washed, and resuspended in 0.2 volumes of phosphate buffered saline (PBS), lysed with a sonicator (Vibra Cell, Sonics & Materials Inc., USA) at a setting of 40%, and centrifuged for 20 min at 20,000×*g* at 4°C. The soluble supernatant was then applied to a glutathione-Sepharose 4B column (Pharmacia Biotech Co., USA) and equilibrated with PBS. After washing the column with equilibration buffer, the proteins were eluted in elution buffer with 50 mM Tris/pH8.0 and 10 mM reduced glutathione (Sigma). The fractions harboring sufficient quantities of active enzyme were pooled, then dialyzed and concentrated with Centricon 10 concentrators (Amicon). Purified prosubtilisin JB1 protein was utilized for SDS-PAGE, Western blotting, and enzyme activity assay.

Preparation of Polyclonal Antibody Against Prosubtilisin JB1

The prosubtilisin JB1 protein (50 μg) was utilized to immunize New Zealand rabbits with complete Freund's adjuvant for the primary injection and with incomplete Freund's adjuvant for three additional booster doses. The sera were then passed over an Econo-Pac protein A cartridge (Bio-Rad, USA), and the IgG fractions were eluted and utilized for the Western blotting of *B. subtilis* JB1 extracellular proteases and recombinant prosubtilisin JB1.

Western Blotting and Immunoblot Analyses of the Native and Recombinant Subtilisin JB1

The molecular mass and purity of the prosubtilisin JB1 protein were analyzed via Western blotting. Prestained molecular weight markers (Fermentas, USA) were run as standards. The electrophoresed samples were transferred to nitrocellulose membranes (Schleicher & Schuell. Co., USA) using a Hoefer transblotting system (Pharmacia. Co., USA). After transfer, the membrane was blocked with 3% BSA in TPBS (200 mM Tris/pH7.0, 1.37 M NaCl, 1% Tween-20) for 1 h at room temperature. The membrane was then incubated with monoclonal anti-GST antibody (Santa Cruz Biotechnology, Inc., USA) or rabbit anti-prosubtilisin JB1 polyclonal antibody (1:200) overnight at 4°C, rinsed and washed as before, then incubated with phosphatase-labeled goat antimouse or rabbit IgG antibody (1:1,000 dilution, Kirkegaard Perry Laboratories, USA) for 90 min at room temperature. The membrane was washed and rinsed as before, and the expressed proteins were visualized with an AP conjugation kit (Kirkegaard Perry Laboratories, USA).

Enzymatic Characterization of Prosubtilisin JB1

In an effort to assess the optimal pH of prosubtilisin JB1, enzyme activity was assayed at 37°C at a variety of pH values. The buffer utilized for the pH ranges of 3.0~10.0 was 100 mM Tris buffer with 2 mM DTT. The effects of enzyme inhibitors on protease activity were evaluated using E-64, E-64c, antipain, leupeptin, pepstatin A, PMSF, chymostatin, EDTA, 1,10-phenanthroline, EGTA, and NEM. The effects of various metal ions (1 and 5 mM) on enzyme activity were assessed using $ZnSO_4$, $CuSO_4$, $CoCl_2$, KCl, $MgSO_4$, $CaCl_2$, and $HgCl_2$. The effects of some surfactants (Triton X-100, Tween 80, and SDS) on enzyme stability were also assessed. The pH dependency, enzyme inhibitor assay, and effects of metal ions and detergents on the activity of prosubtilisin JB1 protease were assessed at a pH of 7.5 using the peptidyl fluorogenic substrate AAF-AMC as described above. All of the experiments were replicated three times. The values provided in the tables and graphical data are represented as the means of three assays (± standard deviation). All analyses were conducted using Microsoft Excel and SigmaPlot software (SPSS Inc., USA).

