Improvement of the Functional Properties of Insoluble Gluten by Pronase Digestion Followed by Dextran Conjugation

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Insoluble gluten was solubilized by Pronase treatment. Pronase-treated gluten (PTG) was conjugated to dextran by dry heating at 60 °C and 79% relative humidity through Maillard reaction between amino groups in gluten peptides and the reducing-end carbonyl group in dextran. The solubility of PTG–dextran conjugates was improved with periods of dry heating. The PTG–dextran conjugate obtained by dry heating for 3 weeks revealed constant solubility at all pH regions (pH 2–12). The emulsifying properties of the PTG–dextran conjugate were greatly increased with dry-heating time. The molecular weight of PTG–dextran conjugates was widely distributed from 0.1 to 2.0 million.

The insolubility of proteins sets limits to their utilization in formulated food systems. Wheat gluten is a typical insoluble protein for which it is desirable to extend its utilization in food application because of its abundant supply as a byproduct of the wheat starch industry. Many researchers have developed methods to change the solubility and functional properties of gluten. Finley (1975) suggested a mild acid treatment of wheat gluten to increase the solubility in fruit-based acidic beverages. Wu et al. (1976) found a significant improvement in the functional properties of gluten by mild acid hydrolysis. Kato et al. (1989) and Matsudomi et al. (1986) reported that proteolytic deamidation of gluten was effective for the improvement of the functional properties. However, considerable amounts of gluten remain insoluble matter, despite an increase in the solubility obtained by the methods described above. Protease digestion is the most promising way to solubilize gluten further effectively. However, the protease digestion causes decreases in the functional properties. Therefore, the reconstitution of gluten peptides should be considered to overcome these demerits. Recently, we have developed a new method to improve the solubility and emulsifying properties of proteins by conjugating proteins with dextran through a naturally occurring Maillard reaction (Kato et al., 1990). In this approach, an attempt was made to reconstitute gluten peptides through conjugation with dextran. This approach may be promising in the utilization of insoluble proteins and the improvement of their functional properties.

MATERIALS AND METHODS

Materials. Dextran (molecular weight, 60 000–90 000) was purchased from Wako Pure Chemical Industries, TSK gel G3000SW was from Toso Co., Sepharose CL-6B was from Pharmacia LKB, and Pronase E was from Sigma Chemical Co. (St. Louis, MO). Wheat gluten was prepared by washing flour dough until the washings were free from soluble proteins. The gluten ball was dialyzed against distilled water and then freeze-dried.

Preparation of Protease-Treated Gluten. Gluten (2 g) was suspended in 200 mL of 0.05 M sodium borate buffer, pH 8.0, and then 20 mg of Pronase E was added. The mixture was incubated at 37 °C for 12 h. After incubation, Pronase E was inactivated by heating at 100 °C for 3 min. Pronase-treated gluten (abbreviated PTG) thus obtained was gel-filtrated on a Sephadex G-25 column (70 × 3 cm) in water and dialyzed against deionized water and then freeze-dried.

PTG–Dextran Conjugation. PTG–dextran mixture in the weight ratio of 1:5 was dissolved in water in 10% (w/v) concentration and freeze-dried. Powdered PTG–dextran mixtures were stored at 60 °C and 79% relative humidity in a desiccator containing saturated KBr solution for a given time (0–3 weeks). To further purify the PTG–dextran conjugate, gel filtration of the conjugate was performed on a column (70 × 3 cm) of Sepharose CL-6B. Elution was carried out with 0.1 M Tris-HCl buffer, pH 7.0, and 3.0-mL fractions were collected.

High-Performance Gel Chromatography of PTG. High-performance gel chromatography of PTG was carried out with a TSK gel G3000SW column (0.75 × 30 cm). Elution was done in 0.2 M phosphate buffer, pH 6.9, with a flow rate of 0.5 mL/min.

Measurement of Solubility. Samples solutions (0.1%) in various pH values were centrifuged at 10 000 rpm for 15 min, and the absorbances at 280 nm were measured to determine the relative solubility. The relative solubilities at various pH values adjusted with the addition of 0.1 N NaOH or HCl were estimated as the percentages of the value at pH 12, which showed the highest solubility.

