Physicochemical Properties of Pronase-Treated Rice Glutelin

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ABSTRACT: Rice glutelin protein was extracted from defatted medium-grain rice by alkali extraction followed by acid precipitation. Extracted glutelin was hydrolyzed with Pronase E, a bacterial protease, and the functional properties of hydrolysates were evaluated. Nitrogen solubility of pronase-treated glutelin protein increased from pH 2 to pH 12. Similarly, foaming and emulsion properties of hydrolyzed protein also showed improved characteristics. The emulsion activity, expressed as the turbidity of diluted emulsions, was significantly greater ($P \leq 0.05$) for hydrolyzed samples. However, turbidity for all samples decreased with increased homogenization time, indicating a decrease in the volume of dispersed oil. There were significant changes in apparent viscosity as a function of shear rate, with viscosity decreasing with increasing shear rate. The viscosity of dispersions of all hydrolyzed samples was significantly lower than that of the native sample at all shear rates tested. Enzymatic hydrolysis of rice endosperm storage glutelin proteins appeared to improve the functional characteristics of the hydrolyzed proteins.

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KEY WORDS: Functionality, hydrolysis, pronase, rice glutelin.

The proteins of rice have been reported to have unique hypoallergenic properties (1) and are thought to have potential uses as food ingredients. Rice proteins also rank high in nutritive quality among the cereal proteins owing to the relatively high levels of lysine (2). The hypoallergenic property as well as the high nutritive quality could make rice protein concentrate a competitive protein replacement in the marketplace as an alternative protein source for development of hypoallergenic infant formulas.

However, the use of rice proteins in food systems is very limited due to poor functional properties. Rice protein consists of four fractions: albumin, globulin, glutelin, and prolamin. Glutelin, the major fractional component, constitutes about 80% of the milled rice protein (1) and is insoluble in water at neutral pH (3). This insolubility results in poor functional properties and limits the use of rice protein in food applications. Modification of the protein could improve its functional properties.

The susceptibility of rice protein to the actions of proteases has been reported. Most of the research in this area pertains to the assessment of *in vitro* digestibility of the rice protein using proteases and various enzymes. Tanaka *et al.* (4) studied the effect of pepsin treatment on the properties of endosperm protein bodies from cooked and uncooked rice flourand reported that, after a 5-h incubation period, 87% of the uncooked rice protein was digested, while only 68% of the cooked rice protein was digested. Buchmann (5) tested six different cereals, including rice, for *in vitro* digestibility using pepsin, pancreatin, and pronase. The protein digestibility of the rice samples was reported to be 90, 46, and 47% by pepsin, pancreatin, and pronase, respectively. Nkonge and Ballance (6) studied the effectiveness of commercial enzymes in solubilizing maize, oat, rye, sorghum, triticale, wheat, and barley proteins and reported that neither trypsin nor α-chymotrypsin was very effective in solubilizing the cereal proteins tested.

Similar studies pertaining to the use of proteolytic enzymes to enhance the functionality of rice proteins for food applications are lacking in the literature, perhaps owing to the fact that the use of rice proteins as food ingredients has not been given much attention. A study on the effect of enzymatic hydrolysis on rice glutelin will help in the future development of food systems with rice proteins as major ingredients. The present study was undertaken to study the effect of enzymatic hydrolysis on selected functional properties of rice endosperm glutelin protein.

EXPERIMENTAL PROCEDURES

Commercially milled medium grain rice flour (cv. Mars) was obtained from Riceland Foods (Stuttgart, AR). Chemicals for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were purchased from Bio-Rad (Richmond, CA). All other chemicals were from Fisher Scientific (Fairlawn, NJ), and were of analytical grade. Deionized water prepared from Milli-Q Water Purification System (Millipore Corp., Milford, MA) was used in all experiments. Pronase E (Type XXV), a bacterial protease obtained from *Streptomyces griseus*, was selected for this study based on preliminary enzyme screening studies. This enzyme has a broad specificity, and was purchased from Sigma Chemical Company (St. Louis, MO).

