Hydrolysis of Soybean 7S and 11S Globulins Using *Bacillus subtilis*

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Soybean protein is an important plant protein source; its two major constituents, 7S and 11S, account for more than 70% of its total protein. In this study, the soybean 7S and 11S fractions were enzymatically hydrolyzed by *Bacillus subtilis* strains from traditional fermented foods, and the hydrolysates were analyzed using immunoblotting and an amino acid analyzer. According to the immunoblotting, the main components and allergens of 7S and 11S were degraded into smaller units. Large amounts of free amino acids (FAA) released in diverse profiles were also detected. *B. subtilis* SB3 and SB5 released more total FAA than other strains. Therefore, these *B. subtilis* strains may have potential for application in the production of hypoallergenic soybean foods rich in FAA.

Keywords: soybean 7S and 11S, *Bacillus subtilis*, free amino acid, immunoblotting, activity

Introduction

Soybean protein is one of the most important plant protein sources, widely recognized for its high nutritional value and health benefits. Soybean 7S (β-conglycinin) and 11S (glycinin), the two major constituents of soybean protein, account for more than 70% of the soybean’s total protein. However, they both were described as the main allergens in soybeans (Zhao *et al.*, 2008). The soybean 7S is a trimeric protein composed of 3 major subunits (α′ = 76 kDa, α = 72 kDa, and β = 54 kDa) (Keum *et al.*, 2006), and the soybean 11S is divided into acidic (approximately 37 to 43 kDa) and basic (approximately 17 to 20 kDa) polypeptides (Lee *et al.*, 2007). *Bacillus subtilis* was reported to give rise to high proteolytic activities in soybean fermentation, and markedly increases the free amino acid content by hydrolysis of protein with proteolytic enzymes (Dajanta *et al.*, 2011; Sarkar *et al.*, 1997). Moreover, some proteolytic enzymes from *B. subtilis* have been proven to degrade wheat allergens (Nagano *et al.*, 2003), hydrolyze soybean protein into functional peptides with anti-angiotensin converting enzyme (Gibbs *et al.*, 2004), and have anti-thrombotic (Omura *et al.*, 2004) and antioxidant properties (Chou *et al.*, 2010; Zhu *et al.*, 2008). Therefore, this study aims to evaluate the hydrolytic influence of *B. subtilis* on soybean 7S and 11S, and to gain a better understanding as an application attempt.

Materials and Methods

**Soybean protein and bacterial strains** Soybean protein 7S and 11S globulins were prepared according to the method of Nagano *et al.* (1992). Nine strains of the same *B. subtilis* species, all isolated from Asian traditional foods, were used in this study. The *B. subtilis* strains SP1, SP2, SB3, SB4 and SB5 were from Thua Nao, a fermented soybean food of Thailand (Phromraksa *et al.*, 2009); strain M2-4 was from the Mantou of Mongolia (Liu and Nagano, 2008); strain JK was from the Mantou of Japan (Phromraksa *et al.*, 2009); and strains CN2 and FS2 were from fish sauces of Vietnam and Laos, respectively (Nagano *et al.*, 2000).

**Media and enzyme production** The media for enzyme
production were prepared according to the method described by Phromraksa et al. (2008). The bacterial enzyme of the B. subtilis strain was prepared according to the method described by Phromraksa et al. (2009). The protein concentration of each enzyme was assayed by the method of Lowry et al. (1951) and adjusted to 20 mg/mL by dilution with mini-Q water (Millipore Co., Japan).

**Enzymatic hydrolysis of 7S and 11S globulin** 0.1% protein samples (both 7S and 11S globulin) were prepared in 0.25 M NaCl solutions. A 0.2-mL protein sample was mixed with the same volume of bacterial enzyme and incubated at 37°C for 24 h. Each hydrolysate was divided into two parts. One part was used for FAA analysis; the enzymatic reaction was stopped by addition of the same volume of 10% TCA solution, and then the mixture was centrifuged at 8000 g for 10 min. The supernatant was filtered through a 0.45-μm membrane (GL Chromatodisc, ATAS GL, Japan). The other part was mixed with the same volume of sample buffer for SDS-PAGE, and then boiled in water at 100°C for 5 min. The mixtures were kept at −20°C. The hydrolysis reaction was repeated three times.

