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Characteristics of a Black Soybean (*Glycine max* L. Merrill) Protein Isolate Partially Hydrolyzed by Alcalase

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Abstract A protein isolate was prepared from black soybean (*Glycine max* L. Merrill) that possessed higher antioxidant activity than ordinary white soy protein isolates. The isolate was partially hydrolyzed by alcalase to reduce the allergenicity of black soybean. Alcalase remarkably reduced the molecular mass of the major soybean allergens that have molecular weights of 53, 38, and 24 kDa. Hydrolytic breakdown occurred more effectively in Gly m Bd 30K than in Gly m Bd 60K or Gly m Bd 28K. Alcalase hydrolysis increased the solubility and hydrophobicity of the black soybean protein isolate. The foaming activity and stability of black soybean proteins were highly increased by the partial hydrolysis.

Keywords: alcalase, black soybean, enzymatic hydrolysis, functional property, hypoallergenic

Introduction

Black soybean (*Glycine max* L. Merrill) has recently received considerable attention because of its high nutritional value and availability as an ingredient in various foods and folk medicines in Asia. Of the supposed medicinal components in black soybean, the common chemical components are anthocyanins (1,2), isoflavones such as phytoestrogens (3,4), oligosaccharides, and saponins (5-7). The pharmaceutical activities of anthocyanins are the main contributors to the antioxidant properties (8-10).

Despite having all of these constituents, soybean is one of 8 significant food allergens. Intolerance to soy protein causes clinical syndromes such as rhinitis, urticaria, asthma, atopic dermatitis, anaphylactic shock, or even death (11). Approximately 15 soybean proteins are recognized in the serum of soybean-sensitive patients who have atopic dermatitis, and Gly m Bd 60K, 30K, and 28K were identified as the major molecular masses of allergens in soybean protein (12,13). Therefore, the characterization of allergens is important to allow the safe use of soybeans and soybean products. Moreover, the poor solubility of soy proteins makes them unsuitable for extraction and functional applications. Hence, in addition to improving protein extractability, the structural modification of soy proteins is necessary to produce functional ingredients and for beverage/infant formula applications. Our objectives were to produce a black soybean protein isolate, reduce the allergenicity, and determine the physicochemical properties of the prepared hypoallergenic protein isolate for the production of hypoallergenic food protein ingredients with high antioxidant properties.

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Materials and Methods

Preparation of black soybean protein isolate and hydrolysates Black soybeans were crushed in a blender (IXM-401; Shinil Co., Seoul, Korea) at high speed for 2 min to produce black soybean flour, which was then defatted by hexane (Merck Co., Darmstadt, Germany) extraction. The resulting defatted black soybean flour was dried for 5 hr in an oven (40°C) and then passed through a 60-mesh sieve. The defatted black soybean flour was mixed with 10 times the volume of water at pH 10.0 for 3 hr to extract the protein, and then centrifuged at 10,000×g for 15 min at 4°C to remove insoluble material. The pH of the resulting supernatant was adjusted to pH 4.5 using 1.0 N HCl to precipitate the proteins; these were washed twice in distilled water. The proteins were redispersed in distilled water (1:10, w/v), adjusted to pH 8.0, and freeze-dried (FD-1000I; Rikakika Co., Tokyo, Japan). The protein content of the isolate was determined using the micro-Kjeldahl method (conversion factor of 6.25) (14).

Hydrolysis of the protein isolate was carried out using alcalase (Alcalase 2.4 L, food grade, Novo Laboratories, Inc., Wilton, CN, USA) according to the manufacturer's instructions. The previously prepared protein isolate was dissolved in distilled water, and the pH was adjusted to the optimum of pH 8.0 using 0.1 N NaOH. This dispersion was incubated in a water bath at 50°C for 10 min. Alcalase (1.5% protein isolate, w/w) was added to the suspension and incubated at 50°C for 30-480 min. The hydrolyzed protein was then heated at 75°C for 5 min in a water bath to inactive the enzyme, allowed to cool to room temperature, freeze-dried, ground, and stored at 5°C until analyzed. The degree of hydrolysis (DH) was determined by measuring the soluble nitrogen content in 10% trichloroacetic acid (TCA, Merck Co.) (15,16). The hydrolyzed protein (0.5 g) was suspended in 50 mL of distilled water. A 1 mL aliquot of the suspension was assayed for nitrogen using the micro-Kjeldahl method (14). A 10 mL aliquot of the

