Improvement in the Functional Properties of Gluten by Protease Digestion or Acid Hydrolysis followed by Microbial Transglutaminase Treatment

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Insoluble gluten was solubilized by proteases (chymotrypsin, papain, Pronase, and pepsin) or mild acid (0.05 N HCl) treatments. The digests and hydrolysate are turbid, but after transglutaminase (TGase) treatment, the turbid mixture was converted to a transparent solution, which was found to be soluble at a wide range of pH. The hydrophobicity was greatly decreased after polymerization by TGase. SDS−PAGE patterns of the digests and hydrolysate with and without TGase treatment showed that the digests (except Pronase) were polymerized by TGase. The emulsifying properties of the polymerized peptides were greatly improved compared to those of the protease digests and acid hydrolysates. The foaming properties of the polymerized digests were also greatly improved. The digests and hydrolysate were found to have a bitter taste, but after polymerization the bitterness completely disappeared.

Keywords: *Functional properties; gluten; protease; hydrolysis; microbial transglutaminase*

INTRODUCTION

The insolubility of proteins sets limits for their utilization in formulated food systems. Wheat gluten is a typical insoluble protein for which it is desirable to extend its utilization in food applications because of its abundant supply as a byproduct of the wheat starch industry. Thus, solubilization of gluten has been attempted to extend its utilization in the food industry. This insoluble property of gluten has been mainly attributed to its amino acid composition (Krull et al., 1966). Large amounts of nonpolar amino acid residues such as proline and leucine tend to be involved in hydrophobic bonding, and the presence of a considerable amount of amide groups in the side chains of amide-type amino acids such as glutamine in gluten plays an important role in stabilizing the protein structure and in promoting the association of gliadin and glutenin molecules through hydrogen bonding. A number of molecular parameters such as molecular mass, conformation, flexibility, net charge, and hydrophobicity of proteins as well as interactions with other food components have been shown to play an important part in both their emulsifying and foaming properties (Nakai et al., 1983; Kato et al., 1985).

The emulsifying properties of proteins were reported to increase in proportion to hydrophobicity (Kato and Nakai, 1980). Although functionality of proteins has generally been improved by solubility, contradictory results were reported with respect to emulsifying properties (Aoki et al., 1981; McWatters et al., 1979). Studies on ovomucin (Kato et al., 1985) and ovalbumin (Kato et al., 1987) showed that the foaming properties were increased with increase in the molecular weight of the protein. It has been reported that deamidation levels as low as 2−6% could enhance the functional properties of proteins (Matsudomi et al., 1981, 1985a). 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) reported that proteolytic deamidation of gluten by chymotrypsin at alkaline pH was effective for the improvement of the functional properties and also (1991a) reported that Pronase digestion is the promising way to effectively solubilize gluten further. However, the protease digestion causes a problem of bitterness due to the presence of peptides enriched with hydrophobic amino acid residues (Arai et al., 1970). Therefore, transglutaminase (TGase) treatment should be considered to modify these peptides and overcome this problem (Tanimoto et al., 1991).

Transglutaminase as a polymerizer has been extensively studied (Folk, 1980; Nio, 1983; Motoki, 1986; Nonaka, 1989; Tanimoto, 1991; Kato, 1991b; Sakamoto, 1994, 1995; Sergo, 1995) and is known to catalyze the acyl transfer reaction between an amide group in a protein-bound glutamine and an ϵ-amino group in a protein-bound lysine side chain, resulting in cross-links between the protein molecules. Therefore, in this paper an attempt was made to polymerize protease digests and acid hydrolysate of gluten through microbial transglutaminase treatment. This approach may be promising for the utilization of unutilized proteins by improving their functional properties.

MATERIALS AND METHODS

Materials. *Enzymes.* Pronase E (4.1 units/mg), chymotrypsin (52 units/mg), papain (14 units/mg), and pepsin (2345 units/mg) were purchased from Sigma Chemical Co. Unless otherwise stated, all reagents used in this study were of reagent grade.

