Preparation of Hypoallergenic Soybean Protein with Processing Functionality by Selective Enzymatic Hydrolysis

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Received September 16, 1998; Accepted December 10, 1998

The enzymatic hydrolysis of soybean protein isolate (SPI) was investigated using commercially available *Bacillus sp.* proteases for reduction of soybean allergenicity. An alkaline protease, Proleather FG-F, was found to be useful for degradation of the major soybean allergens (Gly m Bd 30 K, Gly m Bd 28 K and α -subunit of β -conglycinin), whereas all proteases tested so far preferentially degrade β -conglycinin in native SPI at 70°C. The hydrolysate prepared using Proleather FG-F retained gel-forming ability, while the reactivity with IgE in serum of soybean-sensitive patients was markedly reduced. Thus, this hydrolysate may be especially useful as an ingredient in hypoallergenic foods.

Keywords: Soybean protein, allergen, *Bacillus sp.* protease, Gly m Bd 30 K, Gly m Bd 28 K, β -conglycinin, gelling properties

Soybean is an important protein in the food industry because of its excellent nutritional and functional properties, including those of gelling and emulsification. Food allergies have recently become a serious problem and for allergic patients, avoidance of the foods responsible for allergies is recommended as a conventional therapy. At present, the incidence of allergies to soybean proteins is lower than that of allergies to egg or milk proteins, but the growing consumption of soybean products not only in Japan but all over the world may result in an increase in the incidence of patients with soybean allergy. Thus, the development of hypoallergenic soybean-based foods is desirable.

Three soybean proteins, Gly m Bd 30 K (the same as the 34 kDa oil-body-associated protein (Kalinski *et al.*, 1990)), the α -subunit of β -conglycinin, and Gly m Bd 28 K have been identified as the major allergenic soybean proteins, based on the frequency of binding of these proteins to IgE in the sera of 69 soybean-sensitive patients with atopic dermatitis (Ogawa *et al.*, 1991; 1993). There have been several attempts to lower the allergenicity of soybean proteins by enzymatic (Hosoyama *et al.*, 1994; Yamanishi *et al.*, 1996) or physico-chemical treatments (Obata *et al.*, 1994; Samoto *et al.*, 1994). In the previous report, it was demonstrated that *Bacillus sp.* proteases were effective to hydrolyze Gly m Bd 30 K using autoclaved soybeans as the substrate, although hydrolyzing of all the proteins in soybean was required for the destruction of Gly m Bd 30 K (Yamanishi *et al.*, 1996). Such protease-treated soybeans therefore did not maintain their processing function. Studies on the allergenicity of mutant soybean lacking the α' - and α -subunit of β -conglycinin (Samoto *et al.*, 1997) or mutant soybean with low levels of Gly m Bd 30 K (Obata *et al.*, 1998) have also been reported. However, all of these approaches have some limitations in terms of implementation on an industrial scale, particularly with

respect to the functionality for processing and the production cost. Some authors recently proposed a process for producing hydrolysates with a reduced level of β -conglycinin (Tsumura *et al.*, 1997), and it was found that the level of other allergenic soybean proteins (Gly m Bd 30 K and Gly m Bd 28 K) were also lowered under similar conditions of hydrolysis.

In this paper, we report a new method for preparing soybean protein hydrolysate with processing functionality by selective enzymatic hydrolysis using a conventional soybean cultivar, which is effective in reducing the levels of the three major allergens in soybean.

Materials and Methods

Materials Defatted soybean meal was prepared from ground soybean IOM (Indiana-Ohio-Michigan, 1996 crop) by solvent extraction with *n*-hexane. Subtilisin (EC 3.4.21.62, Sigma No. P-4789, 8.1 units/mg) and thermolysin (EC 3.4.24.27, Sigma No. P-1512, 41 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Proleather FG-F (granulated enzyme from powdered Proleather, 10 units/mg) and Protease N (150 units/mg) were provided by Amano Pharmaceutical Co. (Nagoya). These proteases were used according to the manufacturer's instructions. All other chemicals employed in this study were of reagent grade and were used without further purification.

Preparation of soybean protein isolate (SPI) The following procedures were performed at room temperature. Defatted soybean meal was extracted with a 10-fold volume of water at pH 7.0 for 1 h and centrifuged (5000×*g*, 30 min) to remove the insoluble material. The pH of the extract was adjusted to pH 4.5 with 2 N HCl, and the insoluble fraction was collected by centrifugation (5000×*g*, 10 min). The precipitate obtained was adjusted to pH 7.0 with 2 N NaOH, yielding SPI and the SPI was lyophilized.

