Purification and Characterization of Proteases from *Bacillus amyloliquefaciens* Isolated from Traditional Soybean Fermentation Starter

Seong-Jun Cho,[†,§] Sung-Hoon Oh,[#] R. David Pridmore,[†]
Marcel A. Juillerat,[†] and Cherl-Ho Lee*,[§]

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea;
Department of Food and Biotechnology, Ansan College of Technology, Kyungki-do 425-792, Korea;
and Nestlé Research Center, P.O. Box 44, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

Bacillus amyloliquefaciens FSE-68 isolated from meju, a Korean soybean fermentation starter, was identified on the basis of biophysical tests and 16S rRNA gene sequence. A neutral metalloprotease (NPR68) and an alkaline serine-protease (APR68) were purified by ammonium sulfate precipitation and cation exchange chromatography and identified on the basis of their activities at different pH values and the selective protease inhibitors. The molecular weights of NPR68 and APR68 measured with ESI-MS were 32743 ($\pm$ 0.8) and 27443 ($\pm$ 0.5) Da, respectively. Against oxidized insulin chains, the NPR68 has a cleavage preference at the site where leucine is located as a P1′ residue followed by phenylalanine, and the APR68 has broad specificity and favors leucine at the P1 site. These results indicate that the proteases are natural variants of subtilisin and bacillolysin.

KEYWORDS: *Bacillus amyloliquefaciens*; strain identification; protease; specificity; insulin; purification; meju

INTRODUCTION

Proteins are one of the most important food constituents together with carbohydrates and lipids. The primary role of proteins in the diet is to supply the body adequately with indispensable amino acids, especially the essential amino acids and organic nitrogen. In addition to these basic functions, physiologically active properties of the protein have been increasingly acknowledged. Biologically active peptides, for example, opioid peptides, angiotensin converting enzyme inhibitory peptides, immunostimulating peptides, antithrombotic peptides, antimicrobial peptides, and mineral binding peptides, have been derived from dietary proteins. These peptides are inactive within the sequence of the parent protein and can be produced by enzymatic hydrolysis. Milk proteins are the most studied source of bioactive peptides because milk protein constitutes up to ∼20% of the average food protein intake in the United States and in European countries (*1, 2*). Compared with milk protein, however, little attention has been paid to soy protein as a source of biologically active peptides, although it is a main protein source in Asia. In Korea, fermented soybean products prepared from meju, a traditional soybean fermentation starter, are commonly consumed. These fermented soybean products are processed into condiments such as soybean sauce (kanjang), soybean paste (doenjang), and hot soybean paste (kochujang).

To discover new proteases that are suitable for producing biologically active peptides from soy protein, several bacteria strains were isolated from meju. One very promising strain, which was mainly responsible for meju fermentation, was selected. In this study, we have identified the isolated strain by phenotypic and genetic methods and characterized the two major proteases secreted by this strain.

MATERIALS AND METHODS

Bacterial Strains. Strain 68 used in these experiments was originally isolated from meju, a Korean soybean fermentation starter (*3*). The bacteria were grown in brain heart infusion (BHI) agar at 37 °C for 12 h and stored at 4 °C. *Escherichia coli* strain BZ234 (collection of the Biozentrum, University of Basel, Basel, Switzerland) was grown in Luria–Bertani (LB) broth at 37 °C with shaking. The general *E. coli* vector pK19 (*4*) was used for 16S rRNA gene cloning.

Chromosomal DNA Isolation. Strain 68 was incubated in LB for 16 h to produce a dense culture. The bacteria were pelleted by centrifugation, suspended in 2 mL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) plus 10 mg/mL lysozyme and incubated at 37 °C for 1 h. Sixty microliters of 12 mg/mL proteinase K, 250 μL of 500 mM EDTA, and 500 μL of 10% SDS were added, and the mixture was incubated at 56 °C for 1 h. The mixture was extracted once with an equal volume of phenol/chloroform, and the DNA was precipitated with 2 volumes of 95% ethanol. The DNA precipitate was dissolved in 1 mL of TE buffer containing 10 μg/mL RNaseA (Roche Molecular Biochemicals), incubated at 56 °C for 1 h, and then extracted once

* Corresponding author (telephone 82-2-3290-3414; fax 82-2-927-5201; e-mail chlee@korea.ac.kr).
† Nestlé Research Center.
§ Korea University.
# Ansan College of Technology.

with an equal volume of phenol/chloroform; the DNA was precipitated with 2 volumes of 95% ethanol. The DNA was pooled, washed once with 70% ethanol, and dried. The DNA was finally dissolved in TE buffer.

Phenotypic Characterization. For preliminary characterization, the strain 68 was cultured in BHI broth and sporulation agar (15 g of nutrient agar, 5 g of agar, 0.5 g of glucose, and 0.03 g of MnSO₄ in 1 L of distilled water, pH 7.0) at 37 °C for 24 h. The strain was initially tested for cell and endospore morphology using phase contrast microscopy and catalase reaction by the 3% H_2O_2 method. The strain was further characterized by its substrate utilization pattern using the API-50 CHB and API-20E systems (bioMérieux), and APiLAB Plus version 3.3.3 was used to analyze data from API tests. Other biochemical tests were performed as described by O'Donnel et al. (*5*). For further identification, lactose utilization was checked according to the method of Nakamura (*6*).