Preparation of Microbial Transglutaminase. Microbial transglutaminase was purified from the culture medium of *Streptoverticillium cinnamoneum* subsp. *cinnamoneum* IFO12852. The microorganism was inoculated in 200 mL of 0.2% polypeptone, 0.5% glucose, 0.2% dipotassium hydrogen phosphate, and 0.1% $MgSO_4$ for 8 h at 30 °C. The culture medium was added to 20 L of fresh medium (pH 7.0) composed of 2.0% polypeptone, 2.0% lustergen, 0.2% dipotassium hydrogen phosphate, 0.1% $MgSO_4$, 0.2% yeast extract, and 0.05% Adekanol and then cultured for 3 days. The culture medium

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(pH 6.5) was applied to a column of Amberlite CG-50, and then the adsorbed fraction was eluted with 0.05 M phosphate buffer (pH 6.5) containing 0.5 M NaCl. The fraction having high activity of TGase was collected and then adsorbed to Blue Sepharose CL-6B (Pharmacia Co.). The adsorbed sample was eluted with a gradient of 0−1 M NaCl. The peak of TGase was collected and dialyzed against deionized water (Ando et al., 1989).

Wheat Gluten Preparation. Gluten was prepared by washing flour dough with water until the washings were free from soluble proteins. The gluten ball was dialyzed against distilled water and then freeze-dried in a freeze-dryer (VD-80, Taitec Co., Japan).

Preparation of Protease-Digested Gluten. A freeze-dried sample (4 g) of gluten was suspended in 400 mL of 0.05 M Tris-HCl (pH 8.0) containing 0.05% sodium azide, and then 40 mg of Pronase E was added (Kato et al., 1991a). The mixture was incubated at 37 °C for 24 h. After incubation, Pronase was inactivated by heating at 100 °C for 3 min. The obtained Pronase-treated gluten was centrifuged (8000 rpm for 10 min) to remove a small amount of undigested protein, and then the supernatant was dialyzed (3000−4000, molecular weight cutoff) against distilled water or 0.1 M phosphate buffer (pH 7.0). The former was freeze-dried, and the latter was used for transglutaminase treatment. The chymotrypsin and papain treatments were similar to the Pronase one except that for papain treatment the pH was adjusted to 7.0. Pepsin digestion was carried out by dispersing 5 g of gluten in 400 mL of 0.1 M HCl containing 0.05% sodium azide and 30 mg of pepsin. The mixture was incubated at 37 °C for 18 h. The enzyme was inactivated by heating at 100 °C for 3 min. The digested mixture was centrifuged to remove a small amount of undigested protein and then the supernatant was dialyzed as shown in the Pronase treatment. The yield of protease digests obtained was more than 60−70% after dialysis.

Acid Hydrolysis. To 5 g of gluten was added 200 mL of 0.05 N HCl, and then the mixture was incubated at 120 °C for 60 min. The treated mixture was centrifuged to remove a small amount of unhydrolyzed protein, and then the supernatant was dialyzed (3000−4000, molecular weight cutoff) against distilled water or 0.1 M phosphate buffer (pH 7.0). The former was freeze-dried, and the latter was used for transglutaminase treatment. The yield of acid hydrolysate was more than 80% after dialysis.

Transglutaminase Treatment. The protease-digested or acid-treated gluten which dialyzed against 0.1 M phosphate buffer (pH 7.0) (10 mg/mL) was reacted with TGase (0.5 mg/mL). The mixture was incubated at 55 °C for 60 min. The enzyme was inactivated by *N*-ethylmaleimide (0.1 mL, 0.1%) (Kato et al., 1991b). The treated samples were dialyzed against distilled water and then freeze-dried.

Measurement of Solubility. Freeze-dried samples (0.2%) of protease digests and acid hydrolysate with and without TGase treatment were used for the determination of solubility at various pHs: pH 2−3, 0.05 M citrate buffer; pH 4−5, 0.05 M acetate buffer; pH 6−8, 0.05 M phosphate buffer; pH 9−11, 0.05 M carbonate buffer; and pH 12, 0.05 M NaOH slightly adjusted with 0.05 M HCl. Samples were dissolved in the buffer and shaken with a vortex mixer (Scientific Industries, adjusted on digit 4 to work on touch) for 10 s, and the turbidity was measured at 500 nm. Values obtained are means of triplicate samples.

SDS−Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) of freeze-dried samples was done using the method of Laemmli (1970) with 15% acrylamide separating gel and 5% acrylamide stacking gel containing 0.1% SDS. Samples (20 μL, 0.2%) were prepared in a Tris-glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was done at a current of 10 mA for 1 h and thereafter at 20 mA for 2 h in electrophoretic Tris-glycine buffer containing 0.1% SDS. After electrophoresis, the gel sheets were stained with 0.2% Coomassie brilliant blue-R250 and destained with 10% acetic acid containing 20% methanol for 18 h to remove any traces of the staining solution.