**SDS-PAGE and immunoblotting** The hydrolysates of 7S and 11S were analyzed by 12.5% SDS-PAGE. Protein on the gel was transferred onto a PVDF membrane (GL Chromatodisc, ATAS GL, Japan). The gel was stained with 1% amido black.

**Amino acid composition analysis** The composition of the amino acids released from globulin was analyzed using a Hitachi ELITE LaChrom HPLC system (Hitachi High-Technologies Co., Japan). The system was equipped with an L-2130 pump, an L-2200 autosampler, an L-2350 column oven and an L-2485 FL detector. Chromatographic separation was carried out using a NIN/OPA column (4.0 mm I.D. × 150 mm L.; TR3-2004) with a flow rate of 0.4 mL/min at an operating temperature of 55°C. The injected volume was optimized to 20 μL, and identification and quantification of amino acids were carried out by comparison with a standard mixture of amino acids (type H) (Wako, Japan).

**Assay of bacterial enzyme properties** Gelatin zy-

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Fig. 1. Hydrolysis of soybean 7S and 11S by B. subtilis. Soybean 7S and 11S hydrolysates were analyzed by SDS-PAGE and immunoblotting. The 7S hydrolysates were stained by CBB, as shown in Fig. 1a, and the immunoblotting results are shown in Fig. 1b. The results for the 11S hydrolysates are shown in Figs 1c and 1d. Lane C, the 7S or 11S fraction with no enzyme treatment that was used as the control. Lane M, molecular weight marker; lane 1, B. subtilis SP1; lane 2, B. subtilis SP2; lane 3, B. subtilis SB3; lane 4, B. subtilis SB4; lane 5, B. subtilis SB5; lane 6, B. subtilis M2-4; lane 7, B. subtilis CN2; lane 8, B. subtilis JK; lane 9, B. subtilis FS2.
activity in lane 6 (M2-4). Compared to Fig. 1a, some bands disappeared in Fig. 1b due to the lack of IgE-binding activity.

Figs. 1c and 1d show the corresponding results of 11S hydrolysates. In Fig. 1c, the acidic polypeptides and basic polypeptides were also hydrolyzed by *B. subtilis*. A series of polypeptides with molecular weights less than 20 kDa were also present in lanes 1, 6 and 8. The acidic polypeptides (approximately 20 kDa) did not appear to be completely degraded in lane 6. Although Figs. 1d and 1b show a similar band of approximately 30 kDa, other allergenic bands with a small size can be seen in lanes 1, 6 and 8 in Fig. 1d.

**Diverse FAA profiles released by bacterial enzymes**

The generated FAAs of each hydrolysate are listed in Tables 1 and 2; the obtained FAAs from the 7S and 11S samples without enzymatic treatment were used as controls. A large amount of FAA was released in the 7S hydrolyzing process, as shown in Table 1. From that result, it was observed that the FAA profiles of 7S hydrolysates had a diverse distribution among the 9 bacterial enzymes. First, bacterial enzymes released different amounts of total FAAs from the 7S hydrolysates. For example, *B. subtilis* strains SB3 and SB5 released a higher amount of total FAA than the other strains, with amounts of 90.3 mg/g and 89.42 mg/g, respectively, and *B. subtilis* FS2 released the lowest total amount of FAA among all 7S hydrolysates. Statistically, glutamic acid, arginine, lysine and phenylalanine were detected as the most abundant FAA among the 7S hydrolysates. Glutamic acid was the most abundant in the hydrolysates, with the exception of the hydrolysate of *B. subtilis* FS2. *B. subtilis* FS2 appeared to have a different FAA profile, in which arginine was the most abundant FAA in the hydrolysate. In addition, proline, cysteine and valine were not detected in the 7S hydrolysates of SP1, SB4, FS2, and so on.