Hydrolysis of SPI SPI was dissolved with distilled water at a final concentration of 5% w/v (pH 7.0). Protease solution was then added and the mixture was incubated at the appropriate reaction temperature for 30 min with gentle stirring. After the enzyme reaction, the reaction mixture was immediately boiled to inactivate the enzyme and then analyzed. The degree of hydrolysis (DH) was determined by the following method: A 0.5 ml aliquot of diluted reaction mixture (5 mg/ml in distilled water) and an equal volume of 0.44 M trichloroacetic acid (TCA) solution were mixed and incubated for 30 min at room temperature. Thereafter, the mixture was centrifuged (8000×g, 5 min) to obtain a 0.22 M TCA-soluble fraction. The 0.22 M TCA-soluble fraction and the diluted reaction mixture were each analyzed to determine the protein content by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard protein. The DH value was calculated as the ratio of 0.22 M TCA-soluble protein to total protein in the diluted reaction mixture, expressed as a percentage.

Pilot-scale preparation of the hydrolysate Twenty liters of SPI solution (5% w/v, pH 7.0) was prepared and heated to 70°C. Proleather FG-F dissolved in water (2 g/40 ml) was added, and the mixture was incubated at 70°C for 30 min with gentle stirring. After the enzyme reaction, the reaction mixture was immediately pasteurized with steam at 140°C for 10 s and spray-dried at 75–80°C to yield powdered hydrolysate (940 g). The control SPI was prepared in the same manner, without enzyme treatment.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE was performed by the method of Laemmli (1970) using a 12% polyacrylamide gel (Tefco Co., Tokyo). Molecular weight markers were obtained from Daiichi Pure Chemicals Co. (Tokyo). Protein was stained with Coomassie Brilliant Blue R-250 (CBB) or Amido Black 10B.

Immunoblotting Mouse monoclonal antibodies (mAb) specific for Gly m Bd 30 K (F 5) and Gly m Bd 28 K (C 5) were prepared and used as previously described (Tsuji *et al.*, 1993, 1997). After SDS-PAGE, the proteins in the gel were transferred electrophoretically onto a PVDF membrane (Immobilon-P, Millipore). Detection of Gly m Bd 30 K and Gly m Bd 28 K was performed by the method of Samoto *et al.* (1994) and Tsuji *et al.* (1997), respectively. For evaluation of reactivity with Gly m Bd 30 K-specific IgE antibody by immunoblotting, proteins were treated with patient serum containing Gly m Bd 30K-specific IgE antibody followed by ¹²⁵I-labeled anti-human IgE and the radioactivity was detected by preparing an autoradiogram on X-ray film as described (Ogawa *et al.*, 1991).

Preparation of glucono-δ-lactone (GDL) gel and determination of gel hardness For each of the protein (dissolved in deionized water) solutions to be tested, 500 μl aliquots were placed in wells of a 24-well tissue culture plate (16 mm I.D. × 18 mm height, Becton Dickinson Co., Franklin Lakes, NJ), and a 10% w/v GDL solution, freshly prepared in ice-cold water, was added to each well and mixed well. The final concentration of protein was adjusted to 5.0% w/v, and the final concentration of GDL was adjusted to 0.30%, 0.35% and 0.40%. The wells were tightly sealed with heat-resistant

film, and the plate was placed in a water bath at 80°C for 30 min, cooled, and then kept at room temperature for 1 h before measurement. The compression testing of the gels was carried out using a compression tester KES-G5 (Kato Tech Co., Ltd., Kyoto) with a cylindrical plunger 5.6 mm in diameter. The compression rate was 0.1 mm/s, and the gel hardness was measured as the breaking force.