Measurement of Emulsifying Properties. The emulsifying properties of freeze-dried sample solutions were determined by the method of Pearce and Kinsella (1978). To prepare emulsions, 1.0 mL of corn oil and 3.0 mL of protein solution (0.2%) in 0.1 M phosphate buffer, pH 7.0, were shaken together and homogenized in an Ultra Turrax homogenizer (Hansen & Co., West Germany) at 12 000 rpm for 1 min at 20 °C. A 50 μL sample of emulsion was taken from the bottom of the container at 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 relative emulsifying activity was determined from the absorbance immediately measured after the emulsion formation. The emulsion stability was expressed as the half-time of the initial turbidity of the emulsion (Kato et al., 1991a,b). Values obtained are means of triplicate samples.

Measurement of Foaming Properties. The foaming properties of freeze-dried sample solutions were determined using the conductivity method (Kato et al., 1983). Electric conductivity of foams was measured when air was introduced into 5 mL of a 0.2% protein solution in 0.02 M phosphate buffer, pH 7, in a glass filter (G-4) at a constant flow rate (90 cm^3/min). The conductivity reading was recorded automatically using a recorder connected with a conductivity meter. The foaming properties of the samples were determined from the conductivity curves. The foaming power was expressed as the maximum conductivity during aeration. The foam stability was indicated as the time for the disappearance of the foams, the absence of conductivity. Values obtained are means of triplicate samples.

Determination of Surface Hydrophobicity. The surface hydrophobicity of proteins was determined by the method of Kato and Nakai (1980); 10 μL of 0.1% *cis*-parinaric acid solution in ethanol containing butylated hydroxy toluene (BHT) as an antioxidant was added to 2 mL of protein solution in 0.01 M phosphate buffer, pH 7.4, containing 0.002% dodecyl sulfate. The parinaric acid−protein conjugates were excited at 325 nm, and the relative fluorescence intensity at 420 nm was measured in an Aminco-Bowman spectrophotofluorometer. The slope (S_o), fluorescence intensity/% protein(0.05−0.006), was calculated from the fluorescence intensity vs protein concentration plot. Values obtained are means of triplicate samples.

Sensory Evaluation of Bitterness. Samples of protease- or acid-digested gluten and the TGase-treated ones were used in this test. Each sample was divided into six parts, which were served in random order (randomized block design) to the panelists. The samples were tested at 24 °C by a six-member panel selected from a pool of students and staff members of our department. Initial screening and selection of panelists were based on participant interest, taste acuity, and ability to understand test procedure (Meilgaard et al., 1990). The bitterness score was expressed as the quinine sulfate equivalent (Tanimoto et al., 1991). All the panelists had threshold values for quinine sulfate at about 10^{-4}%. The solution (10^{-4}−10^{-3}%) was used as a control to which the bitterness of the samples was quantitatively estimated. Water for rinsing between samples was provided. The score was estimated on a 9-point scale for 1 (10^{-4}%) to 9 (10^{-3}%). Scores obtained were indicated as means ± standard deviation, $n = 6$.

RESULTS AND DISCUSSION

Changes in the Molecular Mass by Enzymatic Modification of Gluten. Gluten was hydrolyzed by proteases and acid treatment. It is interesting that two bands corresponding to around 40 and 10 kDa are commonly observed in Pronase, chymotrypsin, and papain digests of gluten. The presence of these bands is consistent with the data obtained by HPLC analysis of Pronase digest (Kato et al., 1991a). These results suggest that the protease-resistant peptides exist in gluten. On the other hand, only broad bands less than 10 kDa appear in the pepsin digest, and widely distributed bands over the whole molecular size are observed in acid hydrolysate (Figure 1).

