# Characterization and Functional Properties of Rice Bran Proteins Modified by Commercial Exoproteases and Endoproteases

J.S. HAMADA

**ABSTRACT: Large portions of rice bran protein cannot be solubilized by mild solvents, but endoprotease use increases protein recovery. Bran was treated with 2 commercial proteases to achieve 8% to 9% peptide bond hydrolysis. The exo- plus endprotease was preferred to just using endopeptidases as it allowed the production of protein hydrolysates with enhanced functional properties. Solubility and emulsification activity and stability of hydrolysates produced with the protease blends were greater than that produced with endoprotease alone. These high value hydrolysates, produced from rice bran, an underutilized rice milling coproduct, are suitable for many processed foods, particularly those requiring potent solubility and emulsification at mildly acidic conditions.**

**Key Words: rice bran; proteolysis; functional properties; flavor**



## Introduction

RICE BRAN (12% TO 20% PROTEIN) MAY BE A POTENTIAL SOURCE of inexpensive high-quality protein for new food use (Saunders 1990). Defatted rice bran is typically sold as a low-cost feed but more returns can be collected for the bran by extracting its valuable protein and other highly nutritious or biologically active components from this low-value product. Rice bran contains functional foods that can be an important source of value added products for the food industry. Functional foods are health-promoting, nutritious materials from plant and microbial sources. They are components that have antimicrobial, anticarcinogenic, and other health promoting activities such as dietary fiber, vitamins, essential minerals, phytic acid, proteinase inhibitors, α-amylase inhibitors, and tannins. However, large portions of bran protein cannot be solubilized by ordinary aqueous solvents due to extensive disulfide bonding and aggregation (Hamada, 1997). Recovering this protein will add value to the underutilized rice bran and may lead to obtaining value-added ingredients for the food industry. Proteases have been used to enhance recovery of rice bran proteins from about 60% to 93% and to obtain a wide range of protein hydrolysates (Hamada 1999).

There are 2 classes of proteases, exoproteases, and endoproteases. Exoproteases split single amino acids from either end of the peptide chain. Commercial proteases are mainly endoproteases. They attack peptide bonds in the interior of the polypeptide chain producing a range of polypeptides, which differ in molecular weight, depending on the extent of hydrolysis. Flavourzyme™ has been recently introduced by Novo Nordisk (Pommer 1995) for debittering of bitter protein hydrolysates at low degrees of hydrolysis (10% to 20%) and to enhance flavor for high degrees of hydrolysis (50% or above). Flavourzyme is a mixture of endoproteases and exopeptidases. Undesirable bitter flavors can be produced when using proteases due to the formation of polypeptides of a certain length with hydrophobic peptides at the end of the polypeptide chain. Flavourzyme can cleave off these hydrophobic residues (Pommer 1995).

In this study, exoprotease was included along with endoprotease in bran treatment. The objectives of this research were (1) to determine the yield and biochemical changes of rice bran protein following treatment with a commercial endoprotease and a commercial mixture of endoprotease and exopeptidase for protein hydrolysis to less than 10% degree of hydrolysis and (2) to study the effect of this limited proteolysis using 2 protease preparations on the functional properties of the recovered protein hydrolysates.

## Results and Discussion

### Recovery and analysis of rice bran protein hydrolysates

Protein content of full fat and defatted bran was 12.9% and 15.4%, respectively. Chemical analysis of protein hydrolysates, produced by Alcalase™ and Flavourzyme is presented in Table 1. Lyophilized preparations contained 27.6% and 29.9% protein. Analysis of free amino groups in the protein hydrolysates revealed that the degree of hydrolysis (DH) values of rice bran protein hydrolysates prepared by Alcalase and Flavourzyme were 7.5% and 8.8%, respectively. Percentage of the soluble protein recovered from bran after proteolysis was 81% and 88%, respectively. This is a significant difference in the amounts of proteins obtained by the two enzyme treatments ($P \leq 0.05$). Despite lower protein recovery for Alcalase hydrolysate, both hydrolysates are suitable for many food applications. Protein hydrolysates are widely used for nutritional supplementing, functional ingredients and as flavor enhancers in foods, particularly baked goods, coffee whiteners, whipped toppings, confectionery, fillings, and fortified of soft drinks and juices (Giese 1994.). Protein hydrolysates are also used in soups, sauces, gravies, bouillon, season-

