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Turbidity Profile of Maleylated Glycinin

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Abstract : Glycinin of more than 97% purity was modified using maleic anhydride. Glycinin samples of 0%, 65%, and 95% lysine residue modifications were used to determine the changes in turbidimetric characteristics of the protein due to maleylation. The solubility behavior of the protein as a function of pH was changed with maleylation. The isoelectric point of 65% and 95% modified glycinin shifted to pH 4.0 and pH 3.5-4.0, respectively, as compared to pH 4.6 for native glycinin. Maleylated glycinins exhibited increased solubility at pH above 4.6. Turbidity of native glycinin decreased substantially by the addition of NaCl, but the stabilizing effect of NaCl decreased when the protein was chemically modified. The effect of NaCl on 65% modified glycinin was intermediate between native glycinin and 95% modified sample. Thermal aggregation of native glycinin was completed within 5 min of heating at 80°C. Maleylation contributed significantly to the thermostability of the protein at pH of 7.0 and 9.0, exhibiting little turbidity. Addition of NaCl suppressed thermal aggregation of native glycinin, but turbidity actually increased for the samples of 65% and 95% modification.

Keywords : glycinin, citraconylation, modification, functionality

Introduction

A considerable amounts of researches have been done on the changes in functional properties of food protein using chemical modifications. Among numerous chemical modifications, phosphorylation[1,2] and reversible modifications such as citraconylation[3,4] and maleylation[5,6] are especially promising because of following reasons. Phosphorylated protein such as casein has long been consumed as food and thus phosphorylated protein seems to be nutritionally safe. Reversible modification can be easily reversed by exposing the modified residues to mildly acidic solution and thus nutritional value is not lost. Up-to-date, studies concerning changes in functional properties of reversibly modified glycinin is scarce. Glycinin is of interests to many food chemists because of following reasons: ① glycinin accounts for over 40% of total soybean protein and could be easily fractionated[7,8], ② glycinin contains 3 to 4 times methionine and cysteine per unit protein as compared to other storage proteins of soybean and thus is valuable from a nutritional point of view[9], ③ glycinin has some beneficial health promoting effects such as lowering of blood cholesterol and improving obesity[10,11], ④ glycinin is versatile functionally, an important factor when choosing a food ingredient for food formulation.[12-14] Thus, for successful applications of glycinin as a food additive, still more basic researches are needed. In this study, we report effects of maleylation on turbidimetric characteristics of glycinin with the aim of using the protein as nutritional supplement in beverages with acidic conditions such as juices and sports drinks.

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Materials and Methods

Materials

Soybean (glycine max var. Huanggumkong) was kindly supplied by Choongbuk Agriculture Research and Extension Service. All other chemicals used for the experiment were of analytical grade or HPLC grade.

Glycinin Purification

Defatted soy bean flour was made by grinding

the soybean in a ball mill and immersing the flour in hexane at room temperature for at least 6 hrs. Glycinin was isolated from soybean by the method of Thanh *et al.*[15] Briefly, defatted soybean meal was extracted with 0.03 M Tris-HCl buffer (pH 7.5) containing 0.01 M β-mercaptoethanol and 0.02% sodium azide at room temperature for 1 hr. The extract was adjusted to pH 6.4 and the glycinin fraction was collected by centrifugation at 10,000 × g for 30 min at 6°C. Crude glycinin was washed with distilled water at pH 6.4. and then dispersed in 0.03 M potassium phosphate buffer of pH 7.5 (designated as the "standard buffer" hereafter). NaOH solution was added dropwise while stirring until the protein dissolved (pH 7.8). The protein solution was kept at 3-5°C overnight and traces of precipitate was removed by centrifugation.

Ion Exchange Chromatography

In order to purify the glycinin further, DEAE-Sephadex A-50 column chromatography was performed using 7 × 45 cm column at 4°C. Flow rate was 60 m*l*/hr, and 7 m*l* volume was collected in each fraction. Standard buffer (0.03 M phosphate buffer) containing 0.01 M β-mercaptoethanol and 0.02% sodium azide was used for elution. Ionic strength of the buffer was changed by increasing the concentration of sodium chloride from 0.25-0.65 M. Column effluents were monitored at 280 nm, and protein-containing fractions (absorbance above 1.0) were collected and concentrated by ultrafiltration. Protein fraction corresponding to glycinin was further purified by gel filtration chromatography using Sepharose CL-6B.