aqueous suspension of enzyme-modified soy protein isolates (SPIs, 1%, w/v) was mixed with 10 mL of 20% TCA and centrifuged at 8,900×g for 20 min at 4ºC. The soluble nitrogen in the supernatant was assayed. The DH was expressed as a percentage as follows:

$$\text{DH (\%)} = \frac{\text{Soluble nitrogen in 10\% TCA solution (mg)}}{\text{Total nitrogen (mg)}} \times 100$$

Gel electrophoresis and immunoblotting The molecular weight distribution of the hydrolyzed protein isolates was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples (0.005 g/mL) were prepared in buffer containing 1 M Tris (pH 6.8), 50% glycerol, 10% SDS, and 5% 2-mercaptoethanol. SDS-PAGE was carried out on a slab gel using an SDS-tris-glycine discontinuous buffer system with a 4% stacking gel and a 12% gradient gel to obtain good resolution for lower molecular weight bands. The gels were stained with 0.1% brilliant blue R and then destained until the background staining was removed.

The separated protein isolates on 12% SDS-PAGE were transferred to polyvinylidene fluoride (PDVF) membranes that were blocked with 5% skim milk in Tris-buffered saline-Tween 20 (TBS-T), using the Bio-Rad transblot system. The membranes were then incubated with primary antibodies (polyclonal antibody against soybean protein, purchased from Ab-Frontier, Seoul, Korea) for 2 hr and washed for 1 hr with TBS-T. After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG diluted to 1:1,000 in 5% skim milk for 1 hr at room temperature, washed for 1 hr with 0.1% TBS-T, and stained with ECL reagent (Amersham Co., Arlington Heights, IL, USA).

Antioxidation activity of the isolates The method developed by Lee *et al.* (17) was used to assess the 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA) free radical scavenging activity of the methanol extracts of ordinary white soy protein and black soybean protein isolates. For this, 100 μL of 200 μM DPPH solution was added to 100 μL of methanol extract of various concentrations (1, 5, and 10 mg methanol extract in 1 mL methanol). After incubation for 30 min at 37ºC, the methanol extracts containing DPPH solution were measured spectrophotometically at 540 nm. The scavenging percentage of DPPH was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Surface hydrophobicity The surface hydrophobicity of protein samples was determined using a hydrophobic fluorescence probe method with 1-anillino-8-naphthalene sulfonate (ANS, Aldrich, Milwaukee, WI, USA) (18). The protein was serially diluted with 0.01 M phosphate buffer (pH 7.0) to obtain protein concentrations ranging from 0.00056 to 0.015%. Then, 20 μL of 8.0 mM ANS in 0.01 M phosphate buffer (pH 7.0) was added to 4 mL of the diluted protein solution. The fluorescence intensity of the protein was measured using a spectrofluorometer (Kontron, Model SFM23/B; Kontron Ltd., Zurich, Switzerland). The excitation and emission wavelengths were 390 and 470 nm, respectively. The fluorescence intensity (FI) reading was standardized by adjusting the spectrofluorometer reading for 10 mL of ANS in 5 mL of methanol to 80% of full scale. The slope of the plot of FI vs. percentage protein concentration was calculated using least squares linear regression and used as the surface hydrophobicity.

Protein solubility Protein solubility was determined using the method of Bera and Murkherjee (19). The 50 mg of protein sample was dispersed in 50 mL of distilled water and adjusted to pH 3-11 using either 0.1 N NaOH or HCl. The dispersions were shaken for 60 min at room temperature and then centrifuged at 4,350×g for 20 min. The nitrogen content in the supernatants was determined using the micro-Kjeldahl method (14), and the percentage soluble protein was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Amount of nitrogen in supematant}}{\text{Amount of nitrogen in sample}} \times 100$$