Results and Discussion

Characterization of Extracellular Proteases from *B. subtilis* JB1

B. subtilis JB1 was characterized for protease production. Using skim milk agar, the proteolytic activity could be detected by the observation of a clear zone. The effects of various protease inhibitors of the supernatant from *B. subtilis* JB1 grown in the skim milk assay medium under optimal conditions with the presence of different enzyme inhibitors are described above. In the presence of PMSF (1–3 mM), the proteolytic activities of *B. subtilis* JB1 were inhibited, thereby suggesting that they harbored serine protease. Enzyme activity was also profoundly inhibited by the serine and metalloprotease inhibitors (each 0.1 mM) EGTA, 1,10-phenanthroline, and NEM, whereas the enzyme was not affected by the general cysteine and aspartate protease inhibitors (each 0.1 mM) E-64, leupeptin, pepstatin A, chymostatin, and antipain (results not shown). Thus, the substrate specificity and the effects of the inhibitors show that the expressed enzymes are serine and/or metalloproteases.

Identification of *B. subtilis* JB1 Extracellular Proteases

The extracellular proteases were purified from the culture supernatant of *B. subtilis* JB1. Following concentration with 40–80% ammonium sulfate and the dialysis of the culture supernatant, gelatin zymography yielded eight distinct proteolytic activity bands (results not shown). The enzyme was found to be insensitive to E-64, pepstatin A, and EDTA, thus indicating that it was not a member of the classes of metallo-, aspartic-, or cysteine proteases. However, the PMSF treatment completely inhibited peptidase activity in all eight bands in the gel. The concentrated sample of *B. subtilis* JB1 was divided into five fractions via Sephadex G-200 Gel filtration chromatography, as shown in Fig. 1a. Gelatin zymography also yielded multiple types and sizes of proteases, with eight major clear bands of proteolytic activity in the crude enzyme preparation. As is shown in Table 1, *B. subtilis* JB1 was purified by over 10.02-fold and each of the purified samples evidenced multiple types and sizes of proteases, with clear bands of proteolytic activity on zymography (Fig. 1b). Extracellular proteases from *B. subtilis* JB1 were identified via

