Note

Characterization of *Bacillus Subtilis* Isolated from Asian Fermented Foods

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Eleven *Bacillus subtilis* strains capable of producing protease were isolated from fermented foods and characterized. *B. subtilis* DB and SR were selected for digesting rice proteins. It was found that they could reduce rice allergenicity by hydrolyzing allergenic fragments detected by immunoblotting. Moreover, the fact that *B. subtilis* SR could also digest rice proteins resulted in the increase of glutamic acid, which is the important amino acid for γ -aminobutyric acid (GABA) production. Therefore, *B. subtilis* SR has the potential for application in the production of hypoallergenic fermented rice-noodle with high nutrient availability.

Keywords: *Bacillus subtilis*, proteolytic bacteria, protease, γ -aminobutyric acid (GABA), fermented food

Introduction

Fermented foods have traditionally been produced and consumed for centuries. Food characteristics are unique depending on climate and location. Raw materials commonly come from local agricultural products such as cereals, legumes, vegetables, milk, fish, meat, and so on.

Bacillus strains have been involved in food fermentation processes. They are able to produce high potential proteases, and their main activity during fermentation is enzymatic proteolysis (Ouoba *et al.*, 2004). Most *Bacillus* strains are not harmful to humans (Leonel Ochoa-Solano and Olmos-Soto, 2006). *Bacillus* proteases are very efficient in breaking down proteins into smaller units. Moreover, *Bacillus* fermentation can provide products with high nutrient availability (Kiers *et al.*, 2000). Therefore, the knowledge of how to exploit their potential should be disseminated.

Our previous researches have focused on *Bacillus* strains from fermented cereal foods. In Thailand, a variety of microorganisms, including aerobic bacteria, lactic acid bacteria, fungi and yeast, have been found in fermented rice-noodles

(Kha Nhom Jeen). We recently found numerous aerobic bacteria in soaked rice and rice blocks from production processing of fermented rice-noodles (Kobayashi *et al.*, 2007). *B. subtilis*, isolated from the soaked rice, showed interesting properties that have the potential to reduce allergenicity of wheat allergen (gliadin) (Phromraksa *et al.*, 2008). In Myanmar, Ikeda *et al.* (2005) reported that *B. subtilis*, isolated from soaked rice from fermented rice-noodle (Mohinger) processing, showed high proteolytic activities towards the protein substrates, azocasein and azocollagen, and could digest allergenic rice protein.

The present study aimed to clarify the proteolytic potential of *Bacillus* strains from various Asian fermented foods. Proteolytic bacteria were isolated and a phylogenetic tree was constructed. Because all isolated bacteria were of the same species, *B. subtilis*, in order to examine whether all bacteria also had the same level of proteolytic activity, characterization of bacteria using synthetic substrates was performed. In addition, to investigate the possibility of utilizing the bacteria in rice fermentation, the effects of selected bacteria on rice proteins were examined.

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Materials and Methods

Food samples and bacteria Five kinds of fermented food samples were collected from 5 Asian countries: Thailand, Japan, Mongolia, Laos, and Vietnam. These samples included 1 sample of fermented soybean seeds collected from Thailand in 2003, 3 samples of wheat flour dough (each collected from Thailand in 2003, Japan in 1992 and Mongolia in 1995), 1 sample of soaked rice used for fermented rice-noodle (Kha Nhom Jeen) processing collected from Thailand in 2003, 1 sample of Thai fermented soybean paste collected in 2003, and 2 samples of fish sauce (each collected from Laos in 1995 and Vietnam in 2000).

The samples were suspended in sterilized distilled water, spread on nutrient agar slants (containing 0.5% glucose (Nacalai Tesque, Japan)) and incubated at room temperature. All bacteria were maintained before screening on nutrient agar slants (containing 0.5% glucose) at 4°C.

Media The media for screening proteolytic bacteria and enzyme production were prepared according to the method described by Phromraksa *et al.* (2008).