Rice flour was defatted prior to preparation of glutelin by stirring in petroleum ether (boiling point 35–60°C) in a 1:7.5 (wt/vol) flour-to-solvent ratio for 2 h at ambient temperature. Defatted flour was air-dried under the hood and sieved through an Alpine Airjet sieve with 100 mesh (0.15 mm) screen and

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stored in plastic bags at 4°C prior to use. Compositional analysis of flour samples was performed on duplicate samples. Moisture, Kjeldahl nitrogen, lipid, and ash were determined according to American Association of Cereal Chemists (7) Approved Methods 44-19, 46-11A, 30-10, and 8-10, respectively. Nitrogen was converted to percent protein by multiplying with a factor of 5.95 (8).

Glutelin extraction. The modified Osborne procedure of Cagampang *et al.* (9) was used to extract rice glutelin. Defatted flour was stirred four times at a flour-to-solvent ratio of 1:10 (wt/vol) in each of the extracting solvents for 2 h at room temperature. The solvents used were: 5% NaCl to remove albumin-globulin and 60% (vol/vol) aqueous ethanol to remove prolamin. The residual flour was extracted with 0.05 N NaOH, and the glutelin was precipitated by acidifying with 3 N HCl to pH 4.8. The precipitated glutelin was washed with deionized water and freeze-dried. Glutelin concentrate was analyzed for nitrogen content from which the percentage glutelin was obtained by multiplying by a factor of 5.95. The protein content of the freeze-dried glutelin was determined to be 73.8%. Recovery of glutelin, calculated as g protein in extract/g protein in defatted flour × 100, was 70.9%.

Glutelin hydrolysis. Glutelin dispersions (5%, wt/vol) were prepared in water and adjusted to pH 7.5 with 0.1 N NaOH. The protein solutions were placed in a Lab-Line Orbit Environ Shaker (model 3528; Lab-Line Instruments, Melrose Park, IL) to equilibrate at 37°C for 15 min. After equilibration, an appropriate amount of pronase solution was added to each of the solutions to obtain an enzyme concentration of 0.03% (glutelin basis). The solutions were then incubated under constant shaking at 37°C and sampled at varying times (0, 10, 30, 60, 90, 120, 180, and 300 min). The control sample consisted of native glutelin in water with no enzyme. After the specified times, hydrolysis was terminated by placing the samples in boiling water for 3 min to inactivate the enzyme. Hydrolysates were then freeze-dried to obtain pronase-hydrolyzed glutelin samples that were subsequently used for functionality studies.

SDS-PAGE. SDS-PAGE was performed on hydrolyzed and control glutelin products according to the discontinuous buffer system described by Laemmli (10). The system consisted of 12% acrylamide resolving gel and 4% stacking gel, both containing 0.1% SDS. A Bio-Rad vertical slab gel electrophoresis power supply unit model Xi was used. Freeze-dried samples (5 mg each, protein basis) were dissolved in 100 µL sample buffer containing 10% SDS, 10% glycerol, 5% mercaptoethanol, and 0.05% bromophenol blue before loading 50-µL samples in sample wells. Electrophoresis was carried out at an initial constant current of 13 mA for 1 h to move sample through stacking gel and then increased to 18 mA for 4.5 h. The electrophoretic running buffer consisted of tris-glycine containing 0.1% SDS. Gels were fixed and stained with 0.1% Coomasie Brilliant Blue R-250 in 10% acetic acid and 40% ethanol, destained with 10% acetic acid, 40% ethanol, and dried in a Bio-Rad Model 543 Gel Dryer. Protein bands were quantified using a densitometer.

Nitrogen solubility. Nitrogen solubility profiles of hydrolyzed and native glutelin samples were determined using a modified method of Betschart (11). Samples of 50 mg each (protein basis) were dispersed in 10 mL deionized water to make 0.5% dispersions. The dispersions were adjusted to pH values ranging from 2.0–12.0 with 1 N HCl or NaOH. Samples were then stirred for 30 min and centrifuged at 16,000 × *g* for 30 min. Duplicate aliquots of the supernatant of each sample were analyzed for extracted nitrogen by the Kjeldahl method (7).

