Use of Enzymes for the Separation of Protein from Rice Flour

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ABSTRACT

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When rice flour was treated with heat stable α-amylases, the effectiveness of protein separation increased with increased temperature. Depending on the enzyme, treatment at 90°C for 45 min resulted in protein contents of 47–65% for the insoluble fraction. Prior gelatinization enhanced the effectiveness of the enzyme reaction but was undesirable because the increased viscosity and gelation could cause difficulties in the processing operation. Follow-up treatment with other carbohydrate-hydrolyzing enzymes, such as glucoamylase, cellulase, and hemicellulase further increased the protein content up to 76% for the insoluble fraction. The subunit structure of the isolated proteins, based on electrophoretic analysis, remained practically unchanged after the treatment. The limited solubility and emulsion activity of rice protein were also unchanged.

Rice is the number one food crop in the world. It is also nutritious and hypoallergenic, which makes rice products desirable food ingredients. Recently, methods have been developed to separate, modify, and utilize rice and its components for value-added products. Morita and Kiriyama (1993) partially solubilized rice flour with α-amylase to prepare a protein-rich product with >90% protein. Using rice flour obtained at 70–35% milling, the treatment was conducted at 97°C for 2 hr, and the product was obtained by filtering off the solubles with boiling water. Similar enzymatic methods have been used to produce protein-enriched rice flours (Hansen et al 1981, Chang et al 1986, Griffin et al 1989). Protein content of the products varied depending on the rice flour and the processing conditions.

Of the rice components, rice protein is valuable because it is hypoallergenic and rich in the essential amino acid lysine. The lysine content of 3–4% (≈50% higher than that of wheat) is among the highest in cereal proteins. However, the protein fraction, which is 6–9% of milled rice, compared to starch at 80–85%, is generally a by-product during the starch conversion. Only limited information is available regarding its separation and characterization. There is a need to study and improve the quality and quantity of rice protein as a by-product in the processing of starch.

The present study focused on the separation of rice protein during treatment of rice flour with carbohydrate-hydrolyzing enzymes. The products were characterized by chemical, physical, and functional properties.

MATERIALS AND METHODS

Materials

Long-grain rice flour RL100 (composition shown in Table I) was donated by Rivland Partnership (Houston, TX). Particle-size distributions of the flour are: 95–100% (going through 50 mesh) 55–75% (100 mesh), and 45–60% (140 mesh). Rice Protein Concentrate K, a reference rice protein sample (≈50% protein and 39% carbohydrate), was obtained from California Natural Products (Lathrop, CA). The carbohydrate-hydrolyzing enzymes Taka-Therm L340, Diazyme L200, Cellulase TR, Hemicellulase Concentrate, and Cellulase AC were obtained from Solvay Enzymes (Elkhart, IN), and Termamyl 120L was obtained from Novo Nordisk (Danbury, CT). The proteolytic enzymes Papain 300 and Protease 2A were obtained from International Enzymes (Troy, VA), Pronase E was obtained from Sigma Chemical (St. Louis, MO), and APL-440 was obtained from Solvay Enzymes (Elkhart, IN). All other chemicals used were reagent-grade.

α-Amylase Treatment

Rice flour samples (40 g) were slurried with ≈150 mL of deionized water, adjusted to pH 6.5, mixed with calcium chloride (0.111 g), and diluted to the final processing weight of 200 g with deionized water. The mixture was mechanically stirred in a flask placed on a hot plate with automatic temperature control. Immediately before processing, 0.1% E-S (enzyme-substrate) Termamyl 120L or 0.15% E-S Taka-Therm L340 was added. The mixture was heated quickly in ≈2 min to the desired temperature. In one experiment using the Taka-Therm L340, the flour slurry was gelatinized at 90°C for 30 min before the addition of the enzyme. For all experiments, after incubation with the enzyme for the desired duration, the mixture was adjusted to pH 4 and boiled for 5 min to inactivate the α-amylase. The mixture was then ready for product processing and analysis or for additional enzyme treatment.

For product processing and analysis, the mixture adjusted to pH 4 was diluted with water to 600 mL and then centrifuged at 9,000 × *g* for 15 min at 4°C to separate the residue and the supernatant. The residue, after being washed three times with 600 mL of water at pH 4 and then dispersed in water again, was recovered by lyophilization as the protein-rich fraction.