Gel Filtration Chromatography

The purified glycinin fraction obtained from DEAE-Sephadex A-50 column chromatography was placed on the Sepharose CL-6B column (4 × 90 cm) equilibrated with the standard buffer containing 0.45 M sodium chloride. The elution buffer used was the standard buffer containing 0.45 M sodium chloride and fraction volume and flow rate were 7 m*l*, 15 m*l*/hr, respectively. Column eluates were collected and monitored at 280 nm, and the peak corresponding to glycinin was pooled and diafiltered excessivelly with distilled water and lyophilized for use in consecutive experiments.

Maleylation

The maleylation procedure used was similar to that of Choi *et al.*[6] Briefly, maleic anhydride was added slowly to the protein dissolved in standard buffer and the pH 8.0 was maintained by using pH stat with 1 N NaOH. After the reaction, the sample was exhaustively dialyzed to get rid of salts before lyophilization. Lysine residues modified was determined by using trinitrobenzenesulfonic (TNBS) acid reagents by the method of Adler-Nissen[16], and the samples of 0%, 65%, and 95% lysine residues modification were used for the subsequent experiments. Samples of native and maleylated proteins were quantified using microkjeldahl nitrogen analysis.

pH Effects on Turbidity

To investigate pH effect on turbidity of glycinin, pH 2.0 and 2.5 was adjusted by using KCl-HCl buffer. Citrate-sodium citrate buffer was used in pH adjustment from 3 to 6, tris-HCl buffer from 7.0 to 9.0. Final concentration of buffer solution in which protein was dissolved was 0.06 M and that of protein, otherwise stated, was 0.02%. Effects of NaCl (0.2 M and 0. 6 M) on turbidity of glycinin at various pHs were also determined.

Heating Effects on Turbidity

Heating experiments were carried out in a water bath with 2.5 m*l* of protein solutions in test tubes at 80°C for 5 min. In order to prevent evaporation during heat treatment, crew cap was used. Concentration of the protein was 0.02%. Buffers and protein concentration used were the same as the above experiment. At the end of the heating period, the tubes were removed and immediately cooled in the cold water. The turbidity of the solutions in the test tubes was measured at 500 nm. Before measuring the turbidity, each tube was shaken well to ensure uniform suspension of particle. Salt effects on turbidity was also studied with sodium chloride.

Results and Discussions

Purification and Determination of Molecular Weight of Glycinin

Glycinin of more than 97% purity from soybean flour was obtained after isoelectric point precipitation, DEAE-Sephadex A-50 ion exchange column chroma-

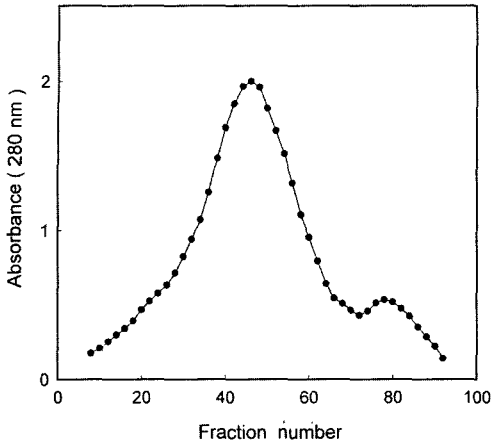

Fig. 1. Elution profiles of 11S soy glycinin on Sepharose CL-6B. Buffer used was 30 mM phosphate buffer containing 0.45 M NaCl and 10 mM β-mercaptoethanol, pH 7.5. Fraction volume and flow rate were 7 m*l*, 15 m*l*/hr, respectively.

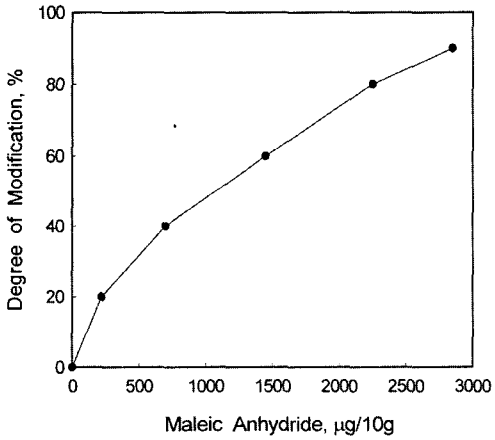

Fig. 2. Modification of glycinin versus concentration of maleic anhydride.

tography, and gel filtration chromatography using Sepharose CL-6B. Purity of the protein was checked by gel filtration chromatography (Fig. 1) and native polyacrylamide gel elecrophoresis. The apparent molecular mass of the native glycinin was estimated to be about 320 kDa by the gel permeation chromatography on Sepharose CL-6B.