Apparent viscosity. The apparent viscosity of 10% (wt/vol) suspensions of native and hydrolyzed glutelin samples was measured at ambient temperature using Brookfield Synchrolectric viscometer, model RVF (Brookfield Engineering Laboratories, Stoughton, MA). The viscometer operates by means of a synchronous induction-type motor that gives a series of constant speeds of rotation. A spindle is attached to a small chuck and when the spindle is immersed in the sample and the motor is switched on, the viscous drag of the fluid on the spindle is registered as torque on a dial. A factor finder scale provided by the manufacturer enables the conversion of the dial reading into apparent viscosity. To measure the apparent viscosity of samples, a 10% (wt/vol) dispersion of sample in water was prepared in 150-mL beaker and allowed to hydrate for 1 h. Viscosity (shear stress) was measured at three speeds of 4, 10, and 20 rpm, using spindle #2 of the viscometer.

Foaming properties. Foaming properties of hydrolysates were performed according to the standard solubilization procedure of Morr *et al.* (12). Sample (3.75 g) was weighed into a 150-mL beaker and deionized water was added with stirring. The volume was brought to 60 mL and the sample was stirred for 30 min. The pH of the solution was adjusted to 7.0 with 0.1 N HCl or NaOH. After an additional 30 min stirring, the pH was readjusted to 7.0 and total volume brought to 75 mL to yield a 5% (wt/vol) sample solution. Foam was formed according to the standard method of Phillips *et al.* (13) and the drainage from foam was monitored at ambient temperature to measure foam stability (13–15).

Emulsifying activity. Emulsion activity was evaluated according to the turbidimetric method of Pearce and Kinsella (16) with some modifications. Five grams of sample for each trial was suspended in 100 mL of water to obtain a 5% (wt/vol) sample dispersion. Aliquot (30 mL) of the dispersion was added to 10 mL of peanut oil and the mixture was homogenized in a 150-mL beaker using Branson model 450 Sonifier (Branson Ultrasonics, Danbury, CT) for 1 min at 25°C at a power setting equivalent to 55 W. The emulsion obtained was held at constant temperature while being stirred gently. At 5-min intervals, 1-mL aliquots were taken and diluted with 100 mL of distilled water. The absorbance of the diluted emulsion was then determined in a 1-cm pathlength cuvette at a wavelength of 500 nm in a Varian series 634 double beam spectrophotometer (Varian, Sunnyvale, CA). The absorbance measured was expressed as the emulsifying activity of the sample.

Statistical analysis. A SAS statistical analysis system (SAS Institute, Cary, NC) was used to analyze data. General

linear model analysis of variance and least significance difference values were determined at the 5% level.

RESULTS AND DISCUSSION

Rice glutelin is essentially composed of two major polypeptide subunits having molecular weights near 40 and 20 kD (17–19). A third minor subunit of 14 kD is also present and is perceived to be a prolamin contaminant of glutelin. Table 1 shows the densitometric scanning data of the 12% SDS-PAGE gel of native and pronase-hydrolyzed glutelin. The data show that in the native glutelin, major polypepetides were present at 12, 19, 22, and 37 kD. Previous studies have shown that the two major polypeptide units of rice glutelin were prominent within the ranges of 28.5–30.8 and 20.6–21.6 kD (20). Zhao *et al.* (21) and Robert *et al.* (22) have also reported ranges of 30–39 and 19–29 kD. Thus, while the 12 kD polypetide found in this study may actually be the 14 kD prolamin contaminant of rice glutelin, or a fragmentation of it, the other polypeptides found were within the previously reported data. The slight variations may also be due to differences in gel preparations and analysis. Fractional components of the respective polypeptides were 15.96, 10.58, 13.16, and 13.40% of total proteins (Table 1). With increasing hydrolysis, some protein bands disappeared, especially at the higher molecular weight range, suggesting that they were completely hydrolyzed by pronase. Protein bands that remained were weaker or lighter than those of native bands, indicating these proteins were hydrolyzed to some extent by pronase. The 60–180 min hydrolysates showed only 5 protein bands each, and all medium-sized (29–64 kD) protein bands were completely hydrolyzed. Since only partially hydrolyzed polypeptides are desirable in food systems, the 30- and 60-min hydrolysates, showing nine and five protein bands, respectively, were selected for functional property studies.