Sequencing of the 16S rRNA Gene. PCR was performed to amplify the 16S rRNA coding region using two oligonucleotide primers, 5′-ATATCCGTCGACAGAGTTYGATYCTGGCT-3′ and 5′-ATATC-CGGATCCTACGGYTACCTTGTTACGACT-3′, introducing *Bam*HI and *Sal*I restriction sites, respectively (underlined) and the *Pwo* thermostable polymerase (Roche Molecular Biochemicals) according to the supplied instructions. Amplification was performed in an Applied Biosystems 9700 PCR machine with a 10 min hold at 95 °C, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 68 °C for 2 min, and finally held at 4 °C. A sample was electrophoresed on a 1% agarose gel and visualized with UV light after the DNA had been stained with ethidium bromide to confirm the presence of amplicon of the predicted size. The amplicon was purified using the Microcon PCR filter device (Millipore) and digested with the enzymes *Bam*HI and *Sal*I (Roche Molecular Biochemicals) at 37 °C. The product was resolved on a 1% agarose gel, and the appropriate DNA fragment was cut out and eluted using the Qiagen gel extraction kit. This DNA fragment was ligated into the previously prepared pK19 vector digested with *Bam*HI plus *Sal*I and dephosphorylated, transformed into BZ234 *E. coli* cells, and plated onto LB plates supplemented with 100 μg/mL kanamycin, 300 ng/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 60 ng/mL isopropyl-β-D-thiogalactoside and incubated at 37 °C. White colonies with a high probability of containing DNA inserts were grown in small-scale cultures, and the plasmid DNA was extracted. Clones were digested with the restriction enzyme *Eco*RI to identify plasmids containing inserts of the expected size and sequenced using the IRD800 labeled fluorescent forward (5′-CTGCAAGGCGATTAAGTTGGG-3′) and reverse (5′-GTTGTGTGGAATTGTGAGCGG-3′) primers and the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia, Uppsala, Sweden). The cycle sequencing was performed on the 9700 PCR machine using the recommended conditions. After cycle sequencing, the sequences were electrophoresed and analyzed on the LiCor model L4000 DNA sequencer.

Production of Proteases. One milliliter of a fresh 10 h preculture was inoculated into 100 mL of the enzyme-producing medium in a 500 mL Erlenmeyer flask, and it was incubated in a rotary shaker (220 rpm) at 37 °C for 22 h. The enzyme-producing medium was prepared with 1.0 g of defatted soybean flour, 1.0 g of lactose, and 0.03 g of CaCl₂ in 100 mL of 0.1 M phosphate buffer (pH 8.0).

Protein Determination. Protein concentration was measured at 595 nm using the Bradford method (*7*), and bovine serum albumin was used as the standard protein.

Protease Activity Assay. The casein−resorufin method (*8*) was used for the protease assay. Fifty microliters of substrate containing 0.4% (w/v) resorufin-labeled casein (Roche Molecular Biochemicals) and 50 μL of incubation buffer (0.2 M Tris-HCl, 20 mM CaCl₂, pH 7.0 for neutral protease assay, and 0.2 M borate buffer, 20 mM EDTA, pH 10.0 for alkaline protease assay) were mixed and preheated at 37 °C. Hydrolysis was initiated by adding 100 μL of a diluted enzyme solution and incubated for 15 min at 37 °C, and the reaction was terminated by adding 200 μL of 7% (w/v) trichloroacetic acid (TCA). The mixture was incubated for 10 min at 37 °C and subsequently centrifuged for 5 min at 20000*g*. The supernatant was mixed with 600 μL of assay buffer (0.5 M Tris-HCl, pH 8.8), and the absorbance was measured at 574

nm. One unit of protease activity was defined as 1 μM liberated resorufin per minute.

Synthetic substrates were also used to confirm the protease activity. Alkaline protease activity was assayed at 25 °C by monitoring at 410 nm the release of *p*-nitroaniline from enzymatic hydrolysis of 1.6 mM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF) in 100 mM Tris-HCl buffer containing 2 mM CaCl₂ (pH 8.6). Neutral protease activity was measured by monitoring the decrease of furylacryloyl-Gly-Leu-NH₂ (FAGLA) absorbance at 345 nm at 25 °C. The assay solution contained 1 mM FAGLA, 10 mM CaCl₂, and 0.1 mM ZnCl₂ in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0).

Purification of Proteases. A crude enzyme solution was obtained by centrifugation of the culture broth at 5000*g* for 15 min and the cell-free supernatant precipitated with 70% saturation of ammonium sulfate. The solution was then kept in an ice bath for 1 h and centrifuged at 15000*g* for 20 min. The precipitate was collected and dissolved in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.7) containing 2 mM CaCl₂ and dialyzed against the same buffer at 4 °C. This preparation was loaded onto a Mono-S HR 5/5 cation exchange column (Amersham Pharmacia) equilibrated with 20 mM MES buffer (pH 6.7) containing 2 mM CaCl₂. Elution was achieved with a linear gradient of NaCl concentration (0−80 mM) at a flow rate of 1 mL/min and eluted protein detected at 280 nm. The fractions were assayed for protease activity, and the active fractions were collected and concentrated by ultrafiltration (MWCO 10000, Millipore).

N-Terminal Sequence. After denaturation and concentration of proteases by deoxycholate (DOC)−TCA precipitation, SDS−polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (*9*) using a 12% polyacrylamide slab gel. The purified enzyme on the gel was transferred to a poly(vinylidene difluoride) membrane (Immobilion-P^SQ, Millipore) by electroblotting in 3-(cyclohexylamino)propanesulfonic acid (CAPS) buffer at pH 11.0 (*10*) and stained with Coomassie blue R-250. The stained portion was excised and used for N-terminal sequencing. The N-terminal sequence was assayed using a 492 cLC protein sequencer (Applied Biosystems), and phenylthiohydantoin (PTH) derivatives of amino acid were analyzed on-line.