SDS Slab Polyacrylamide Gel Electrophoresis. SDS slab polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) using 10% acrylamide separating gel and 3% stacking gel containing 0.1% SDS. Both gels contain 0.27% bis-(acrylamide). Protein samples (20 μL, 0.1%) were prepared in Tris–glycine buffer, pH 8.8, containing 1% SDS and 1% mercaptoethanol. Electrophoreses were carried out at a constant current of 10 mA for 5 h using electrophoretic buffer of Tris–glycine containing 0.1% SDS. Gel sheets were stained for proteins and carbohydrates with 0.025% Coomassie blue G-250 solution and 0.5% periodate–fuchsin solution (Zacharius et al., 1969), respectively.

Determination of the Molecular Weight of the PTG–Dextran Conjugate. PTG–dextran conjugate solution (0.1%) in 67 mM sodium phosphate buffer (pH 7.0) was applied to a high-performance gel chromatography system, connected with a TSK gel G3000SW column (Toso Co., 0.75 × 60 cm) at a flow rate of 0.3 mL/min. Elution from the columns was monitored with a low-angle laser light scattering photometer (LS-8, Toso) and then with a precision differential refractometer (RI-8, Toso). The molecular weight of the PTG–dextran conjugate was estimated from the ratio of total area in the peak of a low-angle laser light scattering photometer (LS) to that of a refractometer (RI) by the equation (Takagi and Hizukuri, 1984)

$$\mathrm{MW} = M_{\mathrm{std}} \frac{(dn/dc)_{\mathrm{std}}(\mathrm{LS/RI})_{\mathrm{sam}}}{(dn/dc)_{\mathrm{sam}}(\mathrm{LS/RI})_{\mathrm{std}}}$$

where M_{std} is the molecular weight of standard protein, $(dn/dc)_{\mathrm{std}}$ is the refractive index increment of standard protein, $(dn/dc)_{\mathrm{sam}}$ is the refractive index increment of the PTG–dextran conjugate, $(\mathrm{LS/RI})_{\mathrm{std}}$ is the ratio of total area in the peak of a low-angle laser light scattering to that of a refractometer of standard protein, and $(\mathrm{LS/RI})_{\mathrm{sam}}$ is that of PTG–dextran

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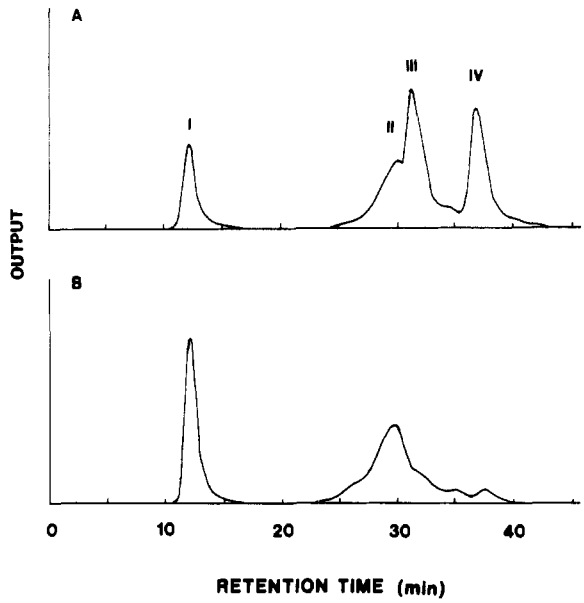

Figure 1. High-performance gel chromatography of PTG. (A) PTG; (B) dialyzed PTG.

conjugate. Ovalbumin was used as a standard protein whose molecular weight was known.

Measurement of Emulsifying Properties. The emulsifying properties were determined by the method of Pearce and Kinsella (1978). To prepare emulsions, 1.0 mL of corn oil and 3 mL of protein solution in 0.1 M phosphate buffer, pH 7.4, were shaken together and homogenized in an Ultra Turrax (Hansen & Co., West Germany) at 12 000 rpm for 1 min at 20 °C. A sample of 50 μL of emulsion was taken from the bottom of the container after different times and diluted with 5 mL of 0.1% sodium dodecyl sulfate solution. The absorbance of the diluted emulsion was then determined at 500 nm. The emulsifying activity was determined from the absorbance measured immediately after emulsion formation. The emulsion stability was estimated by measuring the half-time of the turbidity of emulsion.