TABLE 1
Densitometric Scanning of 12% SDS-PAGE[a] Gel Showing Molecular Weight Distribution and Relative Contents (percentage of total proteins) in Native and Hydrolyzed Glutelin

Molecular weight (kD)	Hydrolysis time (min)							
	0	10	30	60	90	120	180	300
8	5.01	16.78	22.00	35.28	33.69	37.52	43.75	94.68
12	15.96	9.75	9.86	3.35	2.71	1.96	0.59	—
19	10.58	1.71	1.94	4.99	2.60	3.94	7.70	—
22	13.16	17.35	21.16	17.8	18.3	4.24	6.19	—
29	2.08	—[b]	—	—	—	—	—	—
32	1.92	—	—	—	—	—	—	—
37	13.40	7.68	4.61	—	—	—	—	—
42	3.78	3.06	1.68	—	—	—	—	—
45	4.41	2.89	1.82	—	—	—	—	—
50	2.83	—	—	—	—	—	—	—
55	3.34	3.12	1.70	—	—	—	—	—
64	1.87	—	—	—	—	—	—	—
203	9.75	19.17	12.50	9.27	16.89	22.41	13.74	5.32

[a]SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
[b]Absence of band.

Figure 1 shows the increase in glutelin solubilization with varying incubation time at different pronase concentration levels (0, 0.01, and 0.03%, protein basis). Solubilized glutelin after pronase hydrolysis was determined as previously described. Solubilization increased steeply and reached a maximum of 34% after 60 min for an enzyme concentration of 0.03%. There was hardly any change in solubility of the control (0% enzyme concentration) samples. The result indicates that a greater degree of hydrolysis was achieved at 0.03% enzyme concentration at 60 min of incubation. Further hydrolysis did not result in significant change in solubility.

The nitrogen solubility profiles of native and hydrolyzed glutelin are shown in Figure 2. Pronase hydrolysis improved solubility of glutelin. The nitrogen solubility of hydrolyzed glutelin significantly increased over that of native glutelin at all pH levels and showed a maximum of 61.6% at pH 12. At pH 2 solubility increased from 15.6 to over 36% during 60 min of hydrolysis. The corresponding values at pH 12 were 30.1 and 61.6, respectively. Between pH 2 and 5, solubility decreased for both native and hydrolyzed glutelin with a minimum solubility of 8% for the native sample at pH 5, 10.8% for the 30 min digest at pH 5, and 18.5% for the 60 min digest at pH 4. However, while the solubility of hydrolyzed samples increased after pH 5, that of native glutelin remained fairly constant between pH 5 and 10, a region that includes the isoelectric points of 6.6–7.5 and 9.4–10.3 of the two glutelin polypeptides (20). After hydrolysis, the solubility