Gelatin stab medium contained 1.8% nutrient medium (Eiken Chemical, Japan) and 17% gelatin (Nacalai Tesque). The medium was sterilized at 121°C for 15 min and kept at 4°C until use.

Screening of bacteria After bacteria were cultured overnight on nutrient agar slants (containing 0.5% glucose), each bacterium was cultured on a nutrient agar slant (containing 10% NaCl (Wako Pure Chemical Industries Ltd., Japan)) at 37°C for 24 h. Uninoculated identical medium was used as a control.

Enzyme activities towards gelatin were examined in all bacteria cultured on nutrient agar. Each bacterium was stabbed through the center of gelatin stab medium and cultured at 37°C for 72 h. Subsequently, all culture tubes were kept at 4°C overnight. Liquefaction of the medium is an indicator of bacterial enzyme activity. Uninoculated identical medium was used as a control.

Phylogenetic analysis Eleven *B. subtilis* strains were isolated and identified by 16S rDNA sequencing in our previous study (Nagano and To, 2000; Tran and Nagano, 2002; Liu and Nagano, 2008; Phromraksa *et al.*, 2008). The 16S rDNA sequences of bacteria were deposited in the DDBJ database under the accession numbers shown in Table 1.

The multiple alignments of all sequences determined were performed with the MEGA software version 4.0.2 (Tamura *et al.*, 2007). The distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987).

Reference strains used for the homology study were type strains of *B. subtilis* DSM10 (AJ276351), *B. licheniformis* (X68416), and *B. pumilus* DSMZ27 (AY456263).

Preparation of extracellular enzyme Each bacterium was refreshed by aerobic incubation overnight in an enzyme production medium at 37°C. Using this culture as a starter, the bacterium could be aerobically grown in the same medium at 37°C for 48 h. The broth was centrifuged at 10,000 rpm for 5 min. The supernatant was used as bacterial extracellular enzyme.

Preparation of bacterial enzyme Ammonium sulfate

Table 1. Isolated *B. subtilis* strains from Asian fermented foods.

Country	Source	Strain	Accession no.	Salt tolerance	Galatinase activity
Thailand	Soybean seeds	<i>B. subtilis</i> SB3	AB440267	+	+
		<i>B. subtilis</i> SB4	AB440266	+	+
		<i>B. subtilis</i> SB5	AB440268	+	+
	Wheat flour dough	<i>B. subtilis</i> DB	AB440269	+	+
	Rice	<i>B. subtilis</i> SR	AB440270	+	+
	Soybean paste	<i>B. subtilis</i> SP1	AB440271	+	+
		<i>B. subtilis</i> SP2	AB440272	+	+
Japan	Wheat flour dough	<i>B. subtilis</i> JK	AB509381	+	+
Mongolia		<i>B. subtilis</i> M2-4	AB379850	+	+
Laos	Fish sauce	<i>B. subtilis</i> FS-2	AB447487	+	+
Vietnam		<i>B. subtilis</i> CN-2	AB383135	+	+

(Wako Pure Chemical Industries Ltd.) was slowly added to the extracellular enzyme solution until 80% saturation was reached. The solution was gently stirred and kept at 4°C overnight in the refrigerator. The precipitate was separated by centrifugation at 10,000 rpm for 20 min. The precipitated protein was dissolved in 0.1 M potassium phosphate buffer (pH 7) and then dialyzed against distilled water at 4°C for 48 h. The product was used as bacterial enzyme.

