Macromolecular Research, Vol. 15, No. 7, pp 671-675 (2007)

Effect of Maleylation on Physicochemical Properties of Soybean Glycinin

Weon-Sun Shin*

Department of Food and Nutrition, Hanyang University, Seoul 133-791, Korea

Soo-Jin Park

Department of Food and Nutrition, Baewha Women's College, Seoul 110-735, Korea

Chunwuk Park and Kangsung Kim

Department of Food Science and Nutrition, Yong In University, Gyeonggi 449-714, Korea

Received August 6, 2007; Revised August 29, 2007

Abstract: Soybean proteins appear to harbor a great deal of potential as functional ingredients due to the fact that they are composed of highly bioavailable peptides and amino acids. To develop drink- or gel-type foods formulated with soybean protein, the physicochemical properties of intact and chemically modified soy glycinin were assessed. Maleylation to soy glycinin altered the surface charges of glycinin via the modification of lysine residues, and subsequently generated the dissociation of glycinin subunits owing to the increase in charge repulsion. This modification thus improved the solubility of glycinin, particularly under acidic pH conditions. It is worthy of note that maleylation increased the susceptibility of the basic subunits of mTGase and the formation of a substantial quantity of molecules at a low protein solution concentration. The results of dynamic rheological studies indicated that the 5% intact glycinin progressively formed the gel with mTGase treatment in a concentration-dependent manner, but maleylated-glycinin did not.

Keywords: microbial transglutaminase, hwanggumkong glycinin, maleylation, structural susceptibility.

Introduction

Glycinin is the major soy protein about 40% among the soy protein constituents such as α-, β-, γ-conclycinins, glycinin, and other globulins, which can be easily dissolved in salt solution, and fractionated.[1,2] Specifically, recent studies elucidated that health promoting actions of soy proteins are mainly based on glycinin.[3,4] However, soy glycinin restricted to use due to the low solubility, particularly around pH 4.0-7.0, and lack of flexibility owing to the compactness of the protein as exhibited by high enthalpy of denaturation of producing meal pellets.[5] Emerging studies demonstrated that the changes in functional properties of protein through chemical modifications including acetylation, succinylation and maleylation mainly caused the solubility, hydrophobicity, surface conductivity, etc., which stimulated the enhancement of physicochemical functionality like foaming, emulsion, and gelation.[6-9] Despite of these researches for decades, there are limited information on the physicochemical properties of glycinin subunits. Recently, Mo *et al.*[10] have reported that acidic subunits have excellent solubility at pH above 6.0 or below 4.0 with the minimum solubility at pH 5.0, whereas basic subunits have the minimum solubility in the range of pH from 4.5 to 8.0 and have highly hydrophobic amino acids composition.

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) treatment is one of enzymatic treatments to produce soy protein for the improvement in the mechanical properties.[11,12] TGase catalyzes the acyl-transfer reaction, in which γ-carboxlyamide groups of peptide-bound glutamine residues are the acyl donor.[13] When the amino groups of peptide-bound lysine residues are the acyl acceptor, ε-(γ-glutamyl) lysine cross-links are generated.[14] It has been investigated the potent efficacies of TGase from genea pig liver [13] or bovine serum[15] on the modification of functional properties of different protein sources like casein (milk protein), soy globulin, bovine serum albumin,[16] fish protein. In particularly, as the mass production of microbial TGase from *Streptoverticillium mobaraense*,[17] many attempts have been made in meat processing.[18] Recently, Shin *et al.*[19] and Seo *et al.*[20] reported that physical and sensory properties of mTGase-treated noodles made with domestic wheat flour(*Greumil*) or the mixture of rice and wheat flour, respectively, were significantly improved.

*Corresponding Author. E-mail: hime@hanyang.ac.kr

In the present study, we thus purified 11S glycinin from soy protein originated from Korean soybeans (Hwanggumkong variety), one of the most widely cultivated soy in Korea, and maleylated the glycinin fraction to investigate the change of solubility and its efficacy on the enzyme-mediated gelation through microbial transglutaminase.