Table 2 lists the FAA profiles of the 11S hydrolysates that were similar to the 7S hydrolysates. For 11S hydrolysis, *B. subtilis* strains SB3 and SB5 still released higher total amounts of FAA, at 52.64 mg/g and 45.63 mg/g, respectively. Instead of strain FS2, the *B. subtilis* SB4 strain released the lowest total amount of FAA, with 19.8 mg/g in 11S hydrolysates. Glutamic acid, arginine, lysine and phenylalanine were still the dominant FAAs in all of the 11S hydrolysates. However, the total FAA released from 11S was less than that released from 7S for every bacterial enzyme.

**Proteolytic properties in bacterial enzymes**

Our bacterial enzymes produced by the *B. subtilis* strain were separated on an SDS-PAGE gel copolymerized with 0.1% gellan. Clear hydrolyzed bands set against a blue background were visualized on the zymography gel. Different proteolytic properties were observed for all of these *B. subtilis* strains (Fig. 2a). For a better understanding, molecular weights of the main hydrolyzed bands were calculated using Totalab Quant software (Totalab, England) (Fig. 2b). It was difficult to analyze the large molecular weight group (MW, MW > 60 kDa) within the highly intense background. The molecular weights of clear bands less than 60 kDa were detected by Totalab Quant, and divided into 6 groups based on molecular weight range (Fig. 2b). Bands of MW < 21 kDa were relegated to group I. In this group, *B. subtilis* strains showed two
clear hydrolyzed bands, with the exception of \textit{B. subtilis} SB4 and CN2. \textit{B. subtilis} SB4 had three bands and CN2 had only one. The largest band in group I was 20.6 kDa, belonging to the hydrolysate of \textit{B. subtilis} JK, and the smallest band was 14.4 kDa, belonging to the hydrolysate of \textit{B. subtilis} CN2. The band of 28.1 kDa in group II and the band of 38.9 kDa in group III seem to be distinctive. Overall, both \textit{B. subtilis} SB3 and SB5 showed strong signals that contrasted with the background. \textit{B. subtilis} SB4 had 8 bands and JK had 6 bands in the zymography map, which indicated that they had different properties from other strains.

**Discussion**

As one of the most important plant protein sources and nutrient foods, the soybean is widely used to manufacture traditional fermented foods in Asian countries. Proteolytic microorganisms in these foods hydrolyze soybean protein into smaller units through fermentation. All \textit{B. subtilis} strains used in this study were isolated from traditional fermented foods by our group. The soybean 7S β-conglycinin and 11S glycinin were separated from soybean protein, and enzymatically hydrolyzed by 9 strains of \textit{B. subtilis}. Although the proteolytic activities of these \textit{B. subtilis} towards some synthetic substrates have already been reported in 2009 (Phromraksa \textit{et al.}, 2009), our results demonstrated that the 9 strains of \textit{B. subtilis} used can effectively hydrolyze the 7S and 11S fractions. Furthermore, our zymography results directly showed the different properties among these \textit{B. subtilis}. In this sense, it can be inferred that diverse proteolytic activities led to the different proteins and FAA profiles seen in both 7S and 11S hydrolysates.