**Table 1—Chemical analysis of lyophilized protein hydrolysates[a]**

| Analysis | Alcalase | Flavourzyme |
|---|---|---|
| % Protein | 27.6 | 29.9 |
| % Protein recovery | 81.4 | 87.6 |
| % DH | 7.5 | 8.8 |
| Amide (mmol/g protein) | 0.91 | 1.02 |
| Free ammonia (mmol/g protein) | 0.013 (2.0)[b] | 0.056 (5.5)[b] |

[a] Means within each row differ significantly at 95% level of significance.
[b] Values in parenthesis are % Deamidation.

© 2000 Institute of Food Technologists

ings, snacks, and other savory applications.

The protein of defatted bran contained 1.2 millimoles of amide groups per gram. Protein hydrolysate preparations contained 0.91 and 1.02 millimole of amide groups per gram protein for Alcalase and Flavourzyme hydrolysates, respectively (Table 1). The hydrolysates varied significantly in their amide contents. This difference may be due to differences in the type and structure of proteins extracted after protease treatment. Rice bran proteins can be fractionated into albumin, globulin, prolamine, and acid-soluble glutelin based on their solubilities in water, salt, alcohol and acetic acid, respectively. These solvents extract 34%, 15%, 6%, and 11%, of the total bran proteins, respectively (Hamada 1997). Therefore, up to one-third of the total rice bran protein cannot be extracted with these solvents, that is, residue protein or glutelin. From the ammonia values of amino acid analysis of rice bran proteins, brans contain 1.00 to 1.77 millimoles of amide groups per gram protein (Juliano 1972). Glutelin and prolamine had much higher amides than the fractions of albumin and particularly globulin. Thus, it is likely that more prolamine and glutelin were solubilized by the Flavourzyme treatment than the Alcalase treatment of rice bran. Due to relatively high amide contents of hydrolysates, these polypeptides can be an excellent source of flavor enhancing ingredients for food applications after further chemical or enzymatic deamidation (Hamada and others 1998). Glutamic acid in peptides is a potent flavor enhancer as has been shown by several investigators (Kuramitsu and others 1997). Furthermore, during the determination of amide of the lyophilized hydrolysates, we found small amounts of free ammonia in both hydrolysates. Percent deamidation was 2.0% and 5.5% for Alcalase and Flavourzyme hydrolysates, respectively. About 2% deamidation or less of amide groups are common with most food protein and protein hydrolysate samples (Hamada 1991).

### Size exclusion HPLC of extracted rice bran protein hydrolysates

Chromatograms of the size exclusion high performance liquid chromatography (HPLC) separation of the bran protein hydrolysates, with molecular weight range of 1 to 150 kDa, are presented in Fig. 1. Protein hydrolysates were fractionated into 6 major peaks for Alcalase hydrolysates and 1 major peak with a few minor shoulders for Flavourzyme hydrolysates. To compare the chromatograms of these 2 enzymes, this peak was divided into 6 fractions, based on the elution volumes of Alcalase hydrolysates.

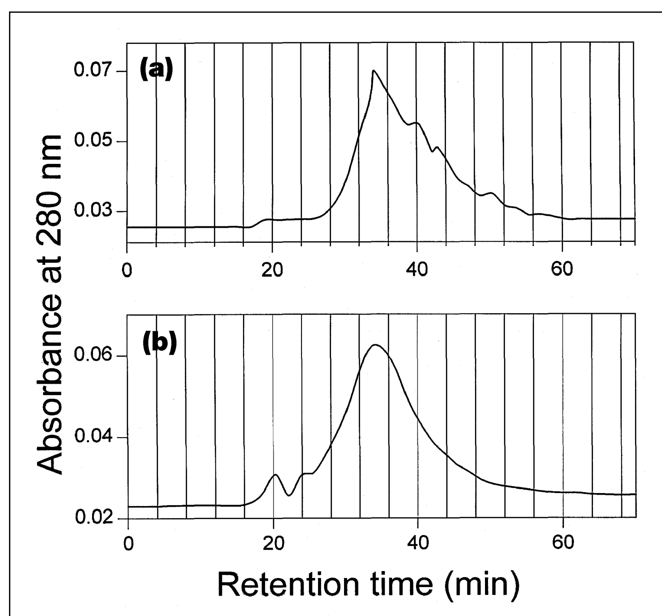


**Fig. 1—Size exclusion HPLC of rice bran protein hydrolysates produced with Alcalase (A) and Flavourzyme (B) at a flow rate of 2.0 mL/min using 2 mg protein loads.**