Experimental

Materials. Soybeans (glycine max var. Hwanggumkong) were purchased from Choongbuk Agriculture Research and Extension Service. Maleic anhydride and all chemicals were obtained from Sigma (St.Louis, MO, USA). mTGase was prepared by the culture of *Streptoverticillium mobaraense*, which was generously given by the department of food science at Cheonbuk University.[21]

Preparation of Glycinin. Glycinin was isolated and fractioned by the method of Thanh *et al.*[22] with a partial modification. Briefly, soybeans were grinded in a ball mill and defatted by immersing in hexane at room temperature for 6 h. Crude glycinin was extracted with 0.03 M Tris buffer (pH 8.0) containing 0.01 M β-mercaptoethanol and sodium azide (0.02%) at room temperature for 2 h. The pH was adjusted to pH 6.4, and the glycinin enriched fraction was collected by centrifugation at 10,000 g for 20 min at 6 °C. The collected glycinin was washed with 1 M Tris-HCl (pH 6.4), and dispersed in 0.03 M potassium phosphate buffer containing 0.01 M β-mercaptoethanol, and stirred until the protein was completely dissolved. This washing step was repeated in three consecutive manners. The glycinin fraction was dialyzed and lyophilized for further study. Protein concentration was measured by the method of manufacture using bovine serum albumin as a standard.

Maleylation of Glycinin. The maleylation procedure was performed by the method of Kawai *et al.*[23] Briefly, maleic anhydride was added slowly to the protein dissolved in standard buffer, and pH 8.0 was maintained with 1 N NaOH. After the reaction, the sample was exhaustively dialyzed to eliminated salts before lyophilization. Lysine residues modification was determined using trinitrobenzensulfonic acid (TNBS) reagents through the method of Adler-Nessen.[24] Samples of 0, 50, 93% lysine residue modifications were used for the subsequent experiments. Samples of native and maleylated protein were quantified through microkjeldahl nitrogen analysis.

Measurement of Solubility. To determine the solubility of glycinin fraction, Clark-Lubs buffer (0.2 M KCl + 0.2 M HCl) was used between pH 1.0 and 2.0, Macllvaine buffer (0.1 M citric acid + 0.2 M Na_2HPO_4) between pH 3.0 to 8.0 glycine-NaCl buffer between pH 9.0 and 10.0 and sodium phosphate buffer between pH 11 and 13. Either half or totally maleylated-glycinin were dissolved at 0.1% (w/v) and the solubility was measured at 280 nm.

Analysis of Lysine. Each sample proteins (approx. 0.1 g) were dissolved in 6 N HCl (5 mL) and dried under N_2, then hydrolyzed at 155 °C for 1 h and cooled to room temperature. After adjusting to 25 mL and filtering, they were analyzed by HPLC according to the method of AccQ-Tag. The column used was Nova-Pak C18 column (3.9 × 150 mm) set at 37 °C, and the separation was achieved by gradient solvent elution using the mobile phase of 0.14 M sodium acetate and 60% acetonitrile at a flow rate of 1 mL/min. Fluorescence detector has excitation wavelength at 250 nm and emission wavelength at 395 nm.

SDS-PAGE. 1% glycinin solution (pH 7.5) was preincubated at 37 °C for 15 min. Enzyme reaction was started by the addition of mTGase to the respective glycinin at 50 °C for 1 h and stopped rapidly in cold water. Polymer samples (0.2 g) were dissolved with 9 M urea at 37 °C for 15 min and diluted with a separating buffer. Diluted samples (15 μg) were separated on the stacking gel (4%) and separating gel (12%) at 10 mA for 3 h according to the method of Leammli[25] and stained by the commassie brilliant blue R 250 and destained by the mixture of acetic acid : methanol : H_2O (1 : 4.5 : 4.5, v/v/v).

Rheological Property. Rheological measurements were carried out using a dynamic oscillatory rheometer (Carrie-Med CSL100, TA instruments, Germany) equipped with peltier-type water circulator. Rheological property was determined by tilting a 1 mL of homogenate solution, that is mTGase-mediated soy glycinin (5%, w/w), sealing with silicone plate to avoid drying. Both the change of storage modulus (G') and loss modulus (G'') and a phase transition point were measured with a probe (angle 2, 4 cm dia.) at a frequency of 1 Hz at 50 °C for 30 min.