Many extracellular proteases from \textit{B. subtilis} have been reported. In comparison with the gelatin zymography results, the 18-kDa metalloprotease (Mine \textit{et al.}, 2005) from \textit{Bacillus} spp. may be related to group I. An enzyme of 27 kDa was reportedly identified from \textit{B. subtilis} CN2 (Uchida \textit{et al.}, 2004), along with a 33-kDa proteolytic enzyme in \textit{B. subtilis} M2-4 (Liu and Nagano, 2008). They may be associated with group II and group III, respectively. Proteases such as subtilisin FS33 (Wang \textit{et al.}, 2006), nattokinase (Fujita \textit{et al.}, 1993), subtilisin E (Wong \textit{et al.}, 1984), and KA38 (Kim \textit{et al.}, 1997) are likely associated with groups II to VI. These hydrolyzed bands were grouped together by gelatin zymography, which offered a platform to analyze these different proteases; gelatin zymography was reportedly an effective method for checking the proteolytic activities of enzymes (Patricia \textit{et al.}, 2005). Our results also indicated that the type of food source that our \textit{B. subtilis} strains were isolated from had no relationship with the different proteolytic activities of the \textit{B. subtilis}; for example, \textit{B. subtilis} SB3 and SB4 from a soybean-based food showed different enzymatic properties, and both \textit{B. subtilis} JK and M2-4 isolated from wheat flour-based foods also exhibited different hydrolyzed band and FAA profiles in this study. All together, the zymography results demonstrated the different proteolytic properties of different \textit{B. subtilis} strains, and also serve as a preview of potential future protease research using \textit{B. subtilis}.

Our results showed that the total amount of FAA released from 11S was less than that released from 7S. An easier enzymatic digestion of β-conglycinin, rather than glycinin, has been reported by some authors (Aguirre L \textit{et al.}, 2011; Hrackova \textit{et al.}, 2002; Marsman \textit{et al.}, 1997). The results of electrophoresis, as presented in this work, indicated that different cleavage sites in 7S and 11S exist between \textit{B. subtilis} enzymes. The cleavage sites of some enzymes were listed by Liu and Nagano (2008), including the enzymes of M2-4 and FS-2; these differences may explain the different properties of the hydrolysates seen in Fig. 1. The dominant amino acids were glutamic acid, arginine, lysine and phenylalanine, as listed in Tables 1 and 2. As shown in lanes 1, 6 and 8 of Fig. 1c, the basic subunits of glycinin did not digest completely, which may indicate that they are more resistant to \textit{in vitro} enzymatic hydrolysis than are acidic polypeptides. This is in agreement with previous findings of some other authors (Kella, \textit{et al.}, 1986; Romagnolo \textit{et al.}, 1990). Furthermore, this suggests that the reduction of disulfide bands caused a decrease in the digestibility of the basic polypeptides, which might be due to the aggregation of the basic polypeptides primarily through hydrophobic interactions. The differential susceptibility of both subunits in hydrolysis was also probably due to the greater hydrophobicity and compactness of the basic polypeptides (Kang \textit{et al.}, 1988).
It was reported that protease N (bacterial enzymes of *B. subtilis*) showed no IgE-binding activity towards soybean-sensitive patient serum (Nagano et al., 2005); bacterial enzymes of strains JK, M2-4, and DB exposed to the same serum by our group also showed a negative result. More than 20 allergenic proteins with IgE-binding activity have been identified in soybeans (Babiker et al., 1998), and both soybean 7S and 11S have been identified as soybean allergens (Maruyama et al., 1998). In this study, most components of 7S and 11S could be degraded. The main allergenic bands of approximately 30 kDa were present in both immunoblotting Fig. 1b and 1d. The molecular weight of these bands was close to the soybean allergen Gly m Bd 28K or Gly m Bd 30K. The Gly m Bd 28K protein is an allergen contained within the 7S globulin fraction (Tsuji et al., 1997). Gly m Bd 30K was reported as belonging neither to 7S globulin nor 11S globulin. Because of the complex structure of Gly m Bd 30K, heat treatment alone cannot reduce its allergenicity. In contrast, the IgE-binding activity of Gly m Bd 30K was reportedly enhanced by autoclave treatment (Yamanashi et al., 1993). Furthermore, some reports showed that Gly m Bd 30K can be markedly decomposed by *Bacillus* natto fermentation or protease N (Yamanashi et al., 1996). Bands of approximately 28 to 30 kDa were also produced by some strains, such as SP1, SB4, M2-4 and JK (Fig. 1b). Meanwhile, some smaller components in the 11S hydrolysates of *B. subtilis* SP1, M2-4, and JK still caused immune reactions in Fig. 1d. The reason for this might be due to the different properties and activities of the different bacterial strains (Fig. 2). Although Keum et al. (2006) confirmed that the 7S globulin was more allergenic than the 11S globulin, our results showed that both 7S and 11S globulins have similar allergenic characteristics after *B. subtilis* enzymatic digestion. However, the data also indicated that our soybean 11S sample was probably contaminated by 7S or some other soybean proteins. Because the negative control of 11S (lane C in Fig. 1c) showed some bands with molecular weights larger than the acidic polypeptides (approximately 37 to 43 kDa), the main bands of 28 to 30 kDa were present in four figures. Wilson et al. (2005) listed a series of soybean protein fractions of different molecular weights with IgE-binding activity, such as the 7S globulin (including M.W. 33 ~ 35, 35 ~ 38, 40 ~ 41, 47 ~ 50, 52 ~ 55, etc.), Glycinin G1 and G2 in the 11S globulin, and so on. However, most of these allergens in 7S and 11S could not be detected in our study, especially the main components of 7S and 11S, indicating its potential application in eliminating food allergens.