Hydrolysis of Oxidized Insulin Chains. Oxidized insulin A- and B-chains were used as substrates to determine protease specificity. Aliquots of 1.0 mg/mL of each insulin chain were dissolved in 20 mM phosphate buffer (pH 7.0) containing either 1 mM pheylmethanesulfonyl fluoride (PMSF) for neutral protease assay or 20 mM borate buffer (pH 10.0) containing 5 mM EDTA for alkaline protease assay. To 200 μL of each substrate was added and mixed 0.1 μg of protease, and the mixture was incubated at 30 °C. An aliquot of 30 μL was removed from the reaction mixture at specific time points and mixed with 20 μL of 0.25% TFA to stop the reaction.

LC-MS and MS/MS Analysis. Peptides were separated using a C₁₈ narrow-bore reverse-phase column (Vydac 218TP52, 5 μm, 2.1 mm i.d.) with a linear increase of solvent B (0.045% TFA/80% acetonitrile in water) in solvent A (0.05% TFA in water) at a flow rate of 0.2 mL/min with protein detection at 215 nm. LC-MS and MS/MS analyses were performed using an LCQ mass spectrometer (Finnigan Co., San Jose, CA) equipped with an electrospray ionization source. MS and MS/MS data acquisition and analyses were performed with Xcalibu software v1.0 (Finnigan Co.), including the Bioworks v1.0 software package for SQUEST database search.

Characterization of Proteases. A buffer containing 50 mM acetic acid, 50 mM MES, and 100 mM Tris was used from pH 4.0 to 9.0, and 50 mM CAPS buffer was used from pH 9.0 to 12.0. The ionic strength of the buffers was adjusted to 0.1 by NaCl. For the stability test, the proteases were dissolved in the buffer system above and incubated at 30 °C for 60 min. Residual activity was measured at pH 7.0 by FAGLA for neutral protease and at pH 8.6 by AAPF for alkaline protease using the standard conditions explained above. To study the effect of pH on the protease activity, neutral and alkaline proteases were dissolved in the above buffer systems at 25 °C and assayed with FAGLA and AAPF, respectively.

Half-lives ($t_{1/2}$) for irreversible thermal inactivation reactions were determined at 60 or 55 °C in 50 mM MOPS, pH 7.0, and 2 mM CaCl₂ with 100 μg/mL enzyme in a total volume of 100 μL. Aliquots removed
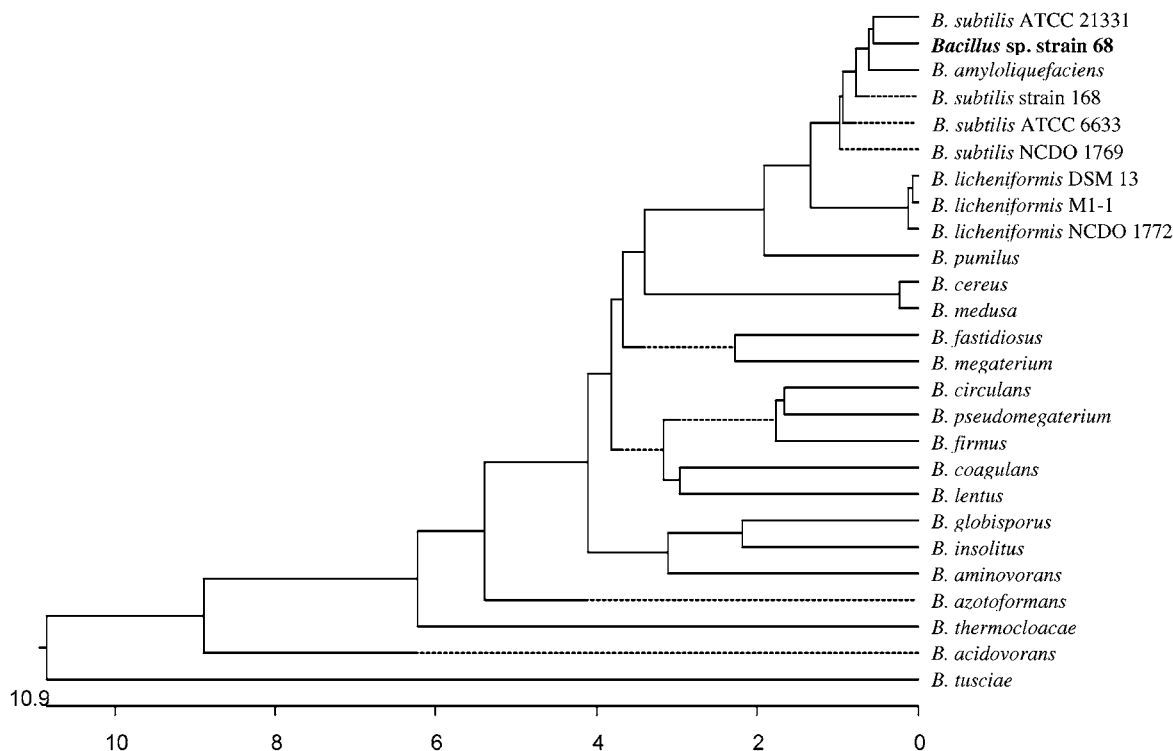
Figure 1. Phylogenetic tree of 16S rRNA genes of the genus *Bacillus. Bacillus* sp. strain 68 is shown in bold. This tree was constructed by the Clustal method using Lasergene software. The origins and GenBank accession numbers are as follows: *B. acidovorans* ATCC 51159, X77789; *B. aminovorans* NCIMB 8292, X62178; *B. amyloliquefaciens* ATCC 23350, X60605; *B. azotoformans* ATCC 29788, X60609; *B. cereus* NTCT 11143, X55063; *B. circulans* NCDO 1775, X60613; *B. coagulans* NCDO 1761, X60614; *B. fastidiosus* DSM, X60615; *B. firmus* NCIMB 9366, X60616; *B. globisporus* NCIMB 11434, X60644; *B. lentus* NCDO 1127, X60601; *B. medusa* NCIMB 10437, X60628; *B. megaterium* DSM 32, X60629; *B. pseudomegaterium* ATCC 49866, X77791; *B. pumilus* NCDO 1766, X60637; *B. subtilis* ATCC9799, AF142574; *B. subtilis* ATCC 21331, AB018487; *B. subtilis* NCDO 1769, X60646; *B. subtilis* strain 168, Z99104; *B. thermocloacae* DSM 5250, Z26939; *B. tusciae* DSM 2912, Z26933; *B. licheniformis* NCDO 1772, X60614; *B. licheniformis* DSM 13, X68416; *B. licheniformis* M1-1, AB039328; *B. insolitus* DSM 5, X60642.