Fig. 2 shows modification degree of lysine residues of glycinin versus maleic anhydride added. Samples of 0%, 65%, and 95% modifications were obtained, which were dialysed excessively against distilled

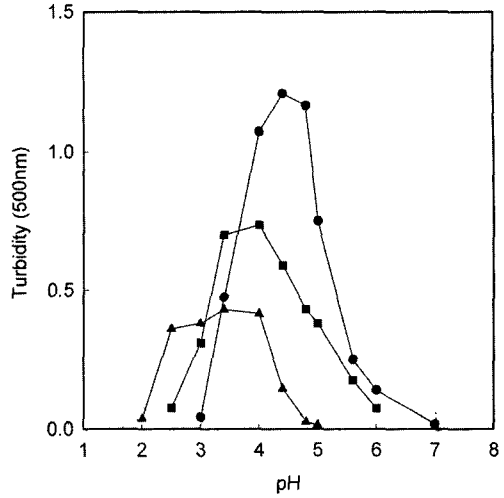

Fig. 3. pH profiles of aggregation of native and maleylated glycinin. Degree of lysine residue modification: 0% (●), 65% (■) and 95% (▲). The protein concentration was 0.02%.

water, lyophilized and were used for the subsequent experiments.

Effect of pH on Turbidity

The solubility behavior of the protein samples as a function of pH is shown in Fig. 3. The replacement of positively charged ε-amino groups at lysine residues with negatively charged maleyl groups should have resulted in a shift in the isoelectric point of glycinin (pH 4.6) to lower pH values: 65% and 95% modified glycinin exhibited isoelectric points of pH 4.0 and pH 3.5-4.0, respectively. As the result, turbidity profile of maleylated glycinin showed increased solubility at pH above 4.6[17]. Aggregation of native glycinin around isoelectric point is known to be mainly due to intermolecular hydrogen bondings because of high amide content of glycinin[18]. However, when the lysine residues are maleylated, hydrogen bondings between protein molecules would be weakened considerably due to blocking of amide residues as well as increase in net negative charges of the protein. Thus, reduction of turbidity and shift of isoelectric point to acidic region was resulted (Fig. 3).

Effect of NaCl on Turbidity

The effect of NaCl on suppressing turbidity of

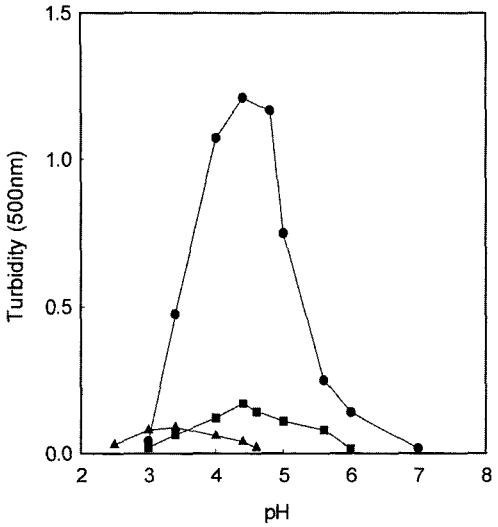

Fig. 4. Effect of NaCl concentration on aggregation of native glycinin. The protein concentration was 0.02%. Concentration of NaCl used were 0 M (●), 0.2 M (■) and 0.6 M (▲).

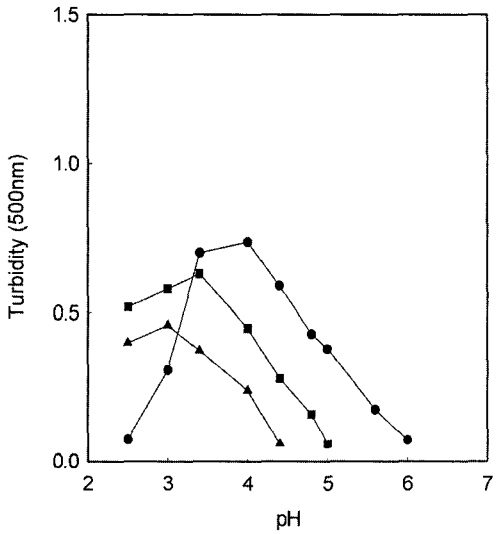

Fig. 5. Effect of NaCl concentration on aggregation of 65% maleylated glycinin. The protein concentration was 0.02%. Concentration of NaCl used were 0 M (●), 0.2 M (■) and 0.6 M (▲).