Results and Discussion

Hydrolysis of SPI using *Bacillus sp.* proteases The degradation of SPI by various *Bacillus sp.* proteases was examined. In the case of subtilisin and thermolysin, DH was 28% and 40%, respectively, after enzymatic hydrolysis at an enzyme to substrate (E/S) ratio of 2 units/g-SPI at 70°C for 30 min. Protein stained on the gel clearly showed that selective hydrolysis of the β-conglycinin in SPI had occurred in both of the hydrolysates (Fig. 1A, lanes 2 and 3). However, immunostaining of the hydrolysates indicated that Gly m Bd 30 K was more effectively degraded by subtilisin although the DH value was a little lower than that by thermolysin (Fig. 1B, lanes 2 and 3). These results suggest that subtilisin is the more effective of the two in degradation of Gly m Bd 30 K. Similarly, two food-grade proteases, Proleather FG-F and Protease N, were also examined. Although the E/S ratio differed, *i.e.*, 20 units/g-SPI in the case of Proleather FG-F and 300 units/g-SPI in the case of Protease N, the DH value was 25% in each instance after enzymatic hydrolysis. Protein stained on the gel showed that selective hydrolysis of the β-conglycinin in SPI had occurred in both hydrolysates (Fig. 1A, lanes 4 and 5). While Protease N was inadequate for degradation of Gly m Bd 30 K, the Gly m Bd 30 K was almost completely degraded by Proleather FG-F judging from the immunostaining of the hydrolysates (Fig. 1B, lanes 4 and 5). Among the four proteases tested, Proleather FG-F was the most effective in degradation of both β-conglycinin and Gly m Bd 30 K, we therefore selected it to reduce the soybean allergenicity, and examined the reaction conditions for

Fig. 1. SDS-PAGE and immunoblotting patterns of the hydrolysate prepared with various *Bacillus sp.* proteases. A 5% w/v of native SPI solution (pH 7.0) was incubated with each protease at 70°C for 30 min. Five micrograms of protein was loaded to each lane for SDS-PAGE. Panel A; staining with CBB. Panel B; staining with anti-Gly m Bd 30 K (F 5). Lane 1, Undigested SPI; lane 2, Subtilisin; lane 3, Thermolysin; lane 4, Proleather FG-F; lane 5, Protease N. The letters α', α and β represent α', α and β-subunits of β-conglycinin, and AS and BS the acidic and basic subunits of glycinin.

enzymatic hydrolysis in detail.

Reaction temperatures in the range of 37–70°C were evaluated using Proleather FG-F at an E/S ratio of 20 units/g-SPI, at pH 7. With regard to the degradation of β -conglycinin in SPI, hydrolysis at about 70°C was required as shown in Fig. 2A, lanes 2–4; however, Gly m Bd 30 K was almost completely degraded at temperatures above 50°C (Fig. 2B, lanes 2–4). Thus, Gly m Bd 30 K in the SPI was more readily hydrolyzed than β -conglycinin, without destruction of glycinin under these conditions. On the other hand, when heat-denatured SPI (which had been incubated in a boiling water bath for 10 min) was subjected to hydrolysis as the substrate, the major storage proteins (β -conglycinin and glycinin) were hydrolyzed randomly, and poor degradation of Gly m Bd 30 K was obtained (Fig. 2A and 2B, lanes 5–6). Yamanishi *et al.* (1996) reported that heat-treatment such as autoclaving was necessary to degrade all proteins and thus promote the penetration of proteases into soybean cotyledons when soybean was used as the substrate. In addition, the previous results indicated that Gly m Bd 30 K was decomposed following the hydrolysis of major storage proteins during enzymatic reaction. These results coincide with the present findings that the digestibility of Gly m Bd 30 K is poor in comparison to that of the major storage proteins using heat-denatured SPI as the substrate. The reason for this is not clear, but there is a plausible explanation that some steric hindrance due to denaturation of Gly m Bd 30 K (*e.g.* aggregation, *etc.*) caused by heating may lead to poor digestibility.

The pH conditions for the reaction using Proleather FG-F were also examined in the range between pH 7 and pH 10. More effective degradation of both β -conglycinin and Gly m Bd 30 K was obtained at pH 7 than at pH 10, although Proleather FG-F is an alkaline protease (data not shown). Thus, for reducing the levels of both β -conglycinin and Gly m Bd 30 K the most desirable reaction conditions were as follows: Proleather FG-F at 70°C and pH 7 using native SPI as substrate. The present results demonstrate that Proleather

FG-F is effective in degradation of both β -conglycinin and Gly m Bd 30 K without the destruction of glycinin under the conditions described above.