Results and Discussion

Effect of Maleylation on the Solubility The solubility of native glycinin and maleylated-glycinin in dependence on the pH was measured (Figure 1). This result is similar as Liu *et al.*'s[26] report of isoelectric point of native glycinin appeared in the range of pH 4.5-4.6. However, the isoelectric point of maleylated glycinin was shifted to the acidic region (pH value of 3.0), and excessive maleylation (93%) reduced the solubility even at the same isoelectric point, indicating lowered interactions of protein molecules with water molecules due to the change in surface charge of maleylated glycinin.

As shown in Figure 2, we observed that the native and maleylated-glycinin are constituted with acid subunits and basic subunits in 35 and 22 kDa, respectively, and the each subunit in maleylated-glycinin was slightly dissociated with the degree of modification.

In addition, we found that the electrophoretic mobility of acidic subunit derived from 50% maleylated glycinin decreased, and the basic subunit had little change in mobility compared to intact glycinin. On the contrary, the mobility shift was not observed on the acidic subunit but on the basic subunit at the

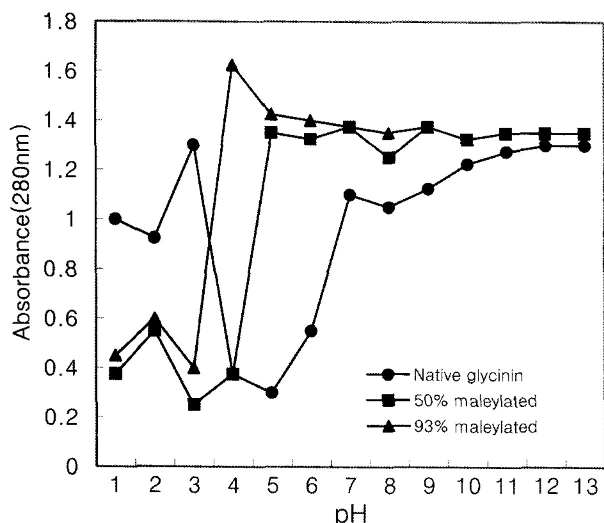

Figure 1. Effect of maleylation on the solubility of soybean glycinin: closed round, native glycinin; closed square, 50% maleylated; closed triangle, 93% maleylated.

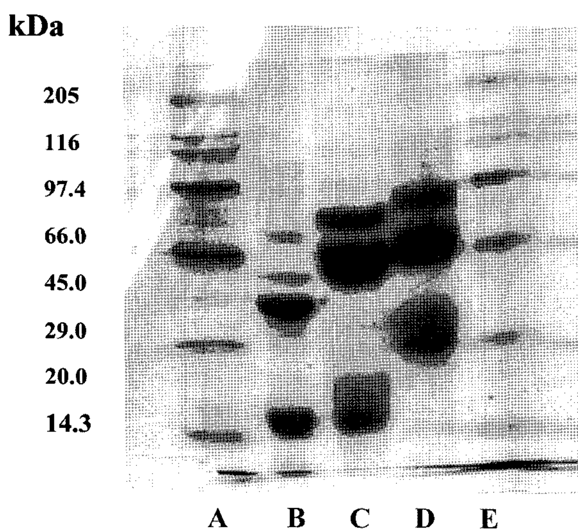

Figure 2. SDS-PAGE pattern for polymerization of the native and maleylated glycinin : lane A and E, molecular weight marker; lane B, native glycinin; lane C, 50% maleylation glycinin; lane D, 93% maleylated glycinin.

level of 93% in maleylation as shown in Lane C (Figure 2).

Generally, the electrophoretic mobility of a protein is determined not only by its molecular weight, but also by its net charge and its shape; that is, SDS-protein complexes have comparable shapes and net charge densities on the polypeptide chain. However, only proteins that are intrinsically very basic or acidic seem to produce complexes with SDS that have somewhat atypical charge densities, reflecting their intrinsic charges.[27] Accordingly, it might be explained that difference in the electrophoretic mobility of each subunit to glycinin was caused by changing in charge balance and tertiary structure due to the degree of maleylation. These results can also account for difference in susceptibility of glycinin subunits on the maleylation step.