Additional Enzyme Treatment

After enzyme deactivation at the end of the α-amylase treatment, the mixture was adjusted to pH 4 and 55°C. Selected carbohydrate-hydrolyzing enzymes were then added to the mixture at E-S ratios of: 0.18% for Diazyme L200, 0.5% for Cellulase TR, 0.2% for Cellulase AC, and 0.5% for Hemicellulase. After incubation for 2 hr, the reaction products were processed and analyzed as described earlier.

TABLE I
Composition of Rice Flour

Composition	g/100 g of flour
Protein (N × 5.95)[a]	8.1 ± 0.8
Carbohydrate[a]	80.9 ± 1.9
Moisture[a]	10.1 ± 1.8
Crude Fiber[b]	0.6
Fat[b]	0.4
Ash[b]	0.6

[a] Laboratory-analyzed data with means of triplicates ± standard deviation.
[b] Data from the manufacturer.

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Proteolysis

Proteolytic enzymes (0.05%, E-S), APL-440, Papain 300, Pronase E, or Protease 2A were added to a dispersion of protein-rich fraction (4 g) in deionized water (80 mL). The mixture was incubated for 4 hr at 50°C and neutral pH, before the enzyme was inactivated by boiling for 5 min. After cooling to room temperature and adjusting to pH 4, the supernatant and residue were separated by centrifugation (9,000 × *g*), washed, and recovered by lyophilization.

Protein Analysis

Nitrogen content of the sample was determined using a nitrogen analyzer (FP-428, LECO Corp., St. Joseph, MI). Protein content in rice products was calculated as N × 5.95. Solubility test for the protein-rich fraction was conducted using a 0.1% dispersion of the sample adjusted to pH 2–9. After stirring for 30 min, the supernatant was analyzed for dissolved protein (Protein Microassay Method 3, Bio-Rad Labs, Hercules, CA). Emulsification activity index (EAI), expressed as interfacial area/unit weight protein (m^2/g), was assessed by the turbidimetric method of Pearce and Kinsella (1978). Subunits of the protein were analyzed by SDS-PAGE according to Laemmli (1970). The Coomassie blue R-250 stained gels (4–20% gradient) were analyzed by densitometry and image analysis (GDS2000 Gel Documentation System, UVP, San Gabriel, CA).

Carbohydrate Analysis

Carbohydrate content was measured using a glucose standard according to Dubois et al (1956). When referring to starch, the value was multiplied by 0.9 to convert to a starch base (McCready et al 1950). Liquefied starch and carbohydrate were calculated as the ratio of carbohydrate found in the liquefied fraction to the total carbohydrate of the flour. Reducing sugar was determined using a glucose standard (AOAC 1990). Dextrose equivalent (DE) of a converted starch was calculated as a percentage of dextrose to the total dry substance.

Statistical Analysis

The samples were analyzed in triplicate. Data were assessed by the one way analysis of variance (ANOVA) and Duncan's multiple range test ($P < 0.05$) (software ver. 6.10, SAS Institute Inc., Cary NC).

RESULTS AND DISCUSSION

Effect of Temperature

The α-amylases used are heat-stable enzymes with optimum reaction temperatures up to 95°C. The effect of temperature using Termamyl 120L on the separation of the rice protein in terms of product composition is shown in Table II. After treatment with the enzyme for 1 hr at 50°C, the protein content of the protein-rich fraction was 16.2%, increased from the 9.0% (dwb) of the intact flour. Treatment at temperatures ≥70°C were more effective. The effectiveness of processing at 90°C was particularly pronounced, with protein content of the protein-rich fraction up to 65%.

The effectiveness of protein separation by the α-amylase treatment at high temperatures, and the lack of it at low temperatures are believed to be related to starch gelatinization (Brooks and Griffin 1987). Gelatinized starch is more accessible to the enzyme. As rice starch gelatinizes at 70–75°C, a greater concentration of gelatinized starch would be available in or above this temperature range, resulting in more effective separation of the rice flour.

Effect of Prior Gelatinization

The Taka-Therm L340 treatment was conducted with and without prior gelatinization of the rice flour. The data are shown in Table III (Treatments B[c] and B, respectively). With prior gelatinization, the protein-rich fraction had a slightly higher protein content. However, the treatment without prior gelatinization was advantageous because it was a one-step operation that saved time and simplified the process by avoiding the formation of a hard-to-stir gelling mass from the gelatinization. Therefore, unless otherwise indicated, all enzymatic treatment of the rice flour was conducted without prior gelatinization.