at the various times were immediately diluted (1:10) with cold 100 mM Tris-HCl buffer containing 2 mM $CaCl_2$ (pH 8.6), and measurement of the residual protease activity was achieved using AAPF in standard condition.

RESULTS

Strain Properties and Identification. Strain 68 used in this study was Gram-positive, rod-shaped, endospore forming, and catalase positive and grew well up to 50 °C and at 10% NaCl. It produces ellipsoidal spores, and the spores did not swell the mother cell; therefore, it was identified to be in the genus *Bacillus* group (*11*). API strips were used to assess the sugar fermentation and enzyme activity capabilities of strain 68. From the results analyzed by APiLAB Plus software, strain 68 was identified as *B. subtilis*, which scored 80.6%, *B. licheniformis* 17.5%, and *B. amyloliquefaciens* 1.7% (*12*). The 16S rRNA gene sequence of strain 68 was aligned with the closest probable relatives as determined by API testing, namely, *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* using Lasergene software (DNASTAR Inc.). Several 16S rRNA genes of *Bacillus* species were taken from GenBank to solve the problems of species identification with closely related bacteria that seem to differ only in a few biochemical properties. There were distinct nucleotide differences between these strains, but there were an equivalent number of differences between the several 16s rRNA genes within the same bacterium (data not shown). It was, therefore, very difficult to separate these bacteria using these criteria. The phylogenetic analysis (*13*), however, showed that

**Table 1.** Summary of Purification of Neutral Protease from *B. amyloliquefaciens* FSE-68 (NPR68)

purification step	total protein (mg)	total activity (units)	specific activity (units/mg)	purifi- cation (fold)	yield (%)
crude enzyme	60.8	97.2	1.60	1	100
ammonium sulfate precipitation	19.9	78.8	3.96	2.5	81.1
Mono-S	5.16	58.4	11.3	7.1	60.1

Bacillus sp. strain 68 was more closely related to *B. subtilis* and *B. amyloliquefaciens* than *B. licheniformis*, and this result was supported by the failure of *Bacillus* sp. strain 68 to grow at 55 °C (**Figure 1**). This strain utilizes lactose very well; therefore, it was classified as *B. amyloliquefaciens* (*6*). On the basis of these data, the strain was named *B. amyloliquefaciens* FSE-68.

Purification of Proteases. Proteases from *B. amyloliquefaciens* FSE-68 were purified by a two-step purification including salting out by ammonium sulfate and cation exchange chromatography. The results of the purification and chromatogram are summarized in **Tables 1** and **2**. Two major peaks were obtained by cation exchange chromatography, and both showed protease activity. By using protease inhibition tests with either PMSF or EDTA, the peak eluting between 30 and 40 mM NaCl was identified as neutral (or metallo) protease and the peak eluting between 45 and 55 mM as alkaline (or serine) protease. They were very well separated, and when purified on the cation

Table 2. Summary of Purification of Alkaline Protease from *B. amyloliquefaciens* FSE-68 (APR68)

purification step	total protein (mg)	total activity (units)	specific activity (units/mg)	purifi-cation (fold)	yield (%)
crude enzyme	60.8	83.2	1.37	1	100
ammonium sulfate precipitation	19.9	58.4	2.93	2.1	70.2
Mono-S	9.03	55.6	6.16	4.5	66.8
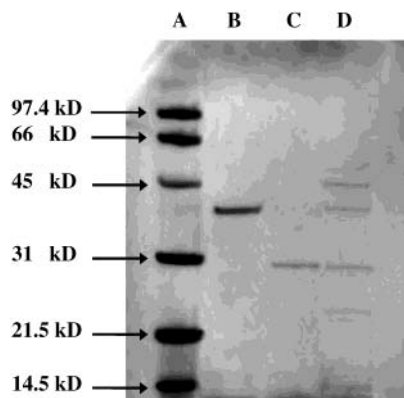
Figure 2. SDS-PAGE of purified proteases and crude enzyme solution: (lane A) molecular weight markers; (lane B) neutral protease (NPR68); (lane C) alkaline protease (APR68); (lane D) crude enzyme of *B. amyloliquefaciens* FSE-68. Samples were analyzed on 12% SDS–polyacrylamide gel.

exchange column, only a strong, single band appeared on SDS-PAGE for each protease. By SDS-PAGE, the molecular mass of neutral protease from *B. amyloliquefaciens* FSE-68 (NPR68) was estimated to be ∼39 kDa and that of alkaline protease (APR68) to be ∼30 kDa (**Figure 2**). However, LC-ESI/MS analysis of NPR68 and APR68 showed molecular weights of 32743 (± 0.8) and 27443 (± 0.5) Da, respectively (**Figure 3**).