Gelation of the Native and Maleylated-glycinin by TGase. Figure 3 showed the dependency of the level of TGase (0.113-0.90 unit) on the gelation of 5% glycinin. The gel in the native glycinin solution begins to be formed with 0.113 unit of mTGase, whereas 50% maleylated-glycinin (protein conc. 5%) was failed to make setting-gel by the addition of 0.225 unit of mTGase at 50 °C for 1 h (Data not shown).

In order to investigate the polymerization process of soybean glycinin by mTGase (0.225 unit), the formation of cross-linked molecules was analyzed as a function of reaction time by SDS-PAGE. As shown in Figure 4(A), polymer fractions with higher molecular weight were formed as the reaction time increased. As polymers were progressively developed, it was observed that only acidic subunits were clearly diminished. Then, the acidic and the basic subunit was further isolated, and each was treated with the same concentration of microbial transglutaminase (0.225 unit). As showed in Figure 4(B), only the acidic subunits treated with mTGase participated in forming the high molecules, whereas the basic subunits did not. This is a good agreement with several reports[28] only the acidic subunits took part in the cross-linking reaction with microbial transglutaminase.

Figures 4(B) and 4(C) show the effect of maleylation on polymerization of glycinin treated with microbial transglutaminase. In these cases, the high molecular polymers was little formed from either 50% or 93% maleylated-glycinin compared to the native glycinin, indicating that the ε-amino acids of lysines saturated with maleic anhydride had no more opportunity to be cross-linked by mTGase.

However, interestingly, the modification of glycinin with maleic anhydride dissociated both the acidic and basic subunit, and the basic subunit was involved in forming the polymers, indicating that the band intensity of the basic sub-

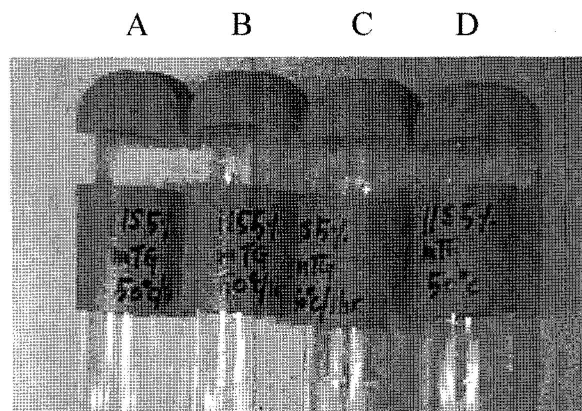

Figure 3. Gelation of 5% soybean glycinin with different concentrations of microbial transglutaminase at 50 °C: A, 0.113 unit; B, 0.225 unit; C, 0.45 unit; and D, 0.90 unit.