Foaming properties The foaming properties were determined using the method of Kato *et al.* (20), with slight modification. The sample dispersions (0.5 g/50 mL) were adjusted to pH 4.0, 7.0, or 9.0 using 0.1 N HCl or NaOH. The foaming capacity of the black soybean protein and the partially hydrolyzed protein was determined and compared by measuring the volume of foams immediately after stirring for 1 min with a homogenizer (High-Flex Homogenizer HF-93; SMT Co. Tokyo, Japan) at 10,000×g. Foaming stability was measured as the foam volume remaining after 20 min. The foaming capacity was expressed by the following equation:

$$\text{Foaming capacity} = \frac{\text{Total volume} - \text{Drainage volume}}{\text{Initial volume of 50 mL}}$$

Foaming stability was determined from the following equation:

$$\text{Foaming stability} = \frac{V_0 \times t}{\Delta V}$$

where V_0 is the initial foam volume and ΔV is the change in foam volume after 20 min (21). All results are the calculated means of triplicate experiments.

Results and Discussion

Production of black soybean protein isolate The protein content of the isolate prepared from black soybean was 93% (dry basis). The yield of protein isolates from defatted black soybean was approximately 53% of the total protein. The isolate produced from black soybean was a grayish color and still possessed high radical scavenging ability (Fig. 1). The methanol extract of the black soybean protein isolate had much higher DPPH radical scavenging ability than did that of a regular soy protein isolate at all tested concentrations (1, 5, and 10 mg of methanol extract in 1 mL of methanol), probably because of the presence of residual antioxidant compounds such as anthocyanins, isoflavones, and/or saponins, even after isolation.

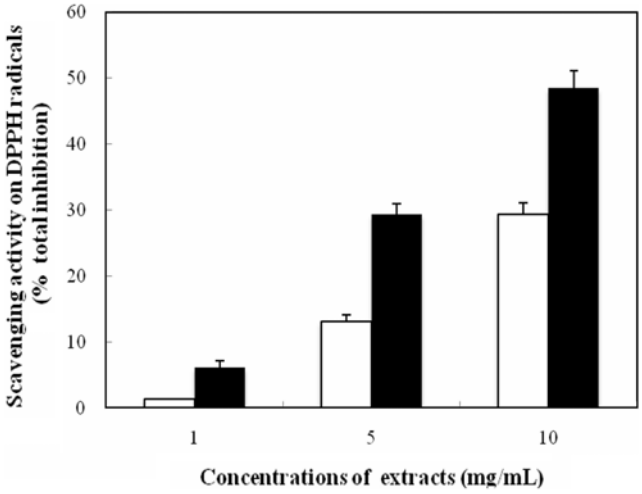

Fig. 1. Radical scavenging activity of methanol extracts obtained from ordinary (□) and black soybean (■) protein isolates.

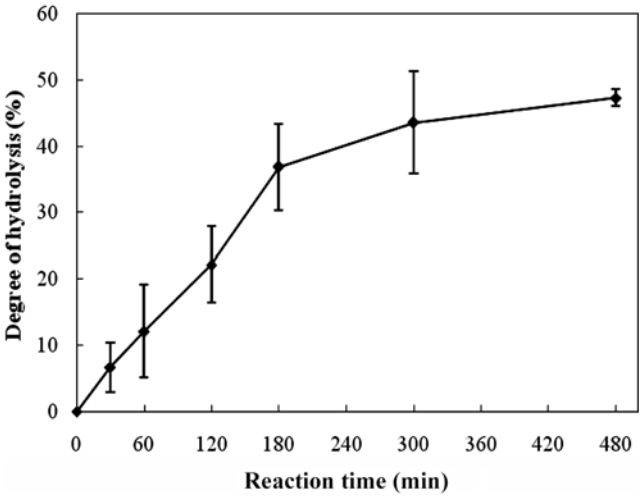

Fig. 2. Changes in the degree of hydrolysis of black soybean protein isolate by reaction with alcalase.