RESULTS

Pronase is used to increase the solubility of gluten. About 90% of the insoluble gluten was solubilized by Pronase digestion. To determine what sizes of peptides resulted from Pronase digestion of gluten, high-performance gel chromatography on a TSK G3000SW column of PTG was done (Figure 1). Four peaks (I–IV) emerged with retention times 12.9, 29.7, 31.1, and 36.3 min, respectively. As peaks III and IV disappeared after dialysis (Figure 1B), these may be peptides and amino acids, respectively. Peak I is eluted in a void volume, corresponding to a molecular weight of several hundred thousand or above, and peak II corresponds to a molecular weight of 10 000–20 000 on the basis of the retention time of standard proteins. When gel filtration was done on a Sepharose CL-6B column, the elution area of the first peak was about 30% (Figure 3). On the other hand, the area of the first peak of HPLC is less than 20% of total peak area. This suggests that some high molecular weight components may adsorb to the HPLC column. Despite exhaustive protease digestion, considerable amounts of high molecular weight components (first and second peaks) seem to remain in PTG. The area of the first and second peaks is about 40% of total peak area. These fractions were used in the experiments for conjugation with dextran.

Although gluten was solubilized by Pronase, the solubility of PTG was greatly decreased at acidic pH. To increase the solubility, the mixture of PTG with dextran

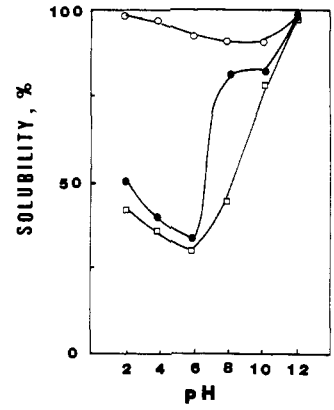

Figure 2. Solubility of PTG–dextran conjugates as various pH values. (□) Native gluten; (●) PTG–dextran mixture without dry heating; (○) PTG–dextran conjugate obtained by dry heating for 3 weeks.

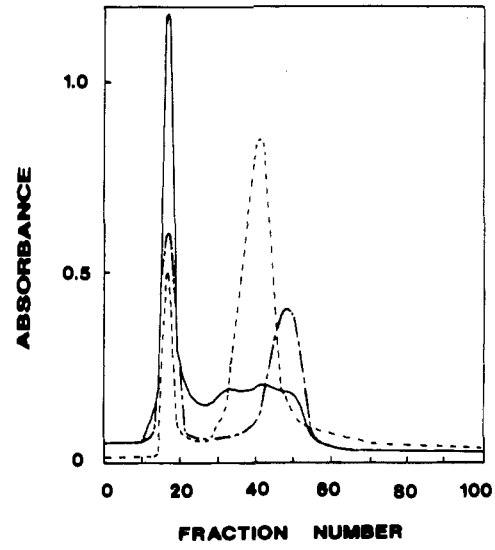

Figure 3. Elution patterns on Sepharose CL-6B of PTG and PTG–dextran conjugate obtained by dry heating for 3 weeks. (- - -) Absorbances at 280 nm of PTG; (—) absorbances at 280 nm of PTG–dextran conjugate; (- - -) absorbances at 490 nm of PTG–dextran conjugate after color development by phenol–sulfate method for carbohydrate detection.

Table I. Increase in Solubility of Gluten Peptides during Conjugation with Dextran in Dry Heating at 60 °C and 79% Relative Humidity

storage time, weeks	solubility, %	storage time, weeks	solubility, %
0	82	2	100
1	93	3	103

was further stored in dry heating at 60 °C and 79% relative humidity for a given time. The solubility of PTG–dextran conjugates was increased with the time of dry heating, as shown in Table I. The pH dependence of the solubility was studied by using PTG–dextran conjugate dry heated for 3 weeks (Figure 2). The solubility of control gluten (alkaline-soluble) and PTG–dextran mixture greatly decreased at acidic pH, while the solubility of PTG–dextran conjugate obtained by dry heating for 3 weeks remained constant and the solubility at acidic pH was slightly higher than that at neutral pH.

Figure 3 shows the gel filtration patterns on Sepharose CL-6B of PTG and the PTG–dextran conjugate obtained by dry heating for 3 weeks. The elution pattern of PTG is almost the same as that of HPLC, emerging in the high molecular weight fraction (fractions 15–20) and in the lower molecular weight fraction (fractions 40–60). On the other