native glycinin is shown in Fig. 4. However, the stabilizing effect of NaCl was found to decrease dramatically with increased modification percentage of the protein (Fig. 5 and Fig. 6). Solubilizing effects

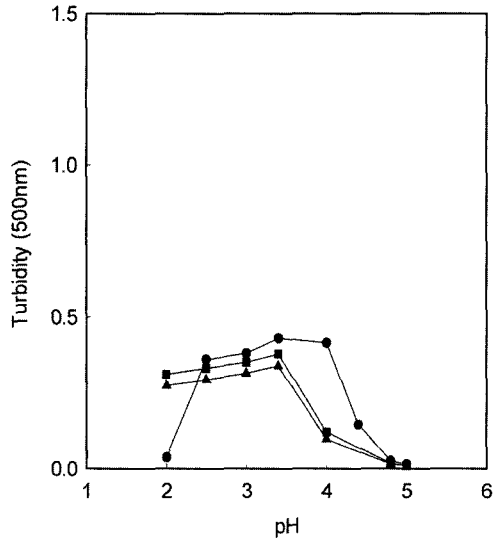

Fig. 6. Effect of NaCl concentration on aggregation of 95% maleylated glycinin. The protein concentration was 0.02%. Concentration of NaCl used were 0 M (●), 0.2 M (■) and 0.6 M (▲).

of NaCl could be due to two possible factors working separately or concordantly: ① increased repulsion between glycinin globulins due to attachment of negatively charged chlorine ions to the protein molecules and ② weakening of hydrogen bonding between protein molecules around isoelectric point due to ion-specific effects on hydrophobic interaction arising from perturbations in bulk water structure, which in turn affect segment-solvent and segment-segment interactions[19]. These interactions are thought to promote protein to more stable conformation such as increase of α-helix or β-sheet and decrease in random coil. Stabilizing effect of NaCl diminished when the protein was maleylated, as net negative charges of the protein is increased with modification reaction and the protein became physicochemically modified.

Effect of Heating on Turbidity

Fig. 7 shows effects of heating on turbidity of native glycinin. Thermal aggregation of 0.05% native glycinin was complete when heated at 80°C for 5 min, which was chosen as the heating condition for the subsequent experiments.

As shown in Fig. 8 and Fig. 9, maleylation contributed significantly to the thermostability of

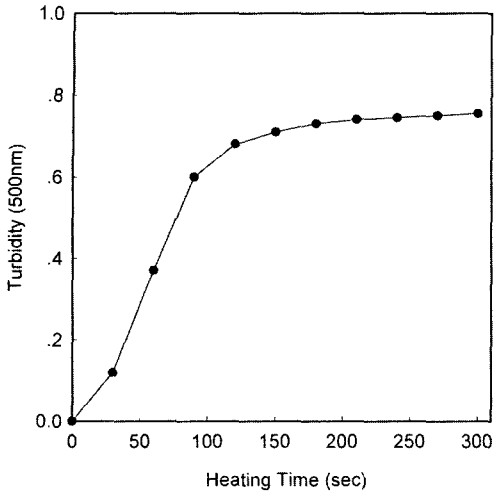

Fig. 7. Effect of heating at 80°C on native glycinin. The protein concentration was 0.05%.

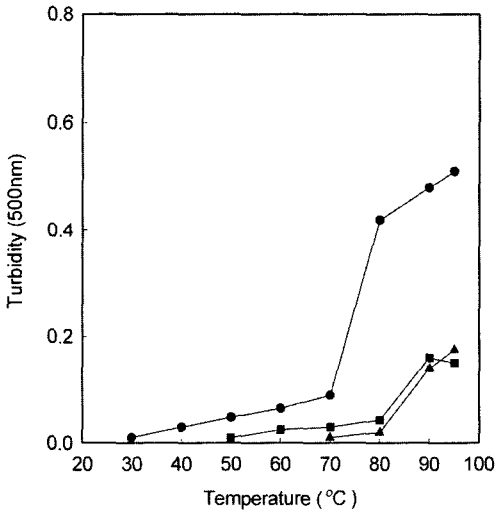

Fig. 8. Effect of temperature on thermal aggregation of 0% (●), 65% (■) and 95% (▲) maleylated glycinin at pH 7.0. The protein concentration was 0.02%, heating time was 5 min.