TABLE II
Effect of Temperature and Reaction Time on Product Composition for the Treatment of Rice Flour with Termamyl 120L

Temperature (°C)	Time (min)	Protein[a] (%)	Carbohydrate[a] (%)
50	15	11.7h	58.0i
	30	12.2h	54.2k
	60	16.2g	53.4k
70	15	18.3f	55.9j
	30	24.4e	53.5k
90	15	45.8d	39.8l
	30	64.0c	24.1m
	45	65.5b	19.4n

[a] Values are dwb. Means of three determinations. Values followed by the same letter are not significantly different ($P < 0.05$).

TABLE III
Composition of Protein-Rich Fraction in the Treatment of Rice Flour with Different Starch-Hydrolyzing Enzymes

Treatment[a]	Protein[b] (%)	Carbohydrate[b] (%)
(A)	65.5g	19.4q
(B)	46.9k	38.6l
(B)[c]	51.1j	35.1m
(B)[c] + Dz	56.0i	32.6n
(A) + TR	71.8f	9.5r
(A) + AC	75.6e	8.5r
(A) + HC	71.6f	8.8r
(A) + (AC & HC)	76.4e	9.2r
(B) + TR	62.2h	21.8p
(B) + AC	60.3h	29.4o
(B) + HC	55.2i	29.2o

[a] Enzyme treatments (A) and (B) with α-amylase Termamyl 120L and Taka-Therm L340, respectively, were conducted at 90°C for 45 min. In multienzyme treatments, the α-amylase process was followed by treatment with the additional enzyme DZ (Diazyme L200), TR (Cellulase TR), AC (Cellulase AC), and HC (Hemicellulose Concentrate) at 55°C for 2 hr.

[b] Values are dwb. Means of three determinations.Values followed by the same letter are not significantly different ($P < 0.05$).

[c] With prior gelatinization of the rice flour.

TABLE IV
Effect of Reaction Conditions on the Degradation and Liquefaction of the Insoluble Fraction

Treatment[a]	Temp. (°C)	Time (min)	Dextrose Equivalent	Carbohydrate Liquefied[b] (%)
(A)	50	15	4.7o	23.4m
(A)	50	30	7.8n	26.6l
(A)	50	60	11.2l	43.9k
(A)	70	15	9.6m	57.4j
(A)	70	30	12.5k	73.2g
(A)	90	15	9.7m	60.2i
(A)	90	30	13.8j	78.0f
(A)	90	45	23.5h	77.7f
(A) + TR	90	45	35.5f	79.7e
(B)	90	45	20.3i	65.4h
(B)[c]	90	45	30.1g	72.7g
(B)[c] + DZ	90	45	60.1e	77.9f

[a] Single enzyme treatments (A) and (B) with α-amylase Termamyl 120L and Taka-Therm L340, respectively, were conducted under the temperature and time conditions indicated. In multienzyme treatments, the α-amylase process was followed by treatment with the enzyme TR (Cellulase TR) or DZ (Diazyme L200) at 55°C for 2 hr.

[b] Values are dwb. Means of three determinations.Values followed by the same letter are not significantly different ($P < 0.05$).

[c] With prior gelatinization of the rice flour.

Multienzyme Treatment

The combination of α-amylase and glucoamylase is particularly effective in the hydrolysis of starch (Chen and Chang 1984, Brook and Griffin 1987). As expected, treatment with the glucoamylase Diazyme L200 after the α-amylosis with Taka-Term L340 (Treatment B^c + Dz, Table III) further enhanced the protein separation. However, the increases were relatively small. Even with a rice flour pretreated for gelatinization, the enzyme treatment produced a relatively low protein content at 56% for the protein-rich fraction.

The combined treatments with α-amylase and glucoamylase may be effective in the saccharification of starch and, therefore, useful for syrup processing, but the process is not particularly effective in the separation of protein from other components such as the insoluble fiber. To enhance the separation, therefore, enzymes such as cellulase and hemicellulase were added after the α-amylosis. The results are also shown in Table III.

With the additional enzyme treatment, the protein-rich fraction had an increase in protein and a decrease in carbohydrate. Cellulase seemed to be more effective than hemicellulase. Of the cellulases used, Cellulase AC was more effective than Cellulase TR. For protein concentration in the protein-rich fraction, the best results were achieved at a protein content of 76% with ≈9% carbohydrate using either Cellulase AC or a mixture of Cellulase AC and Hemicellulase Concentrate after the Termamyl 120L treatment. Apparently, substantial amounts of fibrous material remained attached to the protein in the insoluble fraction. The treatment with additional enzymes only partially hydrolyzed and removed the cellulose and hemicellulose components from the insoluble fraction.