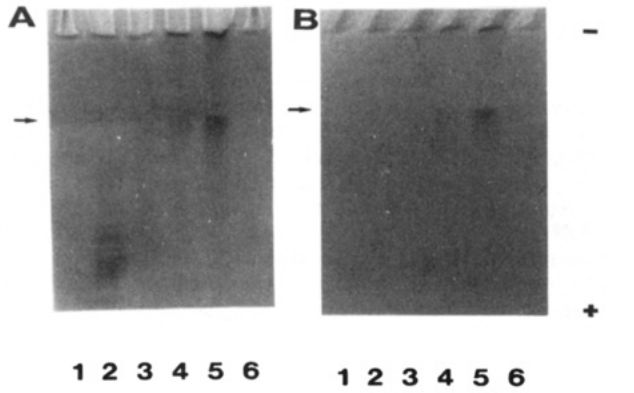

Figure 4. SDS–polyacrylamide gel electrophoresis of PTG–dextran conjugates. (A) Protein stain; (B) carbohydrate stain. (1) Dextran; (2) native gluten; (3) PTG–dextran mixture without dry heating; (4) PTG–dextran conjugate obtained by dry heating for 3 weeks; (5) high molecular weight fraction (fractions 15–20 in Figure 3); (6) lower molecular weight fraction (fractions 30–55 in Figure 3). Arrows indicate the boundary between stacking and separating gels.

hand, the high molecular weight fraction of the PTG–dextran conjugate increased greatly. The overlap of protein peak with carbohydrate peak was observed. This suggested that the high molecular weight fraction apparently revealed the PTG–dextran conjugate covalently attached during dry heating. The lower molecular weight fractions of PTG–dextran conjugate were also slightly shifted to the higher molecular weight side (fractions 30–50).

SDS–polyacrylamide gel electrophoreses of these fractions are shown in Figure 4. The electrophoretic pattern of the high molecular weight fraction in PTG–dextran conjugate showed a dense broad band for protein and carbohydrate stains near the boundary between stacking and separating gels with another higher molecular weight band that was unable to enter into the stacking gel. This suggests the formation of PTG–dextran conjugate by dry heating. As dextran is a branched polysaccharide and several dextrans may be attached to PTG, the conjugate must be unable to enter the separating or stacking gels. On the other hand, no band staining with carbohydrate was observed in the lower molecular weight fraction.

To determine the molecular weight of the PTG–dextran conjugate, the high molecular weight fraction was collected and analyzed by low-angle laser light scattering technique combined with high-performance liquid chromatography. It is very difficult to determine the molecular weight of heterogeneous polymers such as protein– or peptide–dextran conjugates. However, this technique has enabled the determination of the molecular weight of heterogeneous polymers (Takagi and Hizukuri, 1984). An advantage of this method is that the molecular weight of the eluted fraction at each retention time can be determined. The strength of low-angle laser light scattering is proportional to the molecular size and the concentration of eluted samples. The molecular weights are calculated from the equation above. Figure 5 shows the elution patterns of PTG–dextran conjugate obtained with a low-angle laser light scattering photometer, precision differential refractometer, and UV detector. The molecular weight was determined from these absorptions by the method of Takagi and Hizukuri (1984). The molecular weight at each elution position was plotted in Figure 5. The molecular weights are distributed in the range 0.1–2.0 million.

Figure 6 shows the emulsifying properties of PTG–dextran conjugate. The emulsifying properties were increased

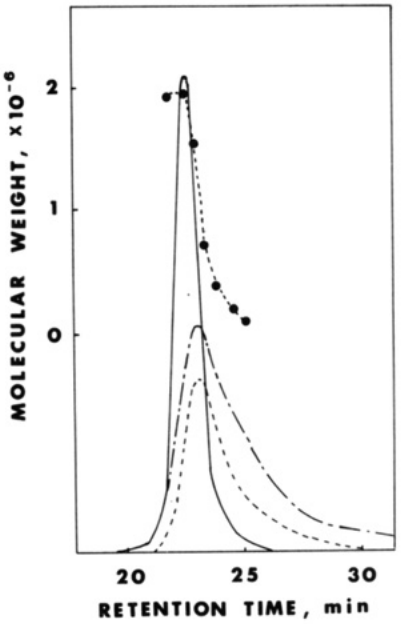

Figure 5. Elution patterns of PTG–dextran conjugate obtained with low-angle laser light scattering photometer (—), precision differential refractometer (- - -), and UV detector (- · -). Molecular weights (●) are plotted at each elution position. A total of 100 μL of a 0.1% PTG–dextran conjugate solution was injected into the HPLC column (G3000SW).