Enzyme activity using synthetic substrate Enzyme activities were measured spectrophotometrically using the synthetic substrates, Suc-AAPF-pNA (Suc-Ala-Ala-Pro-Phe-pNA) and Bz-FVR-pNA (Bz-Phe-Val-Arg-pNA). The 600- μ L mixture, containing 2.63 mg protein/mL of bacterial enzyme and 20 mM Tris-HCl buffer (pH 8), was pre-incubated at 37°C for 2 min. Then, 200 μ L of 0.5 mM Suc-AAPF-pNA (Sigma-Aldrich, USA) or 0.25 mM Bz-FVR-pNA (Sigma-Aldrich) was added to the mixture and incubated at 37°C for 5 min. The amount of *p*-nitroanilide liberated was determined from the absorption at 405 nm. The same mixture, without substrate, was used as a control. Results are expressed as *p*-nitroanilide released calculated by linear regression analysis from the standard curve prepared with a known concentration of *p*-nitroanilide. One unit of activity was defined as *p*-nitroanilide released per amount of enzyme per minute.

The FALGPA (N-(3-[2-Furyl]acryloyl)-Leu- Gly-Pro-Ala) assay was performed as originally described by Van Wart and Steinbrink (1981) with the following modifications. The reaction mixture was composed of 800 μ L of 0.1 mM FALGPA (Sigma-Aldrich) in 50 mM tricine (BIO-RAD Laboratories, USA), 10 mM CaCl₂ (Nacalai Tesque), 400 mM NaCl, pH 7.5, and 3.95 mg protein/mL of bacterial enzyme. The mixtures were incubated at 22°C for 24 h. Hydrolysis of FALGPA was determined by measuring the decrease in absorbance at 324 nm. The same mixture, without FALGPA, was used as a control. One unit of activity was defined as the decrease of 0.1 unit of absorbance at 324 nm in 24 h under assay conditions.

Preparation of rice proteins Rice proteins were prepared according to the method described by Li *et al.* (2007) with slight modifications. Rice seeds were ground to pass through a 250- μ m mesh screen. Protein isolates were prepared from rice flour by extraction with 0.085 M NaOH (Nacalai Tesque) (1:12 w/v flour:water ratio) for 4 h at room temperature. The suspension thus obtained was centrifuged at 3,000 g for 20 min. The supernatant was removed and adjusted to pH 5.5 with 2 N HCl (Nacalai Tesque). The protein precipitate formed was centrifuged at 3,000 g for 20 min, washed twice with distilled water, and then lyophilized. The products were used as rice proteins.

Digestion of rice proteins Each mixture was composed of 500 μ L of 3% rice protein solution and 2.55 mg/mL of extracellular enzyme of each of 2 bacteria (*B. subtilis* DB and SR). The enzyme was added separately. These mixtures were incubated at 37°C for 0 and 24 h, respectively, and the reaction was terminated by boiling for 5 min. The mixtures were used as digested samples. The digested fragments were detected by 15% SDS-PAGE.

Amino acid composition analysis of digested samples was performed by HPLC analysis. The HPLC system used was a Hitachi ELITE LaChrom (Hitachi High-Technologies Corporation, Tokyo, Japan), equipped with an L-2130 pump, an L-2200 autosampler, an L-2350 column oven and an L-2485 FL detector. The column used was a NIN/OPA 4.0 mm I.D x 150 mm L (TR3-2004), operating at 55°C and with a flow rate of 0.4 mL/min. The injected volume was optimized to 20 μ L.

SDS-PAGE and immunoblotting Samples were analyzed using SDS-PAGE (Laemmli 1970). Immunoblotting was performed according to Phromraksa *et al.* (2008) with slight modifications. Proteins on the gel were electrotransferred onto a PVDF membrane (ATTO, Japan) and then blocked overnight at 4°C with 1% skim milk (Difco, USA) in TBS-T (Tris-buffered saline, pH 7.4, containing 0.1% Tween-20). The membrane was incubated for 1 h at 37°C in TBS-T containing a monoclonal antibody specific for 14-16-kDa rice allergens (diluted 1:2000) with gentle shaking. After the membrane was washed with TBS-T, TBS-T containing polyclonal rabbit anti-human IgE/HRP (diluted 1:5000) (Dako Cytomation, Denmark) was added and incubated for 1 h at 37°C with gentle shaking. The membrane was then washed with TBS-T. The reacted protein bands were detected by a light-emitting non-radioactive method (Amersham Biosciences UK Ltd., UK).