N-Terminal Amino Acid Sequence Analysis. The N-terminal amino acid sequences of NPR68 and APR68 were determined by automated Edman degradation and compared with those of neutral proteases (from *B. subtilis* and *B. amyloliquefaciens*) and alkaline proteases (subtilisin E from *B. subtilis*, subtilisin BPN′ from *B. amyloliquefaciens*, and subtilisin Carlsberg from *B. licheniformis*) (**Figure 4**). The N-terminal amino acid sequence of NPR68 was almost identical to that of *B. subtilis* and *B. amyloliquefaciens* in 9 of 10 positions at the amino terminus and differed only at residue 6 (from *B. subtilis*) and at residue 3 (from *B. amyloliquefaciens*). For APR68, its N-terminal amino acid sequence was identical to that of subtilisin BPN′ and subtilisin E in 10 of 10 positions.

Substrate Specificity. Substrate specificity of NPR68 and APR68 was studied with oxidized insulin A- and B-chains. Each hydrolysate of insulin A- and B-chains was analyzed by LC-MS and MS/MS after incubation times ranging from 10 min to 18 h.

By NPR68, the oxidized insulin A-chain was initially cleaved at the bond Gln^{15}–Leu^{16} followed by Ser^{12}–Leu^{13}. Up to 18 h of digestion, the bond Gly^1–Ile^2 was slowly hydrolyzed. In the case of the oxidized insulin B-chain, the bonds His^5–Leu^6, His^{10}–Leu^{11}, Ala^{14}–Leu^{15}, and Tyr^{16}–Leu^{17} were hydrolyzed at the same time, so the corresponding peaks appeared simultaneously. The bond Gly^{23}–Phe^{24} was another feasible bond. After further incubation, hydrolysis at the bonds Phe^{24}–Phe^{25}

and Lys^{29}–Ala^{30} was found. The neutral protease had a cleavage preference at the site where leucine is located as a P1′ residue followed by phenylalanine. The bonds X–Ala and X–Ile were hydrolyzed slowly, and hydrolysis of other bonds was not observed.

In the case of APR68, at the initial state of hydrolysis of insulin A-chain, the major fragments (Gly^1–Leu^{13} and Tyr^{14}–Asp^{21}) and the minor fragments (Gly^1–Leu^{16}, Glu^{17}–Asp^{21}, and Gly^1–Glu^{17}) were found; therefore, the bond Leu^{13}–Tyr^{14} was the most susceptible followed by the bonds Leu^{16}–Glu^{17} and Glu^{17}–Asn^{18}. After 18 h of hydrolysis, the fragment Gly^1–Leu^{13} was cleaved at the bond Cys^{11}–Ser^{12} and the fragment Tyr^{14}–Asp^{21} was hydrolyzed at the bond Tyr^{19}–Cya^{20}. Although the fragments Glu^{17}–Asp^{21}, Ser^{12}–Leu^{16}, and Tyr^{14}–Glu^{17} contained feasible bonds, they were not hydrolyzed. It is suggested that this alkaline protease requires at least four binding sites to cleave peptide bonds. As for oxidized insulin B-chain, the major fragments identified as peaks Phe^1–Leu^{15} and Tyr^{16}–Ala^{30} corresponded to the primary cleavage site of insulin B-chain at the bond Leu^{15}–Tyr^{16} after 10 min of digestion. With increasing digestion times, the fragment Phe^1–Leu^{15} was mainly cleaved at the bonds Gln^4–His^5 and Ser^9–His^{10}, and the fragment Tyr^{16}–Ala^{30} was further cleaved at the bonds Tyr^{26}–Thr^{27} and Phe^{24}–Phe^{25} and slowly at the bonds Glu^{21}–Arg^{22} and Cya^{19}–Gly^{20}. The alkaline protease has broad specificity and favors leucine at the P1 site. The bond Leu–Tyr is the most feasible, but other bonds X–Tyr were not cleaved. All cleavage sites of both proteases are shown in **Figure 5**.

Effect of pH on Protease Activity and Stability. The stability of proteases from *B. amyloliquefaciens* FSE-68 was investigated at various pH values. The enzyme solutions were incubated at 30 °C for 60 min at a given pH value, and the residual activity was then measured at pH 7.0 with FAGLA for NPR68 and with AAPF for APR68 at pH 8.6. To study the effect of pH on the protease activity, the substrates were dissolved in the buffer system and the enzymes were assayed at 25 °C. As shown in **Figure 6**, NPR68 and APR68 were almost 100% stable at pH 5.0–11.0 at 30 °C for 60 min. NPR68 shows optimum activity at pH 6.5 and APR68 at pH 10.0.

Thermostability of Protease. To determine the thermostability, purified enzymes were heated at 60 or 55 °C, and the rate of irreversible inactivation was measured (**Figure 7**). Compared with subtilisin BPN′, APR68 was thermosensitive. The half-life of APR68 at 60 °C was 3.0 (±0.1) min, and that of subtilisin BPN was 13.6 (±0.7) min. When thermal inactivation of APR68 was measured at 55 °C, it showed practically the same curve as that of the subtilisin BPN at 60 °C; the half-life was found to be 13.8 (±0.8) min.