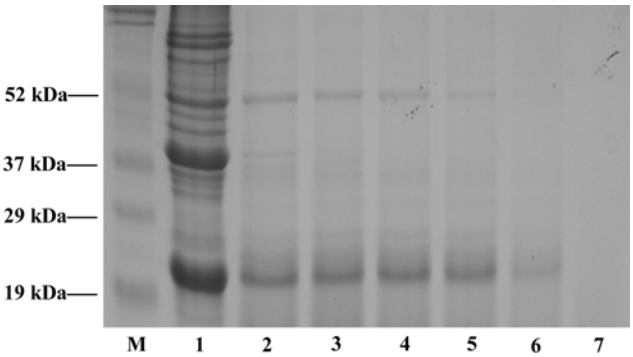

Fig. 3. SDS-PAGE patterns of alcalase-hydrolyzed black soybean proteins. Lane M, marker; lane 1, undigested protein; lane 2, 3, 4, 5, 6, and 7, black soybean proteins alcalase-hydrolyzed for 30 min, 1, 2, 3, 5, and 8 hr, respectively.

Partial hydrolysis of black soybean protein isolate In the previous report (22), Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K have been identified as the major allergenic soybean proteins. Gly m Bd 60K is an α subunit of β-conglycinin well known as a major storage protein. Gly m Bd 30K is also known as a protein associated with the oil bodies. Gly m Bd 28K is a vicilin-like glycoprotein, a minor component fractionated into 7S globulin fraction. These peptides, Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K, which have molecular weights of 53, 38, and 24 kDa, respectively, were identified as the major allergens. Therefore, the hydrolysis of these major allergens was undertaken to reduce the allergenicity of the black soybean protein isolate. Chemical modification is generally more efficient than enzymatic modification with regard to structural and functional changes. However, extensive blocking of essential amino acids and the occurrence of undesirable side reactions may prove to be obstacles to the introduction of chemically modified proteins into food products (23). Therefore, we used an effective and safe method of enzymatic hydrolysis to reduce the major allergens of black soybean protein by random proteolysis using alcalase, which is produced by the submerged fermentation of a selected strain of *Bacillus licheniformis*. Figure 2 shows the typical progression of hydrolysis of the black soybean protein in relation to reaction time with alcalase. The degree of hydrolysis (DH) of the protein increased with increasing reaction time. In particular, the DH was dramatically increased by reaction with alcalase for 120-180 min; however, the rate decreased thereafter, possibly because of substrate exhaustion and end-product inhibition. In addition, the reverse reaction of enzymatic catalysis might have decreased the rate of the reaction, which would become more important as the reaction time increased (24). The changes in molecular weight of the black soy protein isolates after hydrolysis were analyzed by SDS-PAGE (Fig. 3). Our main concern was focused on the major allergens. After 8 hr of incubation with alcalase, the major allergens Gly m Bd 30K, Gly m Bd 28K, and Gly m Bd 60K were fully dissociated (lane 7 in Fig. 3), indicating the effective degradation of the major soybean allergens by alcalase. The β-conglycinin was more feasible to degrade than was Gly m Bd 28K, implying higher resistance of Gly m Bd 28K to the proteolytic hydrolysis. Gly m Bd 28K (24 kDa) was degraded more slowly than Gly m Bd 30K (38 kDa). The relatively high resistance of Gly m Bd 28K to proteolytic hydrolysis is explained by the fact that this polypeptide has a tendency to form a large insoluble complex, which makes it less susceptible to enzymatic hydrolysis (25).

Polyclonal antibodies specific for Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 20K were obtained from rabbit against SPI extracted from black soybean. After SDS-PAGE, each protein sample in the gel was transferred electrophoretically onto a PVDF membrane to determine the hydrolysis of the major allergenic fractions of black soy proteins by immunoblotting with the polyclonal antibody. Previous research has reported that the degradation of the allergenic proteins to <10 kDa effectively reduces their allergenicity (25). Most of the allergenic proteins present in soybean were detected in the black soybean protein isolate (lane 1 of Fig. 4). However, the major allergen bands for Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K were gradually decomposed during the enzymatic reaction,