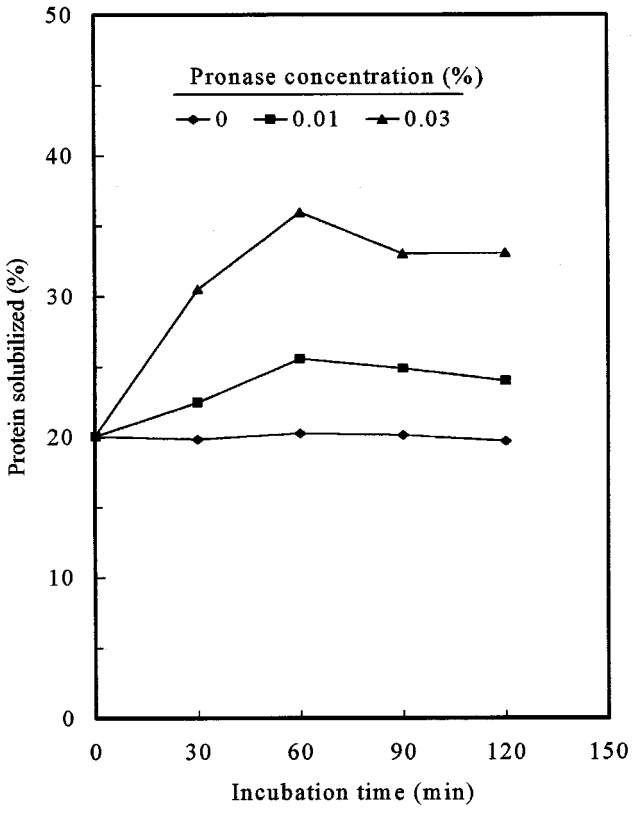

FIG. 1. Effect of pronase concentration (protein basis) on glutelin solubilization.

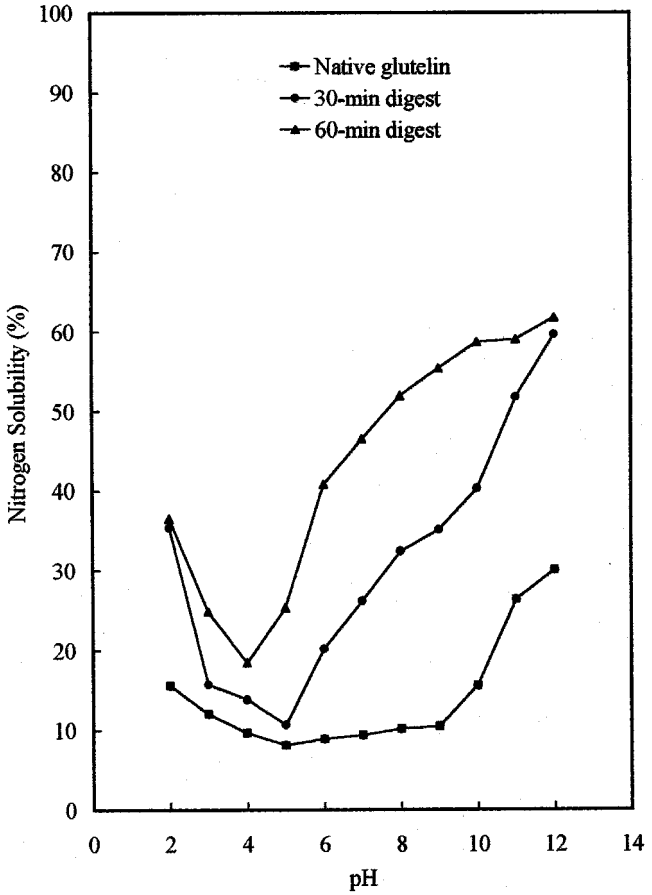

FIG. 2. Nitrogen solubility profiles of native and pronase-hydrolyzed glutelin between pH 2and 12.

minimum shifted from pH 5.0 of the native glutelin to a more acidic pH of 4.0. On the acidic side of the pH spectrum, there was a relatively small increase in solubility, while the increase in solubility on the alkaline side was steep among all treatments. Bera and Mukherjee (23) noted a similar occurrence in the nitrogen solubility of rice bran protein concentrates. It was also observed that the solubility minimum for the native glutelin falls within a much broader range, perhaps due to the composition of native glutelin polypeptide. Rice glutelin has two major polypeptides, an acidic (α) polypeptide and a basic (β) polypeptide. Wen and Luthe (20) reported that the acidic polypeptide has an isoelectric point (pI) of 6.6–7.5, and the basic polypeptide a pI of 9.4–10.3. Below and above the pI, proteins have a positive or negative net-charge which enhances solubility. At the pI, the net charge is zero, attractive forces predominate, and molecules tend to associate, resulting in decreased solubility. The broad nature of the solubility minimum between pH 4 and 10 for the native glutelin may be a reflection of this phenomenon.