Results and Discussion

Phylogeny of isolated B. subtilis Various bacteria have been found in fermented foods, including *Bacillus* spp. Protein food fermentation is usually based on alkaline fermentation by *Bacillus* spp., in which *B. subtilis* is the predominant bacteria responsible. Other species of *Bacillus*, such as *B. licheniformis*, *B. pumilus*, *B. cereus*, *B. megaterium*, *B. badius*, *B. sphaericus* and *B. fusiformis*, are also commonly found in fermented foods (Ouoba *et al.*, 2008). Chantawannakul *et al.* (2002) and Inatsu *et al.* (2006) reported that the highly proteolytic bacteria involved in the Thai traditional fermented soybean, Thua Nao, were *B. subtilis*.

Isolated proteolytic bacteria in the present study are shown in Table 1. These bacteria were pre-screened for salt tolerance and enzyme activity towards gelatin. Eleven bac-

Fig. 1. Phylogenetic tree based on 16S rDNA sequences. Tree was constructed by the neighbor-joining method. The type strain of *B. pumilus* DSMZ27 (AY456263) and *B. licheniformis* (X68416) were used for outgroups. The scale indicates substitutions per site.

teria were able to grow on the 10% NaCl medium, and they were also positive for gelatin liquefaction. The results indicate that these bacteria have the ability to grow under high salt concentration conditions, and have proteolytic activity towards gelatin substances.

A phylogenetic tree, based on the 16S rDNA sequences of 11 isolated bacteria and 3 representative reference strains, is shown in Fig. 1. The isolated bacteria were clearly separated from the reference strains *B. licheniformis* (X68416) and *B. pumilus* DSMZ27 (AY456263), but were grouped together with the reference strains of *B. subtilis* DSM10 (AJ276351). These *Bacillus* isolates were shown to be closely related, by means of phylogenetic analysis. In order to clarify their characteristics, their enzyme activities were assayed.

Enzyme activity using synthetic substrates In our previous research, numerous bacteria were pre-screened by medium plates containing insoluble collagen. Many proteolytic bacteria were isolated and characterized with hydrophilic and hydrophobic protein substrates, azocasein and insoluble collagen. It was found that their enzyme activities varied among these substrates (data not shown). The bacterial enzymes were then characterized using synthetic substrates.

Bacterial enzymes had various enzyme activities on synthetic substrates (Figs. 2, 3, and 4). Figure 2 shows that almost all bacteria, except *B. subtilis* SB4, FS-2 and CN-2, have high enzyme activities towards Suc-AAPF-pNA, a synthetic substrate specific for subtilisin and chymotrypsin (Peng *et al.*, 2003). Moreover, these levels of enzyme activities were the same level as the reported enzyme activity of

B. subtilis DC33, a highly proteolytic bacteria isolated from Chinese fermented soybean (Wang *et al.*, 2006).

Bz-FVR-pNA is a synthetic substrate specific for trypsin (Ahn *et al.*, 2003). The bacterial enzyme activities varied among them, even with the bacteria belong to the same species, *B. subtilis*. Moreover, *B. subtilis* JK and CN-2 were unable to degrade this substrate (Fig. 3).

FALGPA is a good collagenase-specific substrate that can not be hydrolyzed by other enzymes such as chymotrypsin, trypsin, thermolysin, elastase, papain or carboxypeptidase A (Van Wart and Steinbrink, 1981; Jackson *et al.*, 1995). Five bacteria (*B. subtilis* SB5, DB, M2-4, FS-2, and CN-2) exhibited high levels of enzyme activity towards this substrate (Fig. 4).