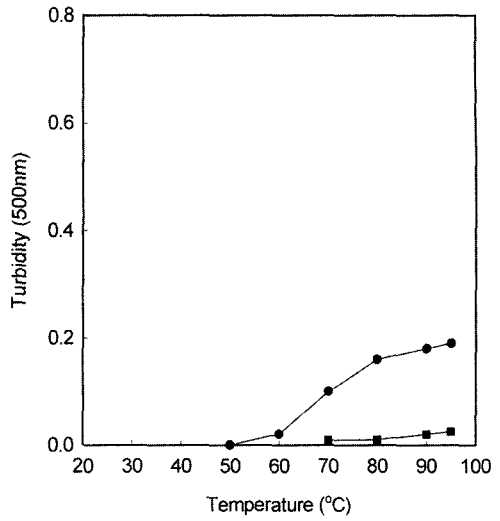

Fig. 9. Effect of temperature on thermal aggregation of 0% (●), 65% (■) maleylated glycinin at pH 9.0. The protein concentration was 0.02%, heating time was 5 min.

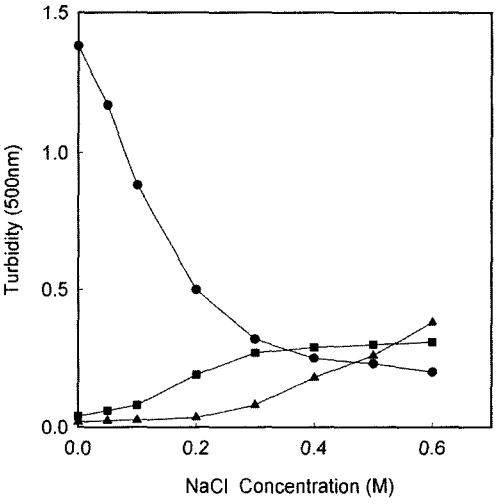

Fig. 10. Effect of NaCl concentration on thermal aggregation of 0% (●), 65% (■) and 95% (▲) maleylated glycinin at pH 7.0. The concentration of protein was 0.06%, heating time was 5 min at 80°C.

the protein at pH of 7.0 and 9.0. According to Hashizume and Watanabe[20], thermal aggregation of native glycinin at pH range 6-9 is due to interaction between basic units of glycinin. They concluded that heating of glycinin solutions resulted in the formation of a transient soluble aggregate consisting of acidic and basic subunits and on subsequent heating, this aggregate disappeared and complete dissociation into acidic and basic subunits took place[20-22]. However when glycinin is excessively modified that accompany change in charge balance, aggregation between basic subunits would be suppressed as the result of charge-charge repulsion, and thus suppression of turbidity is resulted.

Fig. 10 shows the effect of NaCl on the thermal

aggregation of 0.06% glycinin. As ionic strength of NaCl was increased, thermal aggregation decreased sharply for the native glycinin, but turbidity actually increased for the 65% and 95% modified samples. At high ionic strength, together with the neutralization of repulsive charges, the hydrophobic interaction between intermediate subunits in quaternary structure of native glycinin becomes predominant, due to salting out effect, and thus the non-dissociated quaternary structure was maintained[23]. However, for maleylated glycinin, the thermostabilizing effects of NaCl is not evident, and NaCl actually caused turbidity of maleylated protein to be increased.

This study shows that turbidity profile of glycinin could be suppressed by reversible chemical modification at lysine residues of the protein. Thermostabilizing effect of NaCl disappeared when the protein was maleylated suggesting that the physicochemical and functional properties of the protein has been altered. Reversible chemical modification opens new ways for improving usage of under-utilized protein due to poor functionalities, such as leaf proteins and heat treated protein.