Hydrophobic bonding and amino acid composition also affect solubility. Wen and Luthe (20) have reported that the most abundant amino acids in rice glutelin fraction are glutamic acid/glutamine, aspartic acid/asparagine, arginine, glycine, and alanine. The amide groups in the side chains of glutamine and asparagine play an important role in promoting the association of glutelin molecules through hydrogen bonding. The large amounts of nonpolar amino acid residues such as proline and leucine also tend to engage in hydrophobic bonding (24). The enhancement of solubility by treatment with pronase may therefore have been induced by the increased electrostatic repulsion and the decreased hydrogen bonding (24).

Apparent viscosity, foaming overrun, and foam stability values for native and hydrolyzed samples are shown in Table 2. The viscosities of dispersions of all hydrolyzed samples were significantly lower than that of the native sample at all shear rates tested. It is evident that the apparent viscosities of native and hydrolyzed glutelin samples all decreased with increasing shear rate within the range of shear rates tested. This type of fluid flow behavior, known as plastic flow, is characteristic of non-Newtonian fluids. Hence the viscous behavior of 10% dispersions of native and hydrolyzed glutelin at ambient temperatures exhibited non-Newtonian behavior. Kinsella (25) showed that a correlation exists between protein hydration and viscosity of a food system and that this relationship is affected by the concentration of protein and factors such as pH, ionic strength and temperature. A positive relationship also exists between the content of hydrophilic groups, i.e., fewer amides, and the water-binding capacity of the proteins (26). The observed decrease in viscosity of hydrolyzed glutelin over native glutelin indicates less water-binding capacity of hydrolyzed samples.

Foam overrun for the 60-min hydrolyzed glutelin was higher than that of the 30-min hydrolysates and native

TABLE 2
Apparent Viscosity, Foam Overrun and Foaming Stability of Native and Hydrolyzed Glutelin Protein[a]

	Apparent viscosity (cP)			% Overrun[b]			Foam stability (min)[c]		
	Spindle speed (rpm)			Whipping time (min)			Whipping time (min)		
Treatment	4	10	20	5	10	15	5	10	15
Native	166.0	119.5	88.5	335.19	360.33	389.27	20.05	18.85	18.40
30-min	121.0	94.5	76.5	358.09	421.64	457.54	24.35	22.35	20.85
60-min	98.5	86.5	71.5	457.43	508.75	562.20	31.85	25.40	23.20

[a]Values are means of duplicate determinations
[b]Overrun determined according to References 12 and 13.
[c]Foam stability determined according to References 14 and 15

TABLE 3
Effects of Whipping[a] Time on Moisture Loss and Stability of Foams from Native and Hydrolyzed Glutelin[a]

	5 min		10 min		15 min	
Sample treatment	Moisture loss (%)[b]	Stability (min)	Moisture loss (%)	Stability (min)	Moisture loss (%)[b]	Stability (min)
Native glutelin	9.1[a]	20.3[c]	10.4[a]	12.7[c]	12.3[a]	10.9[c]
30-min hydrolyzed glutelin	6.5[b]	22.1[b]	7.2[b]	14.8[b]	9.1[b]	13.4[b]
60-min hydrolyzed glutelin	3.4[c]	24.3[a]	6.8[b]	22.1[a]	7.8[c]	21.8[a]

[a]Values are means of duplicate analysis.
[b]Moisture loss determined as difference in sample weight before and after whipping. Means in the same column followed by different roman letters are significantly different ($P = 0.05$).

glutelin (Table 2), and the rate of volume change was almost constant for both native and hydrolyzed samples. In general, the improvement in foaming capacity of hydrolyzed glutelin as compared to native glutelin was probably due to solubilization of the glutelin molecule as a result of pronase treatment. Tasneem and Subramanian (27) reported higher foaming capacity for protein isolates from detoxified guar meal, as compared to the control defatted meal, and attributed the increase in foam capacity to higher solubility of the detoxified meal isolate.