Amino acids from soybean protein are regarded as having high nutritive value and several health benefits. Glutamic acid was the most abundant FAA in the present study. Glutamic acid and its salts are the principal agents that impart a delicious taste to various Asiatic soybean fermented products, such as miso, koji, and soy sauce (Fukushima et al., 1994). Glutamic acid, arginine and lysine accounted for 40% to 50% of the total 7S hydrolysates and 35% to 42% of the 11S hydrolysates in this study. Increases in glutamic acid, aspartic acid, arginine, and glycine in chungkukjang can improve sweet and savory taste characteristics (Lee et al., 2005). Aspartic acid and glutamic acid belong to the acidic amino acid group, accounting for more than 44% of both 7S and 11S globulins. Conversely, basic amino acids (histidine and arginine), as well as hydrophobic amino acids (valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan and methionine) have a bitter taste and contribute to the maturation of fermented foods. Basic amino acids accounted for more than 39% of all 7S and 11S hydrolysates. Hydrophobic amino acids were regarded to be associated with the proteolytic activities of enzymes. A purified protease from M2-4 (Liu and Nagano, 2008) was proven to digest casein into fragments with hydrophilic and hydrophobic amino acids at the C-terminal. Our data showed that both hydrolyzed 7S and 11S released a large amount of hydrophobic amino acids, with a distribution of 25% to 31% in 7S hydrolysates and 25% to 37% in 11S hydrolysates. Different from fermentation, hydrolyzing 7S and 11S with bacterial enzymes markedly increased the FAA content over a short duration (Aguirre et al., 2011). Although similar FAA profiles can also be found in some traditional foods (Dajanta et al., 2011; Sarkar et al., 1997), enzymatic hydrolysis as a short biochemical process has potential for application in the fields of food, agriculture and industry.

In conclusion, soybean 7S and 11S globulins can be enzymatically hydrolyzed as substrates within 24 h with the high proteolytic activity of bacterial enzymes. Zymography was a simple, but efficient method for comparing the proteolytic enzymes of *B. subtilis* strains. The diverse proteolytic properties of *B. subtilis* bacterial enzymes, as shown in the present study, may be the main reason for the large variation seen in the profiles of released FAAs from soybean 7S and 11S. The digestion of allergens and the generation of FAAs indicated that these *B. subtilis* possess a unique proteolytic characteristic, which may have potential application to the elimination of allergenic foods and the development of healthy foods; this is especially true for some FAAs such as lysine, a limiting essential amino acid found in most cereal flours (Fuller et al., 1994). Therefore, it is necessary to understand more clearly the characteristics of these proteolytic enzymes and their related genes in *B. subtilis* derived from traditional foods; these will be analyzed in a further study.
References


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