Food-grade cellulase and hemicellulase systems often contain small amounts of proteolytic enzymes that could cause partial solubilization of the rice protein. However, as the protein content in the liquefied fraction remained low (<1.8%), the proteolytic activity appeared to be minimal.

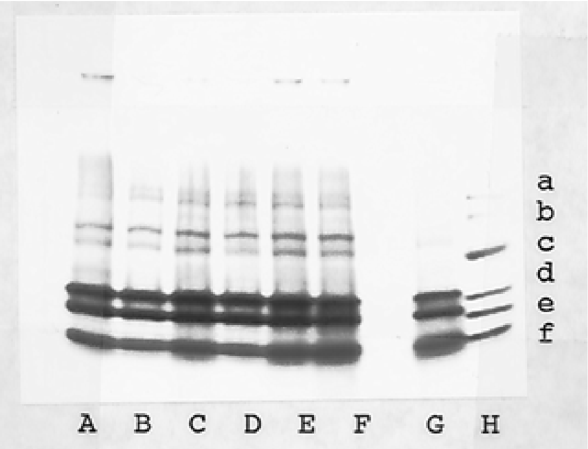

Fig. 1. Electrophoretic profiles for selected rice protein samples from treatments as shown in Table III. Column assignments are: A = B^c + DZ; B = B; C = B + TR; D = A; E = A + AC; F = A + HC & AC; G = rice protein from industrial source; H = molecular weight markers a–f (97,400; 66,200; 45,000; 31,000; 21,500; and 14,400, respectively).

Protein-Rich Fraction

Samples of the protein-rich fraction were analyzed for subunit composition. The electrophoretic profiles for selected protein-enriched samples, including one from an industrial source, are shown in Fig. 1. Subunits of the protein and the profile intensities were practically identical for all laboratory-prepared samples, indicating that the protein remained mostly intact after the treatments. Less intense were the high molecular weight subunits for the industrial sample, indicating protein degradation during industrial processing.

Rice proteins are known to have limited solubility in water. Treatment with heat-stable enzymes at temperature up to 90°C could cause denaturation and cross-linking and further decrease the solubility of the protein. As a result, proteins thus isolated remained mostly insoluble. Fig. 2 shows the pH-solubility profiles for selected samples of the protein-rich fraction, including one from an industrial source as reference. Typically, the rice proteins were more soluble in alkaline or acidic conditions with <3 μg/mL

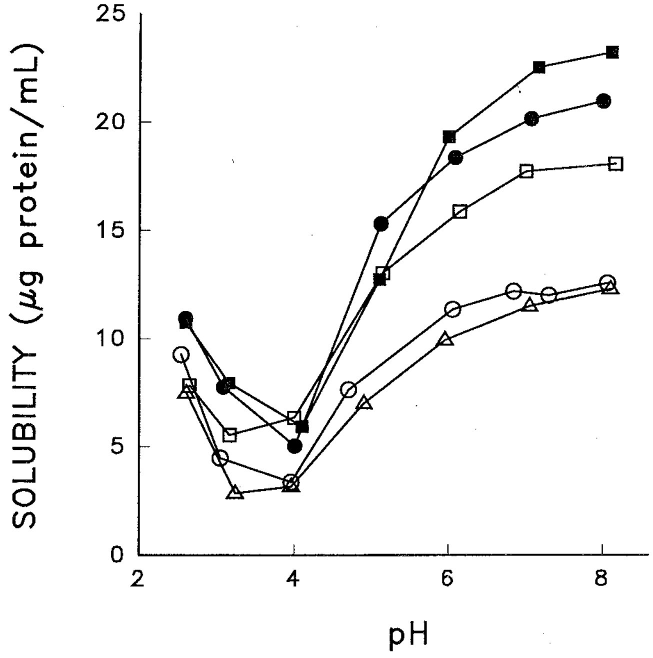

Fig. 2. Solubility of rice proteins as a function of pH. Samples of protein-rich fractions are chosen from treatments as shown in Table III. Protein from Treatment A (□); protein from Treatment A + HC (■); protein from A + AC (○); protein from Treatment A + AC & HC (●); and protein reference from industrial source (Δ).