DISCUSSION

Bacillus species produce a variety of extracellular and intracellular proteases. An alkaline protease (subtilisin family) and a neutral metalloprotease (thermolysin or bacillolysin family) are secreted into the medium, whereas at least two intracellular serine proteases are produced within *Bacillus* species (*14*, *15*). Subtilisins are a family of alkaline serine endoproteases secreted by a wide variety of *Bacillus* species. For example, subtilisin Carlsberg is secreted from *B. licheniformis*; subtilisin E is from *B. subtilis*; and subtilisin amylosacchariticus is from *B. amylosacchariticus* (*16*–*18*). The production of subtilisin has been exploited for laundry detergents, food processing, and other applications (*19*, *20*).

Like other *Bacillus* species, *B. amyloliquefaciens* FSE-68 mainly secreted two proteases, a neutral metalloprotease (NPR68)
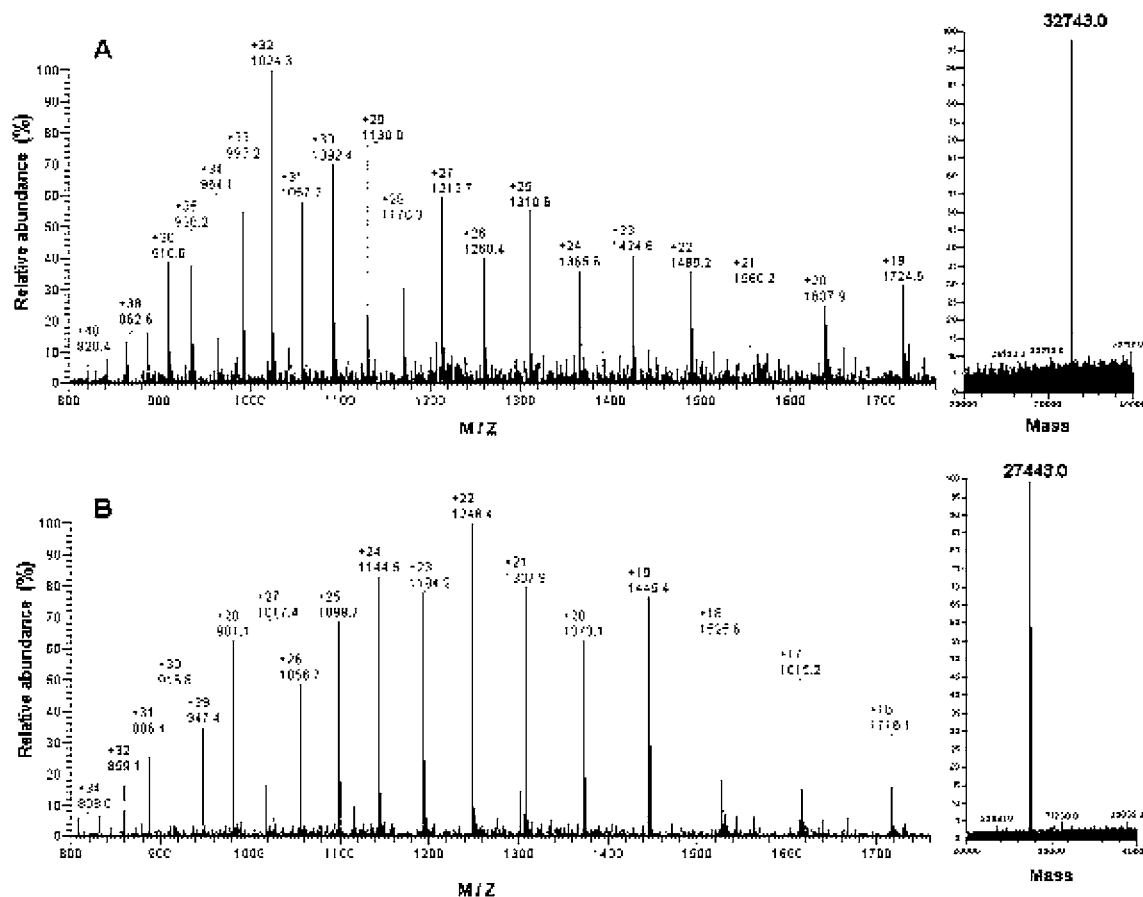
Figure 3. RP-HPLC/ESI-MS analysis of proteases from *B. amyloliquefaciens* FSE-68: mass spectrum showing multiple charged ion signals and molecular weight deconvolution of (A) NPR68 and (B) APR68.

Figure 4. N-terminal amino acid sequences of NPR68 and APR68 from *B. amyloliquefaciens* FSE-68 and those of other proteases: subtilisin BPN′, extracellular protease from *B. amyloliquefaciens*; subtilisin E from *B. subtilis*; subtilisin Carlsberg from *B. licheniformis*.

and an alkaline serine protease (APR68). Judging from the yield and purification folds, a significant proportion of the protease was found in the crude supernatants. They also have characteristic properties from other extracellular proteins, so it can be simply purified by ammonium sulfate precipitation and cation exchange chromatography to produce high-purity proteins. The purity of purified proteases was confirmed during N-terminal sequencing because only one amino acid residue was detected at each stage of sequencing.