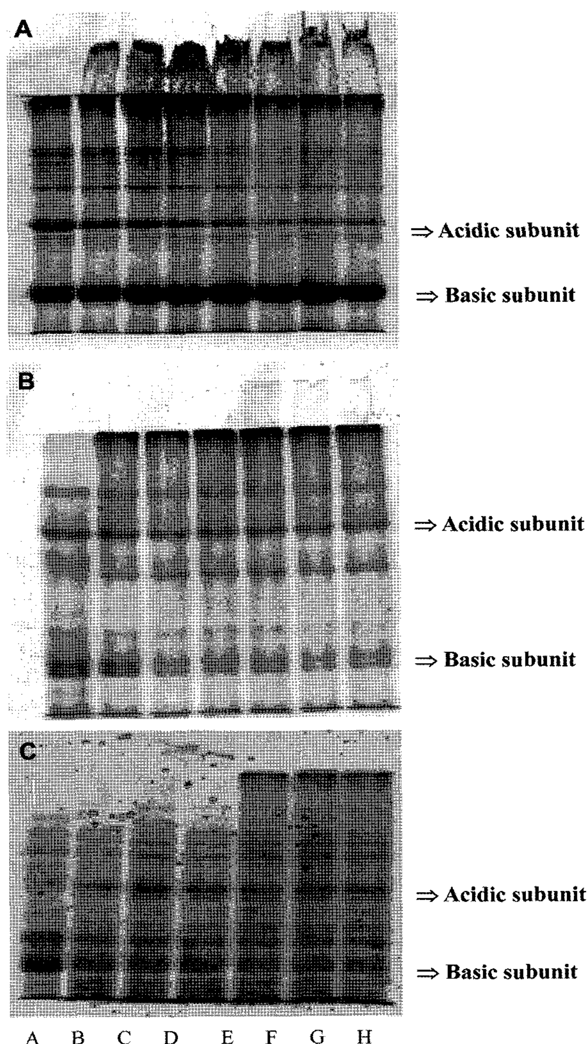

Figure 4. (**A**) Polymerization of the native glycinin treated with mTGase (0.225 unit) without reducing agent at time interval: lane A, 0 min; lane B, 10 min; lane C, 20 min; lane D, 30 min; lane E, 60 min; lane F, 90 min; lane G, 120 min; and lane H, 240 min. (**B**) Polymerization of 50% maleylated glycinin treated with microbial transglutaminase in the presence of different concentrations of reducing agent (DTT) at 50 °C. (**C**) Polymerization of 93% maleylated glycinin treated with microbial transglutaminase in the presence of different concentrations of reducing agent (DTT) at 50 °C: lane A, control; lane B, DTT 0 mM; lane C, DTT 1 mM; lane D, DTT 2 mM; lane E, DTT 3 mM; lane F, DTT 4 mM; lane G, DTT 5 mM.

unit in both 50% and 93% maleylated glycinin was clearly diminished (Figures 4(B), 4(C)). Also, increasing with a concentration of reducing agent (more than 1 mM DTT) promoted the polymerization of the basic subunit catalyzed with mTGase and simultaneously, the band intensity of the basic subunit was gradually diminished. Thus, we confer that the basic subunit buried in the interior of glycinin was exposed to the surface with an excessive maleylation and then, its structure unfolded with a reducing agent, DTT could be reactive (susceptible) to the catalytic enzyme, microbial transglutaminase. These results have good correlation with Kwon *et al.*'s[29] experimental data: slight change in the tertiary structure of maleylated-glycinin at the level of 50% was observed, whereas denaturation and dissociation of subunits in the excessively maleylated glycinin clearly occurred in the spectrophotometric study.

Rheological Change of Maleylated-glycinin Solution. Figure 5 showed that change of rheological properties using a dynamic oscillatory rheometer (CSL2-100, TA, USA) in native or maleylated-glycinin (protein conc. 5%) during TGase mediated-polymerization. The gradual increase in the storage modulus of the native glycinin after incubation with mTGase reflected the occurrence of the polymerization of glycinin, otherwise no change of storage modulus (G') showed in 50% and 93% of maleylated-glycinin, respectively. This result is in a good agreement with the report by Chanyongvorakul *et al.*[28] investigating either the concentration or time dependent - change of G' value in glycinin.

Effect of Enzyme Concentration of Gelation in Native Glycinin. Figure 6 showed that changes in the storage modulus (G') of native glycinin with various concentrations of mTGase were measured as a function of time at a constant temperature (50 °C) for 30 min.

The gradual increase in the G' of 5% glycinin solution after incubation with mTGase reflects the occurrence of the poly-