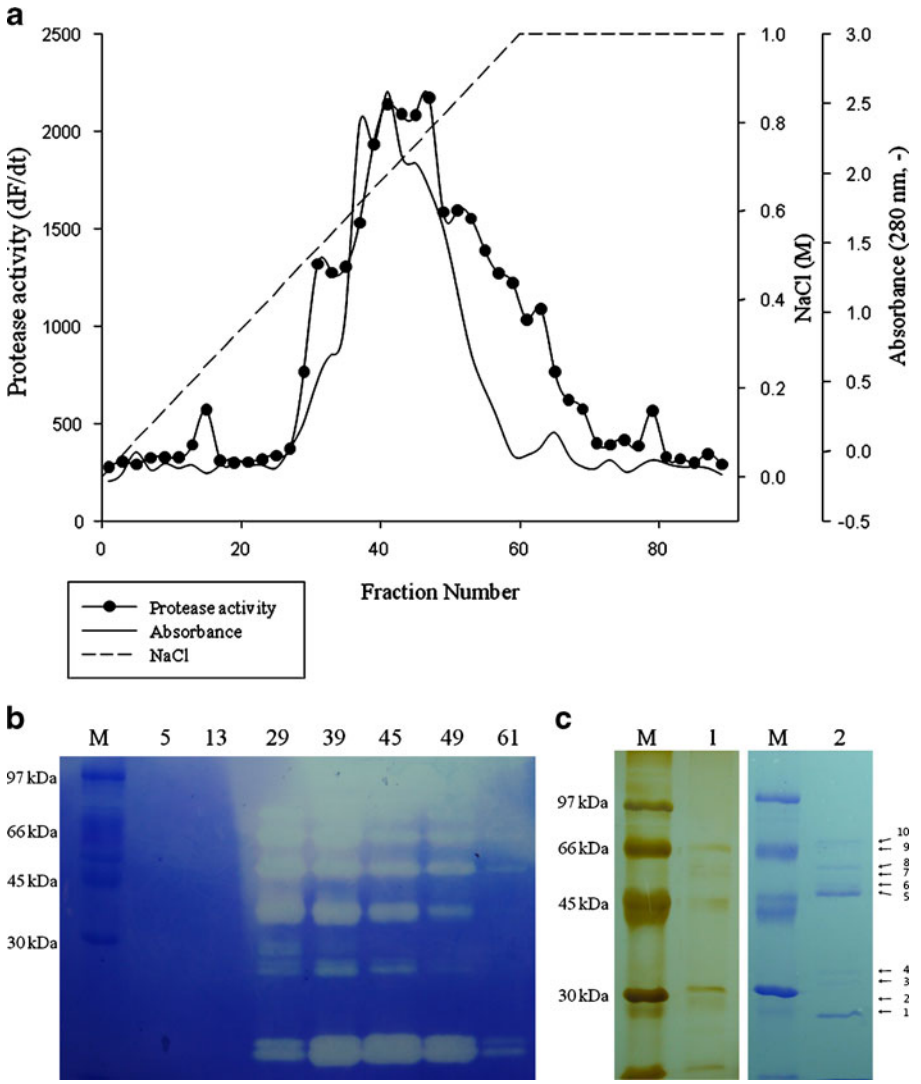

Fig. 1 Purification, gelatin-zymogram analysis, and one-dimensional gel electrophoresis separation of the extracellular proteases from *B. subtilis* JB1 via gel filtration chromatography. **a** Chromatography of extracellular protease activity via gel filtration on Sephadex G-200. The eluted fractions (3 ml) were assayed for protein quantity (*solid line*) and protease activities using AAF-AMC (*dash–circle–dash*) as substrate. Enzymatic activity (*dF*/*dt*) was defined in terms of arbitrary fluorescence units per unit time (*dF*/*dt*). **b** Gelatin zymography of partially purified extracellular proteases from *B. subtilis* JB1. The peak fractions (5, 13, 29, 39, 45, 49, and 61) were subjected to gelatin zymography. **c** For protein identification, the one-dimensional gel separation of fraction number 29 was presented on the gel. *Lane 1*, protein was visualized via silver staining. *Lane 2*, protein was visualized by Coomassie blue. *M* molecular mass standards. The *numbers to the right* indicate the numbers of protein identification using PMF and CAF-MALDI sequencing

one-dimensional SDS-PAGE, MALDI-TOF-MS, PMF analysis, and CAF-MALDI sequencing. A total of ten extracellular proteins were identified, and protein band 2 was identified as serine alkaline protease (subtilisin E; *B. subtilis* subsp. subtilis str. 168, accession no. NP_388911, HPTWTNAQVR), and protein bands 5–9 were identified as

Table 1 Purification of extracellular protease enzymes generated by *B. subtilis* JB1.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	10,617.88	5.834	1,820	1	100
40~80% ammonium sulfate fraction	6,188.12	1.455	4,253	2.33	58
Gel filtration	5,948.20	0.326	18,246	10.02	56

alpha-amylase (*B. subtilis* subsp. subtilis str. 168, NP_388186, LGWAVIASR). Also, endoglucanase (P07983, sample 4), arabinan-endo 1,5-alpha-L-arabinase (NP_390759, sample 3), sn-glycerol-3-phosphate dehydrogenase (NP_417884, sample 10), and hypothetical protein BC0996 (NP_830782, sample 1) were also identified (Fig. 1c). According to these results, it is clear that the extracellular proteases from *B. subtilis* JB1 are principally subtilisin-like serine proteases, rather than a metal-, aspartic-, or cysteine proteases.