Stability of foams of all samples decreased as whipping time increased (Table 2). As whipping progressed, the time required to attain 50% drainage of foams decreased. However, the decrease in stability was more pronounced in the hydrolyzed samples than in the native glutelin. The decreasing stability of foams of both native and hydrolyzed glutelin with respect to whipping time could be explained by the presence of repulsive forces, which tend to weaken the strength and thickness of the film at the interface between air bubbles and water, permitting interactions by hydrophobic bonds (28). It is clear that pronase hydrolysis did not help stabilize the interface between air and water. A similar decreasing foam stability pattern was reported by Ahmed and Ramanathan (29) for sorghum products. There was also a negative correlation between the amount of moisture lost during whipping and the stability of foams formed. The relationship between moisture loss and foam stability was significant ($P \leq 0.05$). As shown in Table 3, higher moisture losses during whipping resulted in lower stability of foams at all whipping times. Native glutelin was less stable and lost more moisture than treated glutelin.

Figure 3 shows the emulsifying activities of native and pronase-hydrolyzed glutelin. Emulsion activity, expressed as the absorbance of diluted emulsion at 500 nm (16), was significantly higher ($P \leq 0.05$) for the 60-min digest than for native glutelin and glutelin treated for 30 min at all measuring times. The increase in emulsifying activity of the hydrolyzed glutelin may be due to induction of an amphiphilic nature by pronase.

After homogenization, the absorbance of emulsions decreased either gradually or rapidly depending on the sample treatment. The emulsifying activity of protein depends on the area of interface-stabilized dispersed oil droplets; therefore, it is a function of the oil volume fraction of emulsion, protein concentration, and type of equipment used to produce the emulsion (16,30). The decrease in absorbance of emulsion within the tested time range therefore suggests that there was a decrease in the amount of oil dispersed during homogenization of samples.

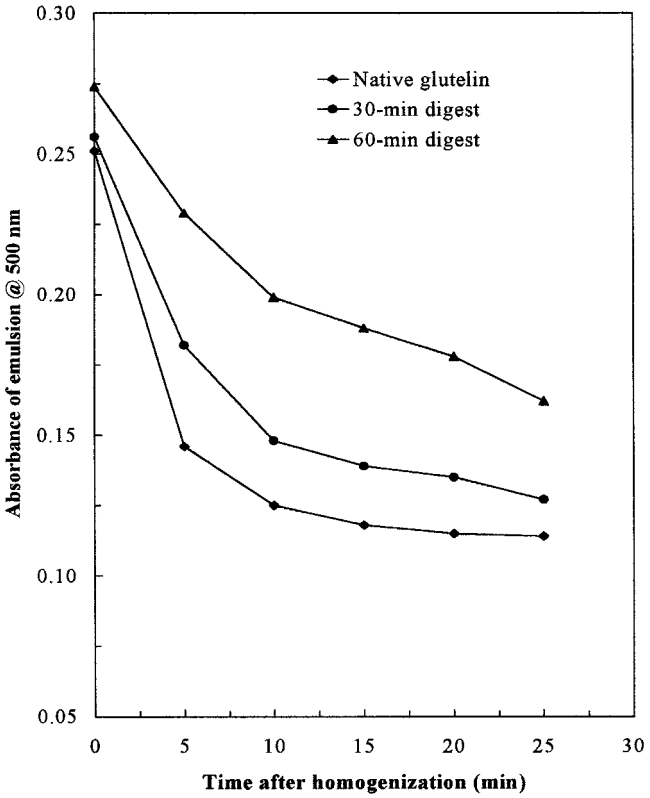

FIG. 3. Emulsion activity of native and pronase-hydrolyzed glutelin, evaluated according to the turbidimetric method of Pearce and Kinsella (16).

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