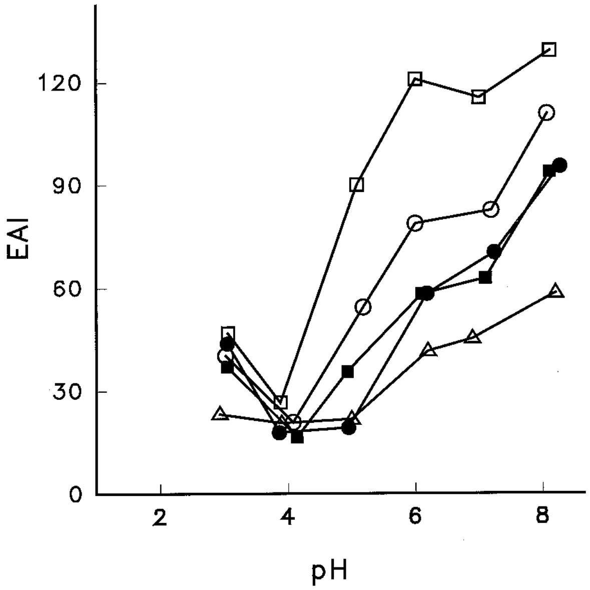

Fig. 3. Emulsifying activity index (EAI) of rice proteins as a function of pH. Samples of protein-rich fractions are chosen from treatments as shown in Table III. Protein from Treatment A (□); protein from Treatment A + HC (■); protein from A + AC (○); protein from Treatment A + AC & HC (●); and protein reference from industrial source (Δ).

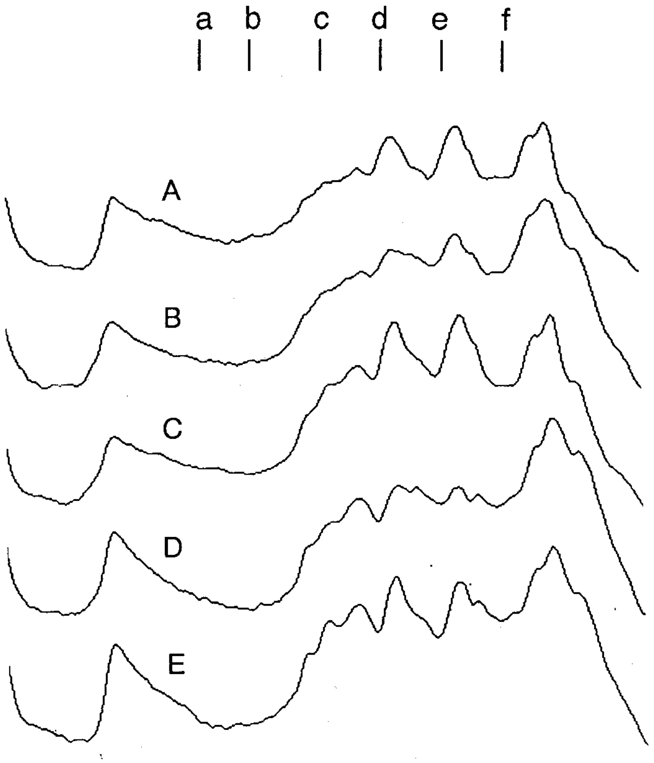

Fig. 4. Scanning electrophoretic profiles for rice protein after proteolysis with no enzyme (A), APL-440 (B), Papain 300 (C), Pronase E (D), and Protease 2A (E). The substrate was the protein-rich fraction from the treatment of rice flour with Termamyl 120L (Treatment A, Table III). Molecular weight markers a–f are: 97,400; 66,200; 45,000; 31,000; 21,500; and 14,400, respectively.