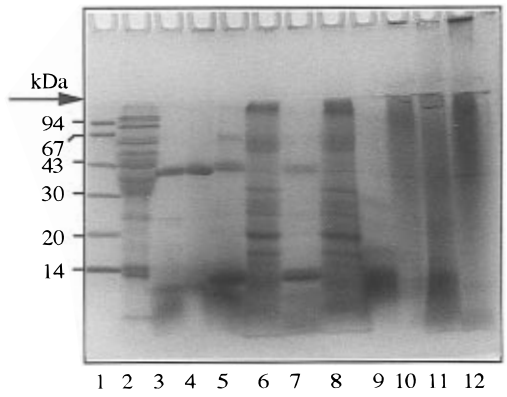

Figure 1. SDS−PAGE patterns of gluten digested by protease or acid treatments followed by TGase treatment: (1) molecular marker, (2) native gluten, (3) Pronase digests, (4) Pronase digests + TGase, (5) chymotrypsin digests, (6) chymotrypsin digests + TGase, (7) papain digests, (8) papain digests + TGase, (9) pepsin digests, (10) pepsin digests + TGase, (11) HCl hydrolysates, (12) HCl hydrolysates + TGase. Arrows indicate the boundary between stacking (upper) and separating (lower) gels.

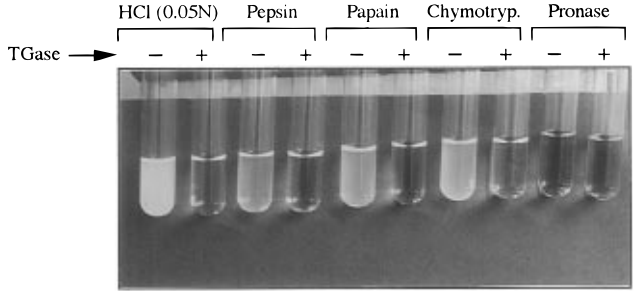

Figure 2. Sample solutions of protease- and acid-treated gluten with and without TGase treatment.

Microbial TGase was used to polymerize the protease digests and acid hydrolysate. As shown in Figure 1, except for Pronase digest, TGase-treated samples were composed of high molecular weight bands near the top of the SDS gel sheet with the disappearance of the 40 and 10 kDa bands. It should be emphasized that the bands of higher molecules were observed in the top of the stacking gel (lanes 10 and 12) or at the boundary between the stacking and separating gels(lanes 6 and 8). The SDS−PAGE patterns also showed that the polymerized materials had a broad molecular distribution. This broad molecular distribution may be attributed to the fact that transglutaminase reacts with various sizes of peptides produced by proteases or acid hydrolysis because, as reported previously, TGase catalyzed the transfer reaction between an amide group in a protein-bound glutamine and an ϵ-amino group in a protein-bound lysine side chain, resulting in various extents of cross-links between the protein molecules. On the other hand, the SDS−PAGE pattern of Pronase digest indicated that it was not cross-linked by TGase. The Pronase is so digestible that the residues involved in the cross-link may be deficient in peptides.

Effect of TGase Treatment on Solubility of Gluten Digests and Hydrolysate. Although gluten was solubilized by proteases and acid treatment, a considerable amount of gluten peptides remained in a turbid form (except Pronase digests) as shown in Figure 2. When the digests and hydrolysate were treated with TGase, the turbid mixture was converted to a transparent solution (Figure 2). The concentration of the digests and hydrolysate after dialysis was adjusted to 0.5%, and then the turbidity was measured at 500 nm (Table 1). The turbidity of the digests and hydrolysate was in the range of 0.04−2.00, and after polymerization with TGase it was decreased in the range of 0.03−0.07. To elucidate the molecular mechanism of this interesting phenomenon, the hydrophobicity of protease- and acid-treated gluten with and without TGase treatment was calculated as the slope of fluorescence intensity vs protein concentration (Table 2). The hydrophobicity of the digests and hydrolysate was found to be in the range of 3.36−9.60, and after polymerization with TGase it was greatly decreased in the range of 0.24−1.76. The results indicated that the exposed hydrophobic residues of the protease digests and acid hydrolysate were buried inside the polymerized molecules.

To investigate the overall effect of TGase treatment on solubility of the digested materials, the pH dependence of the solubility of the protease digests and acid hydrolysate with and without TGase treatment was also measured as shown in Figure 3. The results showed that, except for Pronase digests, the protease- and acid-treated gluten were considerably insoluble at a wide range of pHs, while the polymerized peptides (except

Table 1. Effect of TGase Treatment on the Solubility of Protease Digests and Acid Hydrolysate of Gluten in Distilled Water[a]

	turbidity (OD_{500}), TGase	
sample	−	+
Pronase digest	0.04 (±0.002)	0.03 (±0.001)
papain digest	0.95 (±0.120)	0.05 (±0.006)
chymotrypsin digest	0.90 (±0.210)	0.04 (±0.003)
pepsin digest	0.55 (±0.043)	0.07 (±0.002)
acid hydrolysate	2.00 (±0.310)	0.05 (±0.004)

[a] Values are means (±SD).