Cloning and Expression of *Subtilisin JB1*

In order to identify the subtilisin-like serine protease from *B. subtilis* JB1, two degenerated oligonucleotides, subtilisin-F1 and subtilisin-R1, were employed in the amplification of the open reading frame from the genomic DNA of *B. subtilis* JB1. The *subtilisin JB1* gene of *B. subtilis* JB1 was cloned and its nucleotide sequence was determined (FJ386851). The nucleotide sequence revealed only one open reading frame, composed of 1,143 bp and 381 amino acid residues. The sequence of the protein coding region of *subtilisin JB1* was shown to code for a 29 residue signal peptide, a 77-residue propeptide, and 275 amino acid residues of mature subtilisin JB1. The amino acid sequence of *subtilisin JB1* was compared with the published sequences of other subtilisins and was shown to exhibit an identity of 97% with the subtilisin obtained from *B. subtilis* (CAE18180), nattokinase (AAC60424), subtilisin amylosacchariticus (P00783), subtilisin E (CAA74536), and subtilisin J from *Geobacillus stearothermophilus* (P29142) and 86% and 66% identities with subtilisin BPN from *B. amyloliquefaciens* (P00782) and subtilisin Carlsberg from *B. licheniformis* (P00780). *Subtilisin JB1* was identical to subtilisin, with the exception of 19 amino acid substitutions; three amino acids each were found in the signal peptide and the propeptide, and 13 amino acids were found in the mature subtilisin, as is shown in Table 2. The catalytic residues Asp32, His64, and Ser221 (mature-*subtilisin JB1* numbering) were well conserved. It was reported previously that the prosequence is essential for the in vivo generation of active subtilisin and is autoprocessed upon the completion of the folding of prosubtilisin [16]. Therefore, recombinant subtilisins were expressed with an N-terminal thioredoxin fusion expression system [17], or generated as inclusion bodies and dissolved with the refolded protein [18]. In order to solve this problem, we utilized the GST fusion system. GST from *Schistosoma japonicum*, which is used extensively for the generation of fusion proteins in the cytoplasm of *E. coli*, was used as a functional fusion module that affects the dimer formation of a recombinant protein and simultaneously confers enzymatic reporter activity [19]; thus, it was employed in this study to express *subtilisin JB1*. The plasmid *prosubtilisin JB1*/*pGEX* has been constructed as described above and identified via sequencing, then introduced into *E. coli* DH5αMCR from the expression of the target protein. The overexpressed recombinant prosubtilisin JB1 protein was purified using glutathione affinity chromatography. The purified prosubtilisin JB1, with a molecular size

Table 2 Different amino acids between *subtilisin JB1* and other subtilisins.

Position[a] (amino acid)	*Subtilisin JB1*	Nattokinase	Subtilisin amylosacchariticus	Subtilisin E	Subtilisin J	Subtilisin
3	G[b]	S	S	S	S	S
6	V[b]	L	L	L	L	L
27	A	A	A	V	V	A
77	T[b]	A	A	A	A	A
90	Q	K	K	K	K	Q
104	H	H	H	H	H	L[b]
151	K[b]	R	R	R	R	R
166	D	D	D	D	D	G[b]
167	G	G	G	G	G	R[b]
179	A	A	A	A	A	S[b]
181	L	L	L	L	L	F[b]
184	T[b]	S	S	S	S	S
191	A	A	A	S	S	A
193	N	S	S	S	S	N
236	S	T	S	T	S	S
268	T	T	S	T	S	T
290	N	N	N	N	N	T[b]
298	A	V[b]	A	A	A	A
365	S[b]	N	N	N	N	N

[a] Pre-*subtilisin JB1* numbering

[b] Strain specific amino acid

of approximately 62 kDa, was detected in Western blotting analysis (Fig. 2a). Purified prosubtilisin JB1 was utilized for further enzymatic characterization.

Enzymatic Characterization of Recombinant Prosubtilisin JB1

The effect of pH on prosubtilisin JB1 protease activity was assessed using AAF-AMC as a substrate at various pH values at 37 °C. The pH activity profile of purified prosubtilisin JB1 protease is provided in Fig. 2b. The recombinant prosubtilisin JB1 is active in a pH range of 4.0–8.0, with an optimum occurring at pH7.5. The digestion of various protein substrates via prosubtilisin JB1 was also assessed. It was found to be capable of degrading gelatin, fibrinogen, azocasein, and BSA (Fig. 2c). Using the rabbit antibody, Western blot was developed for the detection of recombinant prosubtilisin JB1 protein and extracellular proteases from the *B. subtilis* JB1 protein. As is shown in Fig. 2d, Western blot analysis revealed that the prosubtilisin JB1-specific polyclonal antibody allowed for the sensitive detection of extracellular proteases from *B. subtilis* JB1 (lane 1, 27 kDa) and the recombinant prosubtilisin JB1 protein (lane 2, 62 kDa). These data indicate that the mass of the native protein band (27 kDa) was consistent with protein band 2, which was identified via one-dimensional SDS-PAGE, MALDI-TOF-MS, PMF analysis, and CAF-MALDI sequencing as serine alkaline protease (subtilisin E; Fig. 1c).