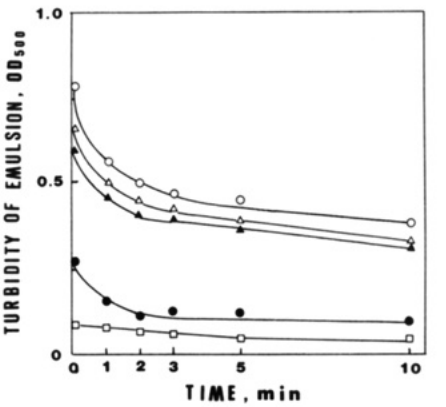

Figure 6. Emulsifying property of PTG–dextran conjugate obtained by dry heating. (□) native gluten; (●) PTG–dextran mixture without dry heating; (▲) PTG–dextran conjugate obtained by dry heating for 1, (△) 2, and (○) 3 weeks.

with storage in dry heating. The emulsifying properties of the purified PTG–dextran conjugate (fractions 15–20) had almost the same values as the crude PTG–dextran conjugate.

DISCUSSION

Although gluten was treated by Pronase, considerable amounts of high molecular weight fractions remained in soluble forms. The Pronase-resistant proteins or peptides were conjugated with dextran. The solubility of PTG–dextran conjugate was increased with storage time in dry heating and improved at acidic pH. The increase in the solubility is apparently due to the attachment of dextran to gluten peptides. We have reported that proteins were covalently attached to dextran through Maillard reaction between amino groups in proteins and reducing-end carbonyl groups in dextran during dry heating at 60 °C and 65–79% relative humidity (Kato et al., 1990; Nakamura et al., 1990).

The molecular weight of PTG–dextran conjugate was widely distributed from 0.1 to 2.0 million. Since dextran

(MW 60 000–90 000) has only one active site per molecule, several dextran molecules may attach around one molecule of gluten peptide. The high molecular weight and its wide distribution of conjugate may be attributed to the heterogeneous attachment of dextran to PTG in addition to the wide molecular weight distribution of PTG.

It is interesting that PTG–dextran conjugation causes not only an increase in the solubility but also an improvement of the emulsifying properties. The remarkable improvement of these functional properties has not been obtained by other modifications of gluten. Thus, Pronase digestion followed by dextran conjugation is one of the most promising methods to utilize insoluble protein sources. Since dextran is expensive for industrial application, other polysaccharides can be substituted for dextran. According to our preliminary attempts, branched polysaccharides are effective for the preparation of the conjugation having better functional properties.

LITERATURE CITED

Finley, J. W. Deamidated Gluten: A Potential Fortifier for Fruit Juices. *J. Food Sci.* **1975**, *40*, 1283–1285.

Kato, A.; Lee, Y.; Kobayashi, K. Deamidation and Functional Properties of Food Proteins by the Treatment with Immobilized Chymotrypsin at Alkaline pH. *J. Food Sci.* **1989**, *54*, 1345–1347.

Kato, A.; Sasaki, Y.; Furuta, R.; Kobayashi, K. Functional Protein-Polysaccharide Conjugate Prepared by Controlled Dry-Heating of Ovalbumin-Dextran Mixtures. *Agric. Biol. Chem.* **1990**, *54*, 107–112.

Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685.

Matsudomi, N.; Tanaka, T.; Kato, A.; Kobayashi, K. Functional Properties of Deamidated Gluten Obtained by Treating with Chymotrypsin at Alkaline pH. *Agric. Biol. Chem.* **1986**, *50*, 1989–1994.

Nakamura, S.; Kato, A.; Kobayashi, K. Novel Bifunctional Lysozyme-Dextran Conjugate That Acts on Both Gram-negative and Gram-positive Bacteria. *Agric. Biol. Chem.* **1990**, *54*, 3057–3059.

Pearce, K. N.; Kinsella, J. E. Emulsifying Properties of Proteins: Evaluation of a Turbidimetric Technique. *J. Agric. Food Chem.* **1978**, *26*, 716–723.

Takagi, T.; Hizukuri, S. Molecular Weight and Related Properties of Lily Amylose Determined by Monitoring of Elution from TSK-GEL PW High Performance Gel Chromatography Columns by the Low-Angle Laser Light Scattering Technique and Precision Differential Refractometry. *J. Biochem.* **1984**, *95*, 1459–1467.

Wu, C. H.; Nakai, S.; Powrie, W. P. Preparation and Properties of Acid-Solubilized Gluten. *J. Agric. Food Chem.* **1976**, *24*, 504–510.

Zacharius, R. M.; Zell, T. E.; Morrison, J. H.; Woodlock, J. J. Glycoprotein Staining Following Electrophoresis on Acrylamide Gels. *Anal. Biochem.* **1969**, *30*, 148–152.

Received for review September 4, 1990. Revised manuscript received January 14, 1991. Accepted January 28, 1991.