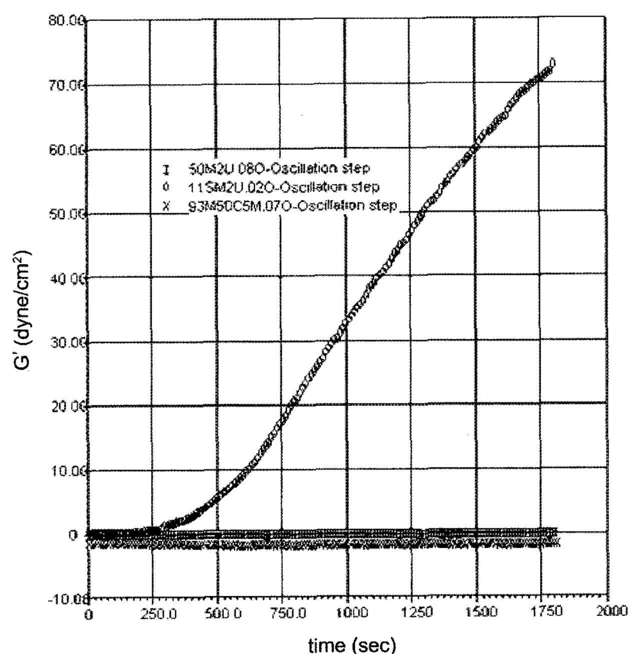

Figure 5. Change in dynamic storage modulus (G') of the native and maleylated glycinin mediated by transglutaminase at a frequency of 1 Hz at 50 °C for 30 min: round symbol, native glycinin; bar symbol, 50% maleylated glycinin; cross symbol, 93% maleylated glycinin.

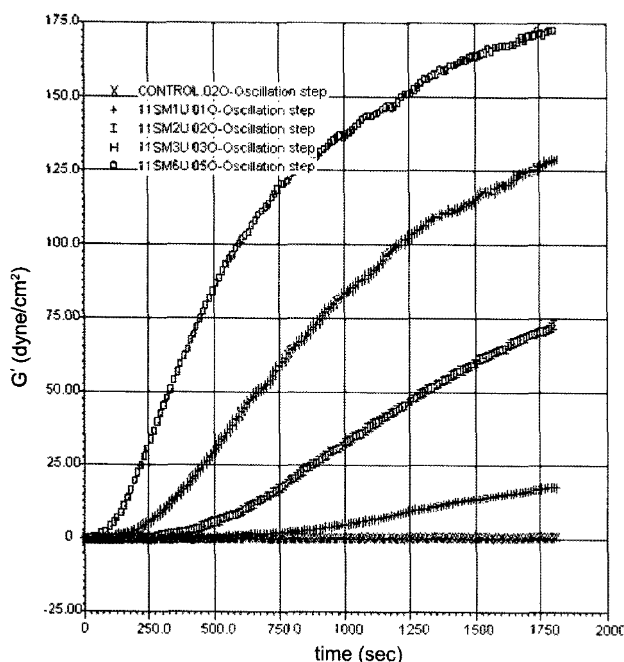

Figure 6. Development of gel elasticity at 50 °C of microbial transglutaminase-treated native soy glycinin gels (5% protein). Dynamic storage modulus (G') at a frequency of 1 Hz at 50 °C is plotted at a time interval.

merization of glycinin, indicating that the formation of cross-linking by mTGase is the concentration-dependent reaction.

Based on change in G' value with 0.5 unit transglutaminase, the polymer formation process appeared to be divided into distinct multiphases: in the first phase, the gradually increased at a low increasing rate at a initial step (0 to 130 sec), in the second phase, the G' increased at a high increasing rate to 600 sec, and then the increasing rate slowed down. This could account for the initial formation of the network through the cross-linking by mTGase, and subsequent stabilization of the network.

Conclusions

This study has proved that maleylation to soybean glycinin improved solubility through isoelectric point shift, especially at acidic pH, and induced dissociation of subunits revealed in SDS-PAGE. Thus, we conferred that this dissociation resulted in molecular unfolding, and caused the basic subunits to be susceptible to an enzymatic reaction, indicating that the basic subunits participated in forming the polymers in the presence of reducing agent (DTT). However, when the soybean glycinin at a protein concentration of 5% was treated with mTGase, the turbid and hard gel was formed in native glycinin, but not in maleylated glycinin. This result was confirmed that mTGase treatment progressively increased G' values in native glycinin, but not in maleylated-glycinin at a measurement of rheological properties.