As expected from the amino acid sequence, the addition of PMSF, a well-known inhibitor of serine protease with prosubtilisin JB1 on gelatin zymography, enzyme activity was also profoundly inhibited; however, no influence on enzyme activity was noted in the

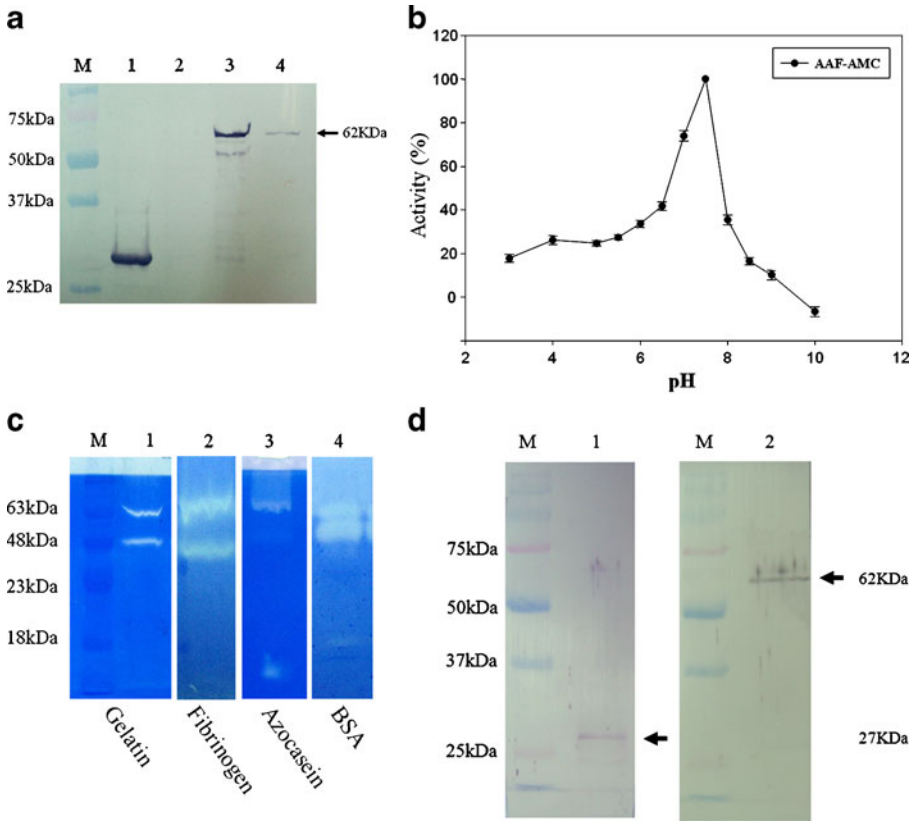

Fig. 2 Enzymatic characterization of prosubtilisin JB1. **a** Western blotting of recombinant prosubtilisin JB1 fused with GST using specific anti-GST antibody. *Lane M*, protein markers; *lane 1*, protein extract of *E. coli* transformed with pGEX-4T-1 after 3 h of induction with IPTG; *lane 2*, protein extracts of *E. coli* transformed with *prosubtilisin JB1*/*pGEX*; *lane 3*, protein extracts of *E. coli* transformed with *prosubtilisin JB1*/*pGEX* after 3 h of induction by IPTG; *lane 4*: purified prosubtilisin JB1. **b** pH dependency of purified prosubtilisin JB1. The purified prosubtilisin JB1 was preincubated for 2 h at 37°C at various pH values. **c** Zymogram analysis of purified prosubtilisin JB1. Proteolytic activity was detected in 10% polyacrylamide gel containing 0.1% substrates including gelatin (*lane 1*), fibrinogen (*lane 2*), azocasein (*lane 3*), and BSA (*lane 4*). **d** Immunoblot analysis of the native and recombinant prosubtilisin JB1 via anti-prosubtilisin JB1 polyclonal antibody. *Lane M*, protein markers; *lane 1*, purified extracellular proteases from *B. subtilis* JB1; *lane 2*, purified prosubtilisin JB1