Protein proportions of the peaks or fractions of these chromatograms, based on both % area (absorbance at 280 nm) and on the protein content determined using bicinchoninic acid (BCA) reagent for each peak or fraction, are shown in Table 2. Flavourzyme hydrolysate had larger polypeptides than Alcalase hydrolysate eluted from the HPLC gel column in the first three fractions. Alcalase protein hydrolysates did not have any polypeptides with a 90 to 150 kDa molecular weight range. Based on the protein content determined using BCA reagent, 68% and 65% of protein hydrolysates, produced by treatment with Alcalase and Flavourzyme, were medium-sized polypeptides ranging in molecular weights from 10 to 90 kDa, respectively (Table 2). Small peptides of 1 to 3 kDa were 5.3% and 3.8% of the protein hydrolysate, prepared with Alcalase and Flavourzyme, respectively (Table 2). The proportion of the peptides with molecular weights smaller than 1 kDa increased more than 2-fold when Flavourzyme was used instead of Alcalase to hydrolyze bran proteins. They were 10.6% and 5.0% of the total protein hydrolysates, respectively.

Rice bran protein hydrolysates had a broad range of medium-sized peptides with generally moderate amounts of high and low-molecular-weight polypeptides and peptides. Typical protein hydrolysates produced with endoproteases consist mainly of small portions of proteins or large polypeptides and great portions of small- and medium-size peptide fractions (Adler-Nissen 1986). Rice bran protein is highly aggregated through hydrogen bonding and/or extensive disulfide bonding resulting in large molecular weights up to the millions (Hamada 1997). During hydrolysis of rice bran protein the protein is opened, and disulfide bonding and extensive aggregation are disrupted. Thus, proteolysis of rice bran proteins is expected to be initially slow since a good portion of bran protein is not easily solubilized. It is likely, therefore, that accessibility of protein substrate for protease accelerates after the protein is released from the bran. This may have caused an even molecular weight distribution and in both cases fragmented the protein to intermediate-molecular-weight polypeptides before further hydrolysis to smaller-molecular-weight peptides with the progress of a hydrolysis.

However, the origin of protease influenced the distribution of high- and low-molecular-weight components in protein hydrolysates prepared with the two proteases used in this study. Flavourzyme hydrolysates had more large polypeptides (90 to > 150 kDa) and small peptides or amino acids (< 1 kDa) than Alcalase hydrolysates. Differences in their contents of low- or high-molecular-weight peptides may be due to the mode of action of each protease. Endoproteases hydrolyze proteins from the interior of the protein and outward. This results in a mixture of polypeptides of varying chain lengths (Pommer 1995). Alcalase protein hydrolysates did not have much of high- or low-molecular-weight polypeptides because all the 8% of peptide bonds hydrolyzed were within the peptide chain. On the other hand, during proteolysis by Flavourzyme, a mixture of endoprotease and exopeptidase, its endo component develops smaller proteins and polypeptides. The combination of polypeptides being formed by the endo component and their subsequent breakdown into amino acids by the exo component resulted in a much greater number of low-molecular-weight components, for example, with less than 1 kDa. Most flavor problems have been attributed to small peptides that are likely to have bitterness (Adler-Nissen 1986). However, this is not a critical issue here since Flavourzyme is declared to be able to cleave off bitter peptides of protein hydrolysates (Pommer 1995). Further, low concentrations of small pep-