Table 2. Effect of TGase Treatment on the Surface Hydrophobicity (S_o) of Protease Digests and Acid Hydrolysate of Gluten[a]

	S_o, TGase	
sample	−	+
Pronase digest	4.00 (±0.80)	1.76 (±0.14)
chymotrypsin digest	4.96 (±0.69)	0.24 (±0.01)
papain digest	3.36 (±0.26)	0.40 (±0.05)
pepsin digest	5.60 (±1.02)	1.60 (±0.06)
acid hydrolysate	9.60 (±1.12)	1.60 (±0.72)

[a] Values are means (±SD).

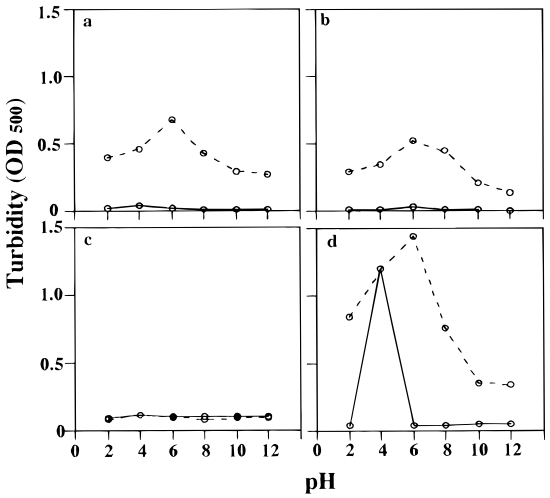

Figure 3. Solubility at different pH levels of gluten digested by (a) chymotrypsin, (b) pepsin, (c) Pronase, or (d) 0.05 N HCl, with (—) and without (− − −) TGase treatment.

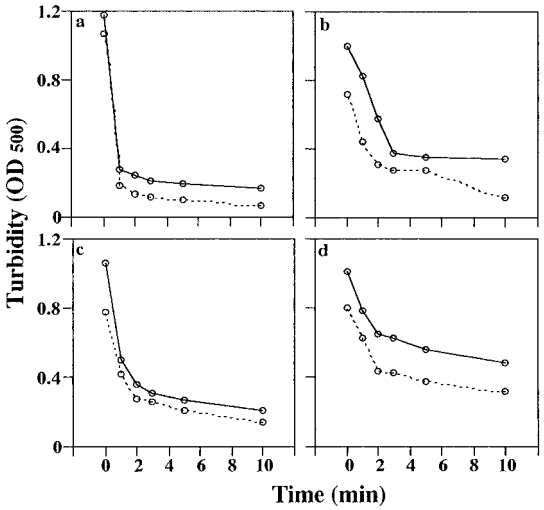

Figure 4. Emulsifying properties of gluten treated with (a) chymotrypsin, (b) pepsin, (c) Pronase, or (d) 0.05 N HCl, with (—) and without (· · ·) transglutaminase treatment.

acid hydrolysate at pH 4) were completely soluble over a wide range of pHs (Figure 3). In addition, the clear shift of maximum insolubility at pH 6 to lower pH 4 was observed in TGase-treated samples, especially in the acid hydrolysate (Figure 4d). Therefore, it seems likely that deamidation of digests and hydrolysate may have also occurred by TGase (Motoki et al., 1986). Solubilization of the protease digests and acid hydrolysate of gluten by TGase is mainly due to the decrease in the hydrophobicity and the increase in the electrostatic repulsion as a result of partial deamidation of glutamine and asparagine.

Effect of TGase Treatment on Surface Properties of Gluten Digests and Hydrolysate. The surface properties, such as the emulsifying and foaming properties of gluten digests, are poor. In order to improve these properties, the effect of TGase treatment on the functional properties was investigated, as shown in Figure 4. The emulsifying properties were improved by TGase treatment for all digests and hydrolysate. The relative emulsifying activity, which is the absorbance (OD_{500}) immediately (0 min) measured after the emulsion formation of the gluten digests and hydrolysate, was found to be in the range of 0.75−1.07 and after polymerization with TGase was increased in the range of 1.00−1.15. The emulsion stability (the half-time of the initial turbidity) was found to be in the range of 0.80−2.10 min and after polymerization with TGase was increased in the range of 1.0−5.0 min. Protease and mild acid treatments were observed to produce an increase in the emulsifying properties, and after polymerization with TGase further increases were observed. Protease treatment of gluten (Matsudoi et al., 1986) and mild acid hydrolysis of ovalbumin (Matsudoi et al., 1985b) caused an increase in the emulsifying properties due to the increase in the negative charge which results from the hydrolysis of the amide groups in glutamine and asparagine. Deamidation and polymerization with TGase, on the other hand, may bring about a more suitable nature to have better emulsifying properties.