B. subtilis DB and M2-4 were found to exhibit high enzyme activities towards these 3 substrates. *B. subtilis* DB and SR were previously reported to have the ability to reduce the allergenicity of wheat allergens, such as gliadin; while *B. subtilis* DB was able to reduce the allergenicity of milk allergens, such as β -lactoglobulin (Phromraksa *et al.*, 2008). Moreover, the results from the present study were expected to support the development of fermented rice-noodle. *B. subtilis* DB and SR were therefore subjected to the next experiment involving digestion of rice proteins.

Digestion of rice proteins The digestion of rice proteins by each enzyme of *B. subtilis* DB and SR is shown in Fig. 5A. After incubating with bacterial extracellular enzyme for 24 h, followed by detection with 15% SDS-PAGE, the enzyme of *B. subtilis* DB (lane 4) and SR (lane 6) were found

Fig. 2. Amidolytic activity of bacterial enzymes towards Suc-AAPF-pNA.

SB3, *B. subtilis* SB3; SB4, *B. subtilis* SB4; SB5, *B. subtilis* SB5; DB, *B. subtilis* DB; SR, *B. subtilis* SR; SP1, *B. subtilis* SP1, SP2, *B. subtilis* SP2; JK, *B. subtilis* JK; M2-4, *B. subtilis* M2-4; FS-2, *B. subtilis* FS-2; CN-2, *B. subtilis* CN-2.

* One unit (U) of activity was defined as μmol *p*-nitroanilide released per gram of enzyme per minute.

Fig. 3. Amidolytic activity of bacterial enzymes towards Bz-FVR-pNA.

Bacteria used in Fig. 3. are the same as in Fig. 2.

** One unit (U) of activity was defined as μmol *p*-nitroanilide released per gram of enzyme per minute.

Fig. 4. Amidolytic activity of bacterial enzymes towards FALGPA.

Bacteria used in Fig. 4. are the same as in Fig. 2.

*** One unit (U) of activity was defined as the decrease of 0.1 unit of absorbance at 324 nm in 24 h under assay conditions.

Fig. 5. SDS-PAGE (A) and immunoblot (B) of rice protein isolate fragments digested by *B. subtilis* DB and SR enzymes.

Lane M, molecular weight marker; lane 1, rice protein isolates at 0 h; lane 2, rice protein isolates at 24 h; lanes 3-4, mixture of rice protein isolates and enzyme of *B. subtilis* DB at 0 and 24 h, respectively; lanes 5-6, mixture of rice protein isolates and enzyme of *B. subtilis* SR at 0 and 24 h, respectively.

to digest the main fragments of rice proteins with molecular masses of about 20 and 33 kDa. However, at before incubation, the low molecular mass protein fragments digested by these enzymes (lanes 3 and 5) were much more than those of controlled mixtures (lane 1), indicating that these enzymes had a high ability to digest rice proteins.

Rice proteins with molecular masses of about 14-16, 26, 33, and 56 kDa were reported to be potentially reactive with IgE antibodies from patients with suspected rice allergy (Izumi *et al.*, 1992). Figure 5B shows that controlled rice proteins cross-reacted with a monoclonal antibody within the molecular mass range of 14-16 kDa (lanes 1 and 2). The 0-h enzyme digested fragments (lanes 3 and 5) had cross-reactivity with a monoclonal antibody at a molecular mass of about 16 kDa. The 24-h enzymatic digested fragments (lanes 4 and 6) did not have cross-reactivity at 16 kDa, but were found to have cross-reactivity at 14 kDa.

The 16-kDa allergenic rice protein was hydrolyzed by these two bacteria, so the allergenicity of rice protein was undetectable at this range. It was demonstrated, using immunoblotting, that these bacterial enzymes could reduce allergenicity of rice protein.

In addition, at the molecular mass of about 14 kDa, the 24-h digested fragments (lanes 4 and 6) had greater cross-reactivity than that of the 0-h digestion (lanes 3 and 5), indicating that digestion by these enzymes for 24 h could change the high molecular mass rice proteins into the 14-kDa rice

Table 2. Amino acid composition of rice proteins digested by isolated *B. subtilis* DB and SR ($\mu\text{g/g}$).