Although the N-terminal sequence of the first 10 amino acids of APR68 was identical to that of subtilisin BPN′, APR68 also had distinct features. APR68 had a higher isoelectric point (p*I*) value than subtilisin BPN′ (p*I* = 7.8) as it was not electroblotted at pH 9.0 but at pH 11.0. The subtilisin-like proteases of NK (*21*), SMCE (*22*), and subtilisin Carlsberg (*23*) showed p*I* values

of 8.3, 9.75, and 7.8, respectively. APR68 was more thermosensitve than subtilisin BPN′. The half-life of APR68 (3 min) at 60 °C was much shorter than that of subtilisin BPN (13.6 min). Furthermore, the molecular weight of APR68 measured with LC-ESI.MS was 27443 Da and that of subtilisin BPN′ was reported to be 27533 Da (*16*). The difference in molecular weight between APR68 and subtilisin BPN′ may be due to the amino acid composition.

Several studies are available on the proteases, particularly alkaline protease from traditional foods, for example, Japanese fermented soybean (natto) (*24*), soybean curd (*22*), fermented soybean from Taiwan (*25*), and two types of Korean fermented soybean, chungkook-jang (*26*) and doen-Jang (*27*). However, for the first time proteases produced by the soybean fermentation starter culture commonly used in Korea were characterized in detail.

Soybean sauce and soybean paste aroused public interest because of their biological activities such as inhibition of angiotensin converting enzyme (*28*) and hypocholesterolemic activity (*29*). Although microorganisms and microbial proteases are greatly needed for improving soybean foods, information about them has been limited. Meju is a starting material for making Korean soybean sauce and soybean paste and contains various microorganisms that are suitable for soybean fermentation. Among several strains in meju, only a few species were involved in the actual fermentation process of meju; the other species were contaminants. Therefore, it is necessary to identify the major strain that is mainly responsible for meju fermentation to find a new type of protease and to produce biologically active soybean food.

Insulin A- chain

```
1        5            10           15        21
GlyIleValGluGlnCyaCyaAlaSerValCyaSerLeuTyrGlnLeuGluAsnTyrCyaAsn
```

NPR68　　↑　　　　　　　　　　　　　　↑　　↑

APR68　　　　　　　　　　↑　↑　　↑↑　↑

Insulin B- chain

```
1        5          10          15           20            25            30
PheValAsnGlnHisLeuCyaGlySerHisLeuValGluAlaLeuTyrLeuValCyaGlyGluArgGlyPhePheTyrThrProLysAla
```

NPR68　　　↑　　　↑　　　↑　↑　　　　↑↑　　　↑

APR68　　↑　　　↑　↑　　↑ ↑ ↑　↑　　　↑　　↑　　↑

Figure 5. Cleavage sites of oxidized insulin A- and B-chain by NPR68 and APR68 from *B. amyloliquefaciens* FSE-68. Thick and thin arrows indicate major and minor cleavage sites, respectively. Cya represents cysteic acid (R = CH_2SO_3H).
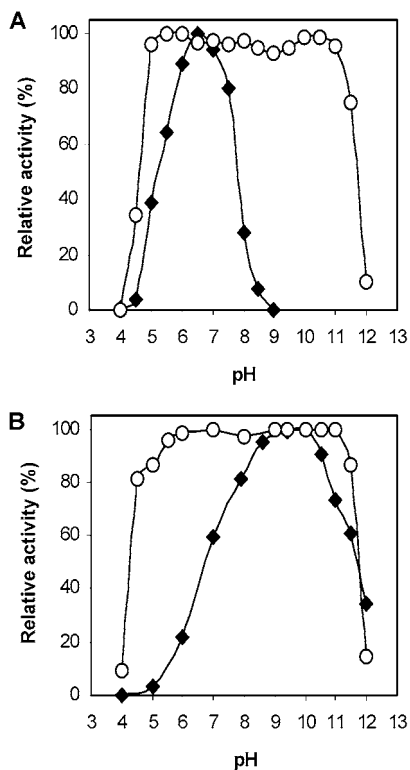

Figure 6. Effect of pH on activity and stability of (A) NPR68 and (B) APR68 from *B. amyloliquefaciens* FSE-68: profile of (♦) pH-dependent activity and (○) pH stability.
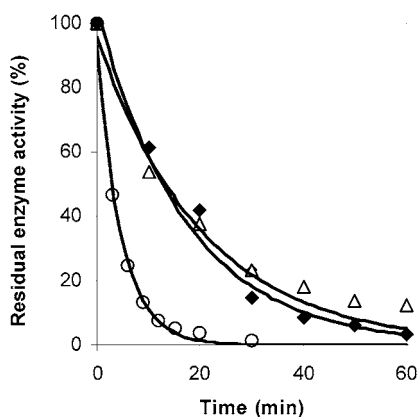

Figure 7. Thermostability of APR68 and subtilisin BPN′. Purified enzyme was incubated in 50 mM MOPS, pH 7.0, and 2 mM CaCl₂ at the given temperature: (○) APR68 at 60 °C; (♦) subtilisin BPN′ at 60 °C; (△) APR68 at 55 °C.

LITERATURE CITED

(1) Schlimme, E.; Meisel, H. Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Nahrung* **1995**, *39*, 1−20.

(2) Korhonen, H.; Pihlanto-Leppälä, A.; Rantamäki, P.; Pasela, T. Impact of processing on bioactive proteins and peptides. *Trends Food Sci. Technol.* **1998**, *9*, 307−319.

(3) Cho, S. J.; Kim, C. H.; Yim, M. H.; Lee, C. H. Large-scale purification of protease produced by *Bacillus* sp. from Meju by consecutive polyethylene glycol/potassium phosphate buffer aqueous two-phase system. *J. Microbiol. Biotechnol.* **1999**, *9*, 498−503.

(4) Pridmore, R. D. New and versatile cloning vectors with kanamycin-resistance marker. *Gene* **1987**, *56*, 309−312.