The foaming properties of gluten digests and hydrolysate with and without TGase treatment were also measured (Figure 5). The foaming properties of gluten digests and hydrolysate were low. The foaming power (maximum conductivity during aeration) of the digests and hydrolysate was found to be in the range of 2.4−4.2 μmho/cm and after polymerization with TGase was

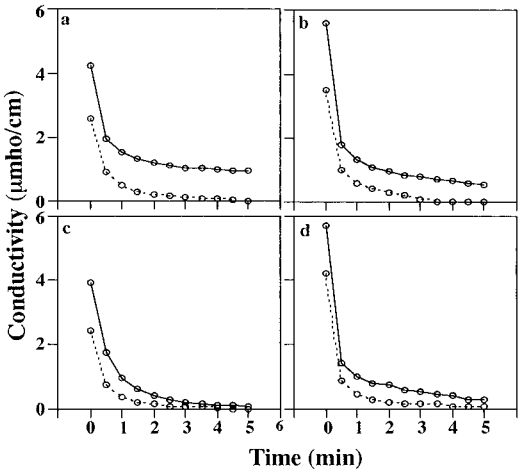

Figure 5. Foaming properties of gluten treated with (a) chymotrypsin, (b) pepsin, (c) Pronase, or (d) 0.05 N HCl, with (—) and without (· · ·) transglutaminase treatment.

Table 3. Bitterness Scores of Protease Digests and Acid Hydrolysate of Gluten with and without TGase Treatment[a]

	bitterness score (quinine sulfate equiv × 10^{-3}%), TGase	
sample	−	+
chymotrypsin digest	7.40 (±1.20)	1.17 (±0.05)
papain digest	7.90 (±1.01)	1.23 (±0.24)
pepsin digest	8.10 (±1.31)	1.13 (±0.07)
Pronase digest	4.40 (±0.83)	2.04 (±0.43)
acid hydrolysate	7.30 (±1.32)	1.34 (±0.10)

[a] Values are means (±SD).

increased in the range of 3.9−5.9 μmho/cm. The foam stability was found to be in the range of 4.5−7.0 min, and after polymerization with TGase it was increased in the range of 0.8−20.0 min. The improvement in foaming properties of gluten peptides by TGase treatment reflects the importance of protein association as a structural factor governing the foaming properties (Kato et al., 1985).

Effect of TGase Treatment on the Bitterness of Gluten Digests and Hydrolysate. It is well known that protease digests are bitter due to the exposed hydrophobic peptides (Arai, 1970). The bitterness scores of sample solutions of gluten digests and hydrolysate with and without TGase treatment are shown in Table 3. The bitterness scores of the digests and hydrolysate were found to be in the range of 4.4−8.1 and after polymerization with TGase were greatly decreased in the range of 1.13−2.04. The results indicated that the protease digests and acid hydrolysate were bitter, and after polymerization the bitterness completely disappeared in all digests and hydrolysate. This interesting phenomenon can be accounted for as follows. The protease or acid treatments resulted in the peptides being enriched with hydrophobic amino acid residues which tend to increase the bitterness (Arai et al., 1970). The polymerization with TGase seems to decrease the exposed hydrophobic peptides, as indicated by the decrease in the surface hydrophobicity (Table 2), as a result of the cross-links between the hydrophilic and hydrophobic peptides (Tanimoto et al., 1991) of the digests and hydrolysate.

In conclusion, although gluten was solubilized by proteases and acid treatments, problems such as insolubility and bitterness remained to be solved in food application. Therefore, polymerization of the gluten

digests by TGase should be done to avoid such problems. In addition to the bitterness reduction, we found that the polymerization with TGase also improved the solubility and surface functional properties. Despite the decrease in the surface hydrophobicity, the emulsifying properties were improved by TGase treatment of gluten peptides, suggesting that the molecular size is also involved in the emulsifying properties.

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