References

1. Ross, L.F. and Bhatnagar, D. : Enzymatic phosphorylation of soybean proteins. *J. Agric. Food Chem.*, **37**, 841-844, 1989.
2. Matheis, G. and Whitaker, J.R. : Chemical phosphorylation of food proteins: An overview and a prospectus. *J. Agric. Food Chem.*, **32**, 699-705, 1984.
3. Yuno, N., Matoba, T. and Hasegawa, K. : Emulsifying properties of native and citraconylated sesame 13S globulins. *Agric. Biol. Chem.*, **52**, 685-692, 1988.
4. Barber, K.J. and Warthesen, J.J. : Some functional properties of acylated wheat gluten. *J. Agric. Food Chem.*, **30**, 930-934, 1982.
5. Kawai, Y., Fujimura, S. and Takahashi, K. : Functional properties of chemically modified proteins of sardine meal defatted with 1-propanol. *Int. J. Food Sci. Technol.*, **33**, 385-391, 1998.
6. Choi, Y.R., Lusas, E.W. and Rhee, K.C. : Effects of acylation of defatted cottonseed flour with various acid anhydrides on protein extractability and functional properties of resulting protein isolates. *J. Food Sci.*, **47**, 1,713-1,716, 1982.
7. Wolf, W.J. and Sly, D.A. : Cryoprecipitation of the 11S component of soybean protein. *Cereal Chem.*, **44**, 653-658, 1987.
8. Briggs, D.R. and Wolf, W.J. : Studies on the cold-insoluble fraction of the water-extractable soybean proteins. I. Polymerization of the 11S component through reactions of sulfhydryl groups to from disulfide bonds. *Arch. Biochem. Biophys.*, **72**, 127-114, 1957.
9. Kitamura, K. : Genetic improvement nutritional and food processing quality in soybean. *Jap. Agric. Res. Quart.*, **29**, 1-8, 1995.
10. Makino, S., Nakashima, H., Minami, K., Moriyama, R. and Takao, S. : Bile acid bonding protein from soybean seed. *Agric. Biol. Chem.*, **52**, 803-809, 1988.
11. Samoto, M. and Kawamura, Y. : Development of low allergenic soybean protein. *The Food Industry*, **39**, 76-86, 1996.
12. Kim, K.S., Kim, S.J. and Rhee, J.S. : Effect of acetylation on turbidity of glycinin. *J. Agric. Food Chem.*, **39**, 1578-1582, 1991.
13. Kim, S.H. and Kinsella, J.E. : Effect of reduction with dithiothreitol on some molecular properties of soy glycinin. *J. Agric. Food Chem.*, **13**, 187-199, 1986.
14. Kim, K.S. and Rhee, J.S. : Effects of acetylation on physicochemical properties of 11S soy protein. *J. Food Biochem.*, **13**, 187-199, 1989.
15. Thanh, V.H. and Shibasaki, K. : Major proteins of soybean seeds. A straightforward fractionation and their characterization. *J. Agric. Food Chem.*, **24**, 1117-1121, 1976.
16. Adler-Nissen, J. : Determination of the degree of hydrolysis of food protein hydrolysates by trinotrobenzenesulfonic acid. *J. Agric. Food Chem.*, **27**, 1,256-1,262, 1979.
17. Barman, B.G., Hansen, J.R. and Mossey, A.R. : Modification of physical properties of soy protein isolate by acetylation. *J. Agric. Food Chem.*, **25**, 638-641, 1977.
18. Catsimpoolas, N., Berg, T. and Watanabe, T. : Gelation phenomena of soybean globulins. *Agric. Biol. Chem.*, **35**, 890-895, 1971.
19. von Hippel, P.H. and Wong, K.Y. : On the conformational stability of globular proteins. *J. Biol. Chem.*, **210**, 3,909-3,923, 1965.
20. Hashizume, K. and Watanabe, T. : Influence of heating temperature on conformational changes of soybean protein. *Agric. Biol. Chem.*, **43**, 683-686, 1979.
21. German, B., Damodaran, S. and Kinsella, J.E. : Thermal dissociation and association behavior of soy proteins. *J. Agric. Food Chem.*, **30**, 807-810, 1982.
22. Yamaghishi, T., Yamauchi, F. and Shibasaki, K. : Isolation and partial characterization of heat-denatured products of soybean 11S globulin and their analysis by electrphoresis. *J. Agric. Food Chem.*, **44**, 1,575-1,580, 1980.
23. Iwabuchi, S. and Yamauchi, F. : Effects of heat and ionic strength upon dissociation-association of soybean protein fractions. *J. Food Sci.*, **49**, 1289-1292, 1984.