References

(1) W. J. Wolf and D. A. Sly, *Cereal Chem.*, **44**, 653 (1987).
(2) D. R. Briggs and W. J. Wolf, *Arch. Biochem. Biophys.*, **72**, 127 (1957).
(3) M. Samoto and Y. Kawamura, *The Food Industry*, **39**, 76 (1996).
(4) S. Makino, H. Nakashima, K. Minami, R. Moriyama, and S. Takao, *Agric. Biol. Chem.*, **52**, 803 (1988).
(5) N. Hettiarachchy and U. Kalapathy, in *Soybeans: Chemistry, Technology and Utilization*, L. Keshun, Ed., Chanpman & Hall, New York, 1997, pp 379-411.
(6) M. Friedman and D. L. Brandon, *J. Agric. Food Chem.*, **49**, 1069 (2001).
(7) W. L. Boatright and N. S. Hetiarachchy, *J. Food Sci.*, **60**, 806 (1995).
(8) Y. R. Choi, E. W. Lusas, and K. C. Rhee, *J. Food Sci.*, **47**, 1713 (1982).
(9) A. H. Clark and C. D. Lee-Tuffnell, in *Functional Properties of Food Macromolecules*, J. R. Mitchell and D. A. Ledward, Eds., Elsevier, London, U.K., 1986, pp 203-272.
(10) X. Mo, Z. Zhong, D. Wang, and X. Sun, *J. Agric. Food Chem.*, **54**, 7589 (2006).
(11) Y. Zhu, A. Rinzema, J. Tramper, and J. Bol, *Appl. Microbiol. Biotechnol.*, **44**, 277 (1995).
(12) H. Sakamoto, Y. Kumazawa, S. Toiguchi, K. Seguro, T. Soeda, and M. Motoki, *J. Food Sci.*, **59**, 866 (1995).
(13) K. Ikura, R. Sasaki, and M. Motoki, *Agric. Food Chem.*, **2**, 389 (1992).
(14) A. Mizuno, M. Mitsuiki, and M. Motoki, *J. Agric. Food Chem.*, **48**, 3286 (2000).
(15) L. Kurth and P. J. Rogers, *J. Food Sci.*, **49**, 573 (1984).
(16) Y. Kang, H. J. Kim, W. S. Shin, G. J. Woo, and T. W. Moon, *J. Food Sci.*, **58**, 2215 (2003).
(17) H. Ando, M. Adachi, K. Umeda, A. Matsuura, M. Nonaka, R. Uchio, H. Tanaka, and M. Motoki, *Agric. Biol. Chem.*, **53**, 2613 (1989).
(18) M. Motoki and K. Seguro, *Food Sci. Technol.*, **9**, 204 (1998).
(19) W. S. Shin, H. S. Seo, G. J. Woo, and Y. S. Jeong, *J. Korean Soc. Food Sci. Nutri.*, **34**, 1434 (2005).
(20) H. S. Seo, W. S. Shin, S. Yoon, and S. J. Lee, *Food Sci. Biotechnol.*, **12**, 1 (2003).
(21) J. S. Yoo, W. S. Shin, G. J. Woo, Y. S. Kim, and Y. S. Jeong, *Korean J. Food Sci. Technol.*, **35**, 260 (2003).
(22) V. H. Thanh, K. Okubo, and K. Shibasaki, *Plant Physiol.*, **56**, 19 (1975).
(23) Y. Kawai, S. Fujimura, and K. Takahashi, *Int. J. Food Sci. Technol.*, **33**, 385 (1998).
(24) J. Adler-Nessen, *J. Agric. Food Chem.*, **27**, 1256 (1979).
(25) U. K. Laemmli, *Nature*, **227**, 680 (1970).
(26) M. Liu, D. S. Lee, and S. Damodaran, *J. Agric. Food Chem.*, **47**, 4970 (1999).
(27) T. E. Creighton, in *Protein: Structure and Molecular Peroperties*, 2nd Eds., Freeman, U.S.A., 1993.
(28) Y. Chanyongvorakul, Y. Matsumura, M. Nonaka, M. Motoki, and T. Mori, *J. Food Sci.*, **60**, 483 (1995).
(29) D. Y. Kwon, S. Kim, H. Y. L. Kim, and K. S. Kim, *Food Sci. Biotechnol.*, **12**, 122 (2003).