Characterization of hydrolysate prepared on a pilot scale using Proleather FG-F Based on the results described above, we attempted preparation of soybean protein hydrolysate on a pilot scale using Proleather FG-F. As shown in Fig. 3, β -conglycinin and Gly m Bd 30 K were markedly degraded by Proleather FG-F judging from SDS-PAGE and immunoblotting analyses. Dot-blot analysis of the hydrolysate and undigested SPI was performed using mAb specific for Gly m Bd 30 K. The antigenicity of the hydrolysate was considerably reduced compared to that of undigested SPI. The extent of reduction of the level of Gly m Bd 30 K was 99.2% compared with undigested SPI as determined by densitometer. Similarly, the allergenicity of the hydrolysate was evaluated using patient serum containing IgE antibody specific for Gly m Bd 30 K. As shown in Fig. 4, there was no band of radioactivity detected in the lanes containing the hydrolysate, indicating that almost all of the Gly m Bd 30 K capable of reacting with the IgE antibody had been elimi-

Fig. 2. SDS-PAGE and immunoblotting patterns of the hydrolysate prepared with Proleather FG-F at various reaction temperatures. A 5% w/v of native SPI solution (pH 7.0) was incubated with 20 units/g-SPI of Proleather FG-F at each reaction temperature for 30 min. Hydrolysate of denatured SPI solution is shown in lanes 5 and 6. Other experimental procedures are described in the legend of Fig. 1. Lane 1, Undigested SPI; lane 2, 37°C; lane 3, 50°C; lane 4, 70°C; lane 5, 37°C (denatured SPI); lane 6, 70°C (denatured SPI).

Fig. 3. SDS-PAGE and immunoblotting patterns of the hydrolysate prepared on a pilot scale. Five micrograms of protein was loaded to each lane for SDS-PAGE. Panel A; staining with CBB. Panel B; staining with anti-Gly m Bd 30 K (F 5). Lane 1, Undigested SPI; lane 2, Hydrolysate with Proleather FG-F.

Fig. 4. Immunoblotting of the hydrolysate prepared on a pilot scale with patient's serum. Ten micrograms of protein was loaded to each lane for SDS-PAGE. Panel A; staining with Amido Black 10 B. Panel B; staining with patient's serum and ^{125}I -labeled anti-human IgE. Lane 1, Undigested SPI; lane 2, Hydrolysate with Proleather FG-F; M, Marker protein.

Fig. 5. Immunoblotting pattern of Gly m Bd 28 K in the hydrolysate prepared on a pilot scale. A; staining with Amido Black 10 B. B; staining with anti-Gly m Bd 28 K (C 5). In each panel: Lane 1, purified Gly m Bd 28 K (1 µg); Lane 2, Undigested SPI (100 µg); lane 3, Hydrolysate (100 µg).

Fig. 6. Hardness of the GDL gels prepared from the hydrolysate and SPI. The experimental procedure is described in the text. Hydrolysate, (○); SPI, (●).

nated. Subsequently, the antigenicity of Gly m Bd 28 K in the hydrolysate was examined. As shown in Fig. 5, Gly m Bd 28 K was also markedly degraded by Proleather FG-F as judged from immunoblotting analysis. These results demonstrate that the levels of the three major allergenic proteins in this hydrolysate were reduced. Recently, Samoto *et al.* (1997) reported that essentially complete removal of these three major allergenic soybean proteins was achieved using a mutant soybean cultivar. In contrast to their method, our procedure is a simpler means of reducing the levels of the three major allergenic soybean proteins in SPI prepared from a conventional soybean cultivar.

To confirm the applicability of this hydrolysate as a foodstuff, its gel forming ability was examined. We produced a tofu gel (soybean curd) from the hydrolysate, and compared it with gel prepared from undigested SPI. A firm, self-supporting gel could be prepared from the hydrolysate using GDL as the coagulant. As shown in Fig. 6, the breaking strength of the gel prepared from the hydrolysate was almost the same as that

of the gel prepared from undigested SPI, indicating that the hydrolysate retained gel forming ability. The hardness of tofu gel prepared using GDL as the coagulant reportedly depends on the nature of the glycinin (Hashizume *et al.*, 1975; Kohyama & Nishinari, 1993).

We have established an effective means of enzymatic treatment to reduce the allergenicity of soybean protein by selective proteolysis using SPI obtained from a conventional soybean cultivar as the substrate. This hydrolysate retained gel forming ability, whereas its protein constituents were digested to the extent that the reactivity with IgE in serum from soybean-sensitive patients was markedly reduced. The results of this study suggest that the present hydrolysate may become widely used as a hypoallergenic food material to produce various fabricated foods. Further studies, such as skin and/or oral challenge tests for patients, are required to evaluate the hypoallergenicity of this hydrolysate and these are now in progress.

Acknowledgements The authors are grateful to Dr. Y. Morita for his valuable comments. The authors also thank Ms. K. Hoshino and Mr. T. Saito for their technical assistance.

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