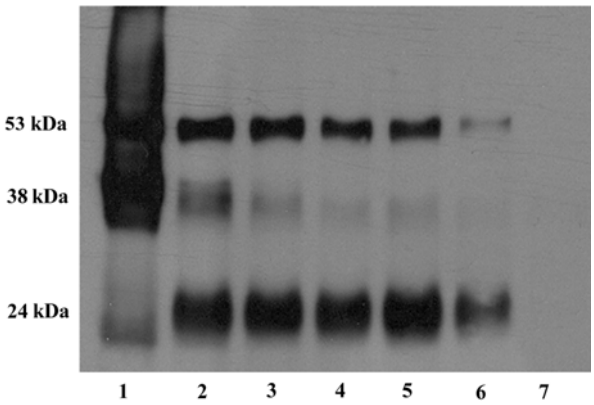

Fig. 4. Immunoblotting patterns of alcalase-hydrolyzed black soybean proteins. Lane 1, undigested protein; lane 2, 3, 4, 5, 6, and 7, black soybean proteins alcalase-hydrolyzed for 0.5, 1, 2, 3, 5, and 8 hr, respectively.

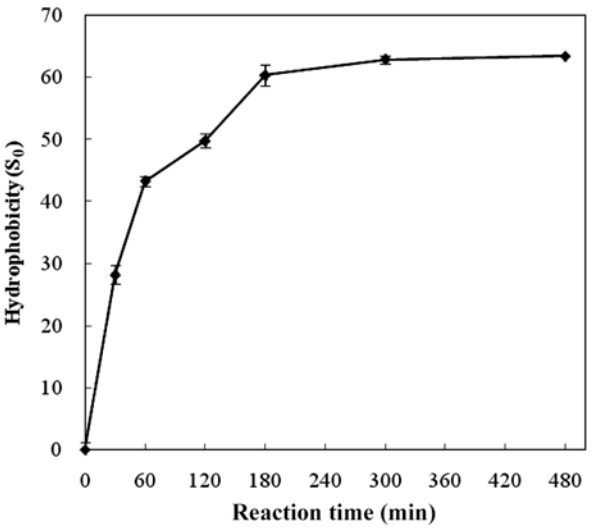

Fig. 5. Surface hydrophobicity (S_0) of black soybean proteins alcalase-hydrolyzed for various reaction periods.

indicating the removal of the major allergenicity of black soybean proteins.

Physicochemical properties of partially hydrolyzed black soybean protein isolate Soy protein isolate is the most refined soy protein product; it has many functional properties and is used in a wide range of food applications, including processed meat, nutritional beverages, infant formulas, and dairy product replacements. Therefore, we wanted to obtain further information on the effects of proteolytic modification on the functionality of black soybean proteins. Alcalase hydrolysis increased the surface hydrophobicity of black soybean proteins (Fig. 5), as found previously (26). In a native protein molecule, hydrophobic groups are buried inside the core of the folded structure. Alcalase randomly cleaved the peptide chains and thus exposed the hydrophobic groups, resulting in increases in surface hydrophobicity. The surface hydrophobicity of the black soybean proteins gradually increased with increasing reaction period for the first 3 hr, but remained relatively unchanged thereafter. Although hydrolysis increased the surface hydrophobicity of the black soybean proteins, it also significantly increased the protein solubility, even at the isoelectric point of black soybean proteins (Fig. 6). The reduced molecular size of the proteins produced by enzymatic hydrolysis resulted in increases in solubility, which overcame the decrease in solubility caused by the increase in surface hydrophobicity.

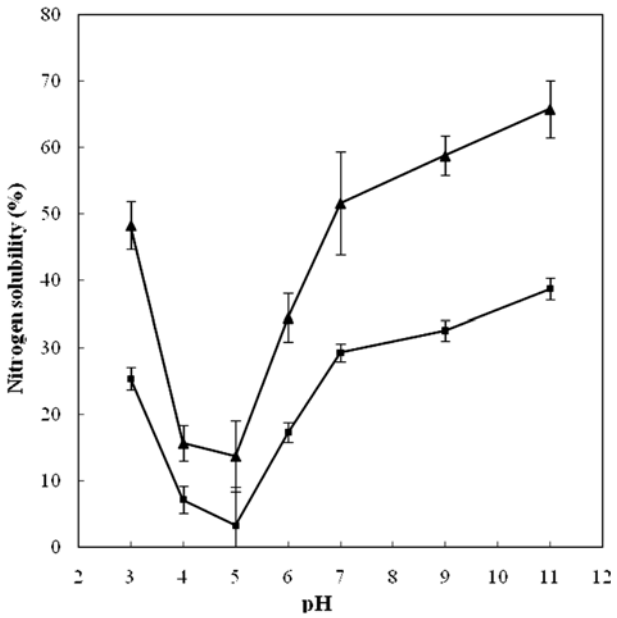

Fig. 6. Protein solubility of black soybean protein isolate (■) and black soybean protein isolate alcalase-hydrolyzed for 8 hr (▲).