(5) O'Donnell, A. G.; Norris, J. R.; Berkeley, R. C. W.; Claus, D.; Kaneko, T.; Logan, N. A.; Nozaki, R. Characterization of *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* by pyrolysis gas−liquid chromatography, deoxyribonucleic acid−deoxyrinonucleic acid hybridization, biochemical tests, and API system. *Int. J. Syst. Bacteriol.* **1980**, *30*, 448−459.

(6) Nakamura, L. K. Deoxyribonucleic acid relatedness of lactose-positive *Bacillus subtilis* strains and *Bacillus amyloliquefaciens*. *Int. J. Syst. Bacteriol.* **1987**, *30*, 444−445.

(7) Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−54.

(8) Schickaneder, E.; Hösel, W.; Eltz, H. v. d.; Geu, U. Casein-resorufin, a new substrate for a highly sensitive protease assay. *Fresenius' Z. Anal. Chem.* **1988**, *330*, 360−360.

(9) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(10) Matsudaira, P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **1987**, *262*, 10035−10038.

(11) Arbige, M. V.; Bulthuis, B. A.; Schultz, J.; Crabb, D. Fermentation of *Bacillus*. In *Bacillus subtilis and Other Gram-Positive Bacteria*; Sonenshein, A. L., Ed.; American Society for Microbiology: Washington, DC, 1993; pp 871−895.

(12) Priest, F. G.; Goodfellow, M.; Shute, L. A.; Berkeley, R. C. W. *Bacillus amyloliquefaciens* sp. Nov., nom. rev. *Int. J. Syst. Bacteriol.* **1987**, *37*, 69−71.

(13) Ash, C.; Farrow, J. A. E.; Wallbanks, S.; Collins, M. D. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequence. *Lett. Appl. Microbiol.* **1991**, *13*, 202−206.

(14) Hirushi, S.; Kadota, H. Intracellular protease of *Bacillus subtilis*. *Agric. Biol. Chem.* **1976**, *40,* 1047−1049.

(15) Mantsala, P.; Zalkim, H. Extracellular and membrane-bound proteases from *Bacillus subtilis*. *J. Bacteriol.* **1980**, *141,* 493−501.

(16) Smith, E. L.; DeLange, R. I.; Evans, W. H.; Landon, M.; Markland, F. S. Subtilisin Carlsberg. V. The complete sequence; comparison with subtilisin BPN′; evolutionary relationships. *J. Biol. Chem.* **1986**, *243,* 2184−2191.

(17) Stahl, M. L.; Ferrari, E. Replacement of the *Bacillus subtilis* subtilisin structural gene with an in vitro-derived deletion mutation. *J. Bacteriol.* **1984**, *158,* 411−418.

(18) Nedkov, P.; Oberthur, W.; Braunitzer, G. Determination of the complete amino acid sequence of subtilisin DY and its comparison with the primary structures of the subtilisins BPN, Carlsberg and Amylosacchariticus. *Biol. Chem. Hoppe Seyler* **1985**, *366,* 421−430.

(19) Godfrey, T.; Reichelt, J. R. *Industrial Enzymology*; Nature Press: New York, 1983; pp 108−118.

(20) Kame, M.; Koda, H.; Kato, A.; Koma, T. Detergency and mechanism of soil removal in detergent-enzyme system. *J. Am. Oil Chem. Soc.* **1973**, *11,* 464−469.

(21) Sumi, H.; Hamada, H.; Tsushima, H.; Mihara, H.; Muroki, H. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese natto; a typical and popular soybean food in the Japanese diet. *Experientia* **1987**, *43,* 1110−1111.

(22) Yasuda, M.; Aoyama, M.; Sakaguchi, M.; Nakachi, K.; Kobamoto, N. Purification and characterization of a soybean-milk-coagulating enzyme from *Bacillus pumilus* Tyo-67. *Appl. Microbiol. Biotechnol.* **1999**, *51,* 474−479.

(23) Ottesen, M.; Svendsen, I. The subtilisins. In *Methods in Enzymology*; Academic Press: New York, 1970; Vol. 19, pp 199−215.

(24) Fujita, M.; Nomura, K.; Hong, K.; Ito, Y.; Asada, A.; Nishimuro, S. Purification and characterization of a strong fibrinolytic enzyme (Nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. *Biochem. Biophys. Res. Commun.* **1993**, *197,* 1340−1347.

(25) Chang, C. T.; Fan, M. H.; Kuo, F. C.; Sung, H. Y. Potent fibrinolytic enzyme from a mutant of *Bacillus subtilis* IMR-NK1. *J. Agric. Food Chem.* **2000**, *48,* 3210−3216.

(26) Kim, W.; Choi, K.; Kim, Y.; Park, H.; Choi, J.; Lee, Y.; Oh, H.; Kwon, I.; Lee, S. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. *Appl. Environ. Microbiol.* **1996**, *62,* 2482−2488.

(27) Kim, S. H.; Choi, N. S. Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp. strain DJ-4 Screened from *Doen-Jang*. *Biosci., Biotechnol., Biochem.* **2000**, *64,* 1722−1725.

(28) Suh, H. J.; Suh, D. B.; Whang, J. H.; Sung, H. J.; Yang, H. C. Purification of ACE inhibitor from soybean paste. *Agric. Chem. Biotechnol.* **1994**, *37,* 441−446.

(29) Kim, J. D.; Lee, Y. I.; Kim, B. R.; Choi, Y. S.; Lee, S. Y. Effects of Meju powder supplementation on lipid metabolism in rats fed hypercholesterolemic diet. *J. Korean Soc. Food Sci. Nutr.* **1997**, *26,* 314−318.