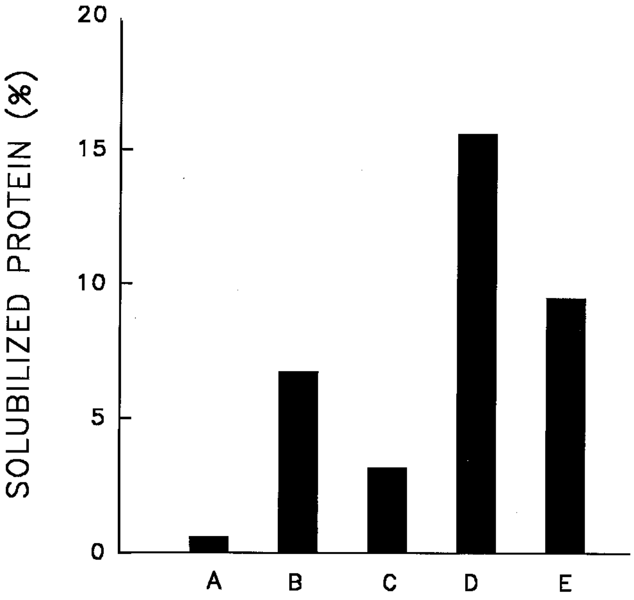

Fig. 5. Solubility of rice protein after proteolysis with no enzyme (A), APL-440 (B), Papain 300 (C), Pronase E (D), and Protease 2A (E). The substrate was the protein-rich fraction from the treatment of rice flour with Termamyl 120L (Treatment A, Table III).

solubility at the isoelectric point (pH 4.6). The profiles for EAI, as a function of pH, are shown in Fig. 3. Again, they follow the same pattern for all samples. As the ability to emulsify oil for a protein is normally closely related to solubility, the values of EAI for the samples increased with increased pH and, at a given pH, they varied only slightly among samples.

Effect of Proteolysis

The effect of proteolysis on the structure and solubility of the rice protein was also investigated. The protein-rich fraction from the Termamyl 120L treatment was analyzed by electrophoresis before and after additional treatments with various food-grade proteases. The scanning profiles of the subunits are shown in Fig. 4. The modification affected mostly subunits with molecular weights ranging from 20,000 to 45,000. With the treatment of APL-440, for instance, the intensity of bands within this range of molecular weights decreased, as compared with the control, whereas bands with molecular weights <15,000 increased. Depending on the protease, the changes ranged from small for Papain 300 to relatively more extensive for Pronase E. Accordingly, solubility of the protein increased with increased proteolysis from <1% for the untreated control to >15% for the Pronase E treatment (Fig. 5).

Liquefied Carbohydrate Fraction

The soluble fraction of the rice flour after treatment with carbohydrate-hydrolyzing enzymes contained mostly maltodextrins. The extent of carbohydrate degradation, indicated by the reducing value or DE, increased with increased hydrolysis. Table IV shows the effect of reaction conditions on starch liquefaction and degradation. At 50°C, liquefaction using the enzyme Termamyl 120L was ineffective. Less than 44% of the starch and carbohydrate was liquefied after 1 hr. Temperatures >70°C were required to achieve extensive (>50%) starch and carbohydrate liquefaction. Depending on the enzyme, temperature, and time, carbohydrate degradation was relatively extensive with DE values up to 60 and was relatively small with DE values down to 5. Normally, in the separation process, extensive starch and carbohydrate hydrolysis enhances protein isolation. However, there is a limit of ≈76% protein enrichment during starch and carbohydrate liquefaction. Notably, in the process involving glucoamylase, in spite of its markedly high level of starch degradation a DE value of 60, the protein fraction contained only 56% protein, with the balance consisting of mostly insoluble fibers.

For uses other than syrup manufacture, excessively hydrolyzed starch with DE values >15 is undesirable sometimes because of reduced food-use functionality such as the film-forming properties. As can be seen in Table IV, effective liquefaction with low degradation was achieved under various conditions. For instance, the treatment with Termamyl 120L at 90°C for 30 min produced 78% liquefied carbohydrate with a DE value of 14. Thus, in addition to the highly protein-enriched insoluble product discussed earlier, the process also generated a soluble product containing maltodextrins with relatively low DE values. A longer reaction time (45 min) for the same treatment resulted in a much higher DE value of 24 for the liquefied starch, but the same protein content at 65% for the residue.

CONCLUSIONS

Protein-enriched rice product with 65% protein can be produced by reacting regular rice flour with the α-amylase Termamyl 120L at 90°C for 30 min. Physicochemical and functional properties of the isolated protein remain practically unchanged. The treatment also liquefies ≈78% of the rice carbohydrates with limited degradation as indicated by the DE value of 14. Additional treatments with carbohydrate-hydrolyzing enzymes can raise the protein content of the protein-rich fraction to 70% and higher, but also increase degradation of the liquefied starch to DE values >15. The treatments are effective in hydrolyzing the starch component but less effective in hydrolyzing the cellulose and hemicellulose. Consequently, the insoluble fraction contains substantial amounts of insoluble fibers in addition to the protein.

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