Amino acid	Controlled rice protein	Enzyme of <i>B. subtilis</i> DB	Enzyme of <i>B. subtilis</i> SR
Aspartic acid	0.64	31.46	81.91
Threonine	—	725.20	622.21
Serine	0.93	396.22	115.52
Glutamic acid	—	440.89	2201.40
Proline	—	—	—
Glycine	1.95	452.81	439.70
Alanine	—	2228.28	3452.93
Cysteine	—	—	—
Valine	—	8052.24	15341.37
Methionine	—	1530.51	2920.06
Isoleucine	4.27	3682.96	6459.26
Leucine	2.02	7682.39	18176.48
Tyrosine	—	2754.00	4104.57
Phenylalanine	—	3258.66	5724.56
GABA	179.27	1119.18	1357.26
Lysine	—	1284.88	2525.12
Histidine	0.58	754.97	818.36
Arginine	—	1728.51	3614.85
Total	189.66	36123.16	67955.56

allergenic substances.

Table 2 shows the amino acid composition of rice proteins digested by *B. subtilis* DB and SR. Rice proteins digested by each enzyme released a high amount of several kinds of amino acids, especially valine and leucine, compared with the controlled rice proteins. This indicates that these bacteria could digest rice proteins into smaller units, i.e., amino acids. Moreover, almost all the amino acids released from digestion by *B. subtilis* SR were of larger quantity than those by *B. subtilis* DB. However, proline and cysteine were not found in the digested proteins. It may be concluded that these enzymes could not cleave rice protein at amino acid positions of proline and cysteine.

We previously found that aerobic bacteria, including *Bacillus* spp., naturally have the ability to produce large amounts of certain amino acids in fermented rice from Thai fermented rice-noodle processing (Kobayashi *et al.*, 2007). These amino acids were glutamic acid, alanine, valine, leucine, phenylalanine, and γ -aminobutyric acid (GABA). The

present study also found large amounts of the same kind of amino acids in digested rice proteins (Table 2). Additionally, microorganisms, including aerobic bacteria, lactic acid bacteria, fungi, and yeast, were present during the processing of Thai fermented rice-noodle (Kha Nhom Jeen) (Kobayashi *et al.*, 2007). The amount of glutamic acid in fermented rice increased during the intermediate process that involved predominantly aerobic bacteria. Thereafter, the amount of GABA was also increased in the next process that was found to be predominant in lactic acid bacteria (Kobayashi *et al.*, 2007). Therefore, we hypothesized that aerobic bacteria, such as *Bacillus* strains, digested rice proteins, leading to the increase in glutamic acid, with lactic acid bacteria subsequently producing GABA from this glutamic acid. The present study can be used as a basic experiment for utilizing isolated *B. subtilis* in the production of fermented rice products with high nutrient availability.

All isolated bacteria in this study were in the same species of *B. subtilis*, although they come from different food

sources and locations. They showed enzyme activities towards synthetic substrates with various potentials, indicating each bacterium had specificity to each substrate even though they were of the same species. The use of *B. subtilis* in the food industry is recommended by the U.S. Food and Drug Administration as one of the GRAS (generally recognized as safe) microorganisms (Kramer and Gilbert, 1989; Denner and Gillanders, 1996). *B. subtilis* SR has the potential to reduce rice allergenicity and produce a large amount of glutamic acid from rice proteins, which is an important raw material for GABA production. It shows promise in its application for the production of hypoallergenic rice products, or use as a starter culture for improving the amount of GABA in the production of fermented rice products. Future study on the application of *B. subtilis* SR, together with lactic acid bacteria, in Thai fermented rice-noodle processing is needed to confirm and evaluate its capacity for producing nutrient-rich fermented rice products, such rice-noodle.

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