presence of the metallo-, aspartic-, and cysteine protease inhibitors EDTA, pepstatin A, or E-64 (data not shown). The effects of variety of inhibitors on fluorogenic peptide substrates associated with prosubtilisin JB1 activity were summarized in Table 3. A significant inhibition of over 70% was observed with the serine protease inhibitors PMSF, chymostatin, and antipain and was partially inhibited by the metalloprotease inhibitors EDTA, EGTA, and 1,10-phenanthroline. The effects of various metal ions, detergents, and reducing agents exerted no significant influence on the stability of recombinant prosubtilisin JB1 (Table 4). Interestingly, Zn^{2+} at concentrations in excess of 1 mM inhibited enzyme activity, with 5 mM Zn^{2+} resulting in an almost complete reduction of prosubtilisin JB1 activity, which is similar to what was observed with a metalloprotease from the *Vibrio harveyi* AP6 strain [20]. However, we were unable to locate the zinc-binding motif, HEXXH-E (HEXXH-19aa-E), which is known to be highly conserved in the

Table 3 Inhibition of prosubtilisin JB1 by various proteinase inhibitors.

Inhibitors	Specificity	Concentration (mM)	Inhibition (%)
Control	–	–	0
Chymostatin	Serine and cysteine protease	0.1	94.70±7.95
PMSF	Serine protease	0.1	78.59±4.20
EDTA	Metalloprotease	0.1	51.80±5.25
EGTA	Metalloprotease	0.1	34.35±4.66
1,10-Phenanthroline	Metalloprotease	0.1	39.53±4.78
NEM	Serine protease	0.1	14.75±5.49
E-64c	All cysteine protease	0.1	24.67±5.66
E-64	All cysteine protease	0.1	31.04±2.89
Leupeptin	Cysteine and trypsin-like serine protease	0.1	0.00±3.15
Pepstatin A	Aspartic protease	0.1	48.46±4.23
Antipain	Serine and cysteine protease	0.1	74.90±2.25

zinc-metalloproteases [21, 22] via the alignment of other zinc metalloproteases with *subtilisin JB1*.

In this study, we report the purification and characterization of extracellular proteases from the newly isolated *B. subtilis* JB1. The *B. subtilis* JB1 extracellular proteases evidenced significant inhibition with PMSF and could be classified as serine proteases. Herein, we also cloned the *subtilisin JB1* gene and determined its nucleotide sequence, which evidenced an identity of 97% with subtilisin, nattokinase, and subtilisin E from different *Bacillus* species. Recombinant prosubtilisin JB1 was expressed with a pGEX-4T-1 vector system, and its gelatinolytic and fibrinolytic activities were evaluated. Therefore, *B. subtilis* JB1, a strong protease-producing bacterium, as well as its recombinant prosubtilisin JB1, have the potential

Table 4 Effects of metal ions and various detergents on prosubtilisin JB1 activity.

Agent	Relative activity (%)			
	1 mM	0.01%	5 mM	0.05%
Control	100		100	
$ZnSO_4$	29.97±5.67		ND	
$CuSO_4$	ND		18.66±6.04	
$CoCl_2$	13.72±4.12		23.77±4.20	
KCl	ND		53.34±6.58	
$MgSO_4$	78.74±5.08		47.84±3.31	
$CaCl_2$	57.23±2.23		45.08±4.59	
$HgCl_2$	32.60±2.77		21.91±5.75	
Brij 35		30.47±3.35		70.16±8.30
Tween 20		53.11±4.72		70.32±5.57
Triton X-100		92.08±9.95		69.41±6.72
SDS		52.30±9.12		29.13±4.02

ND not detected

for use in industrial proteolytic enzyme and microorganism applications for fishery waste degradation and marine by-product processing.

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