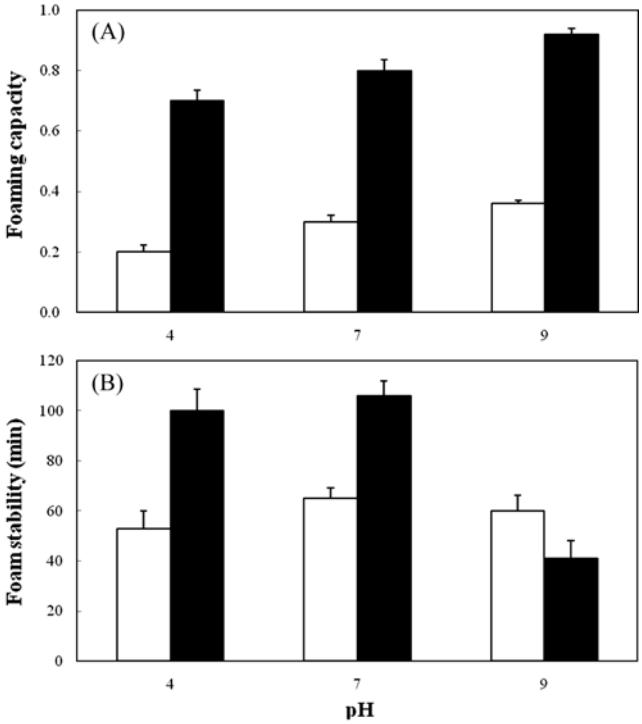

Fig. 7. Foaming capacity (A) and foam stability (B) of black soybean protein isolate (□) and black soybean protein isolate alcalase-hydrolyzed (■) for 8 hr at pH 4, 7, and 9.

The enzymatic hydrolysis greatly enhanced the foaming capacity of black soybean proteins at pH 4, 7, and 9 because of increases in the surface hydrophobicity and solubility of the proteins (Fig. 7). Rapid adsorption of protein at the air-water interface during whipping or bubbling, the ability to undergo rapid conformational change, and rearrangement at the interface are required for high foaming capacity. The reduced molecular size and enhanced solubility of the proteins caused by enzymatic hydrolysis led to the rapid adsorption of the proteins at the air-water interface. Hydrolysis also increased the foaming stability somewhat at pH 4 and 7, but decreased it at pH 9 (Fig. 7). Two macroscopic processes in foams affect the kinetic stability of protein-stabilized foams: the rate of liquid drainage from the lamellae, and film rupture. An increase in the hydrophobicity of proteins negatively contributes to foam stability because the attractive hydrophobic interactions between protein layers usually promote film thinning. Despite the increase in hydrophobicity, one reason for increased foam stability at pH 4 and 7 could be that the highly increased viscosity of the protein solution caused by the enzymatic hydrolysis contributed to foam stability, overcoming the negative effect of hydrophobicity. However, electrostatic repulsion contributes to foam stability at pH 7 (Fig. 7), which is far from the isoelectric pH of the protein, where proteins carry a net negative charge. The charge repulsion between protein layers tends to oppose the thinning of the lamella. However, excessive repulsion between protein molecules within the layer impaired the integrity of the protein layer and caused a decrease in foam stability at pH 9 (27-29).

We successfully developed a new black soybean protein isolate that has lower allergenicity, higher antioxidant properties, and better functional properties such as solubility and foaming than ordinary soy protein isolates. Therefore, we hope that the newly developed protein isolate may be widely used as a hypoallergenic food source to produce fabricated foods for geriatrics, high-energy supplements, weight-control diets, and infant formulas.

Acknowledgments

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