**Table 2—Molecular weight distribution of rice bran protein hydrolysates by size exclusion chromatography** [a]

| Peak or fraction number | Retention time (min) | | Molecular weight range (kDa)[b] | Percent protein | | | |
|---|---|---|---|---|---|---|---|
| | | | | Alcalase hydrolysates | | Flavourzyme hydrolysates | |
| | Start | End | | %Area | Reagent[c] | %Area | Reagent[c] |
| 1 | 17 | 24 | >150 | 3.0 | 4.5 | 6.1 | 5.6 |
| 2 | 24 | 27 | 150-90 | 0.0 | 0.0 | 1.7 | 1.6 |
| 3 | 27 | 34 | 90-50 | 31.3 | 30.2 | 36.5 | 31.2 |
| 4 | 34 | 41 | 50-10 | 39.9 | 37.6 | 38.5 | 32.9 |
| 5 | 41 | 46 | 10-3 | 20.1 | 17.5 | 15.0 | 14.5 |
| 6 | 46 | 53 | 3-1 | 5.7 | 5.3 | 2.3 | 3.8 |
| None | 53 | 66 | <1 | ND[b] | 5.0 | ND[d] | 10.6 |

[a] Chromatograms are presented in Fig. 1.
[b] Linear regression equation for the relationship between molecular weight and elution was log $M_r = 6.965 - 1.773 * V_e/V_o$.
[c] Method of Lowry and others (1951)
[d] ND = not determined, outside column fractionation range.

tides and increased proportions of the large- to medium-sized peptides during protein hydrolysis may have a significant impact on the functional properties and the flavor of protein hydrolysates. Adler-Nissen and Olsen (1979) reported the average peptide chain length as a foremost determinant for taste and the functional properties of protein hydrolysates including solubility, foaming and emulsification.

## Anion exchange separation of extracted rice bran protein hydrolysates

Alcalase and Flavourzyme protein hydrolysates were separated into 12 and 10 major peaks, respectively, on a QM anion-exchange column using 0.1 *M* Tris-HCl (pH 8.0) and 0 to 0.25 *M* KCl gradient as the eluent (Fig. 2). Peaks are considered major peaks when they contain more than 3% of the injected protein, based on % area. Comparing the retention times of the peaks of both chromatograms, the peaks of Flavourzyme hydrolysate were retained longer in the column. Delay in retention times show the latter sample had more negative charges. This confirms that deamidation has taken place in Flavourzyme hydrolysate, as was concluded from the higher free ammonia concentration in this hydrolysate. Deamidation created more negative charges on the protein hydrolysate leading to better resolution and increased retention on the anion-exchange column.

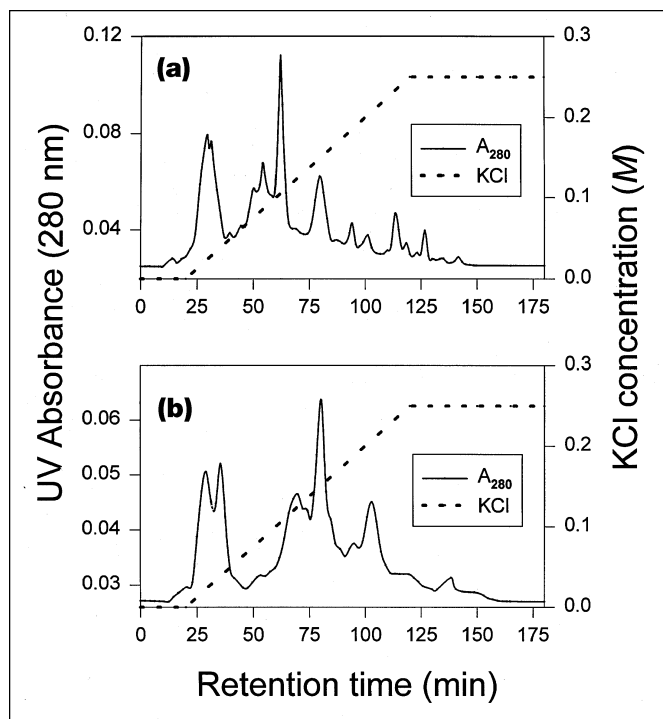


**Fig. 2—Anion exchange separation of protein hydrolysates produced with Alcalase (A) and Flavourzyme (B) using 0.1 M Tris-HCl buffer (pH 8.0) and KCl gradient at 6.0 mL/min and 25 mg protein load.**

## Rate of deamidation by Flavourzyme using 2 synthetic substrates

Alcalase did not have any deamidation activity toward either CBZ-L-glutamine (CBZ-Gln) or t-BOC-glutaminyl-L-proline (BOC-Gln-Pro). Deamidating activity of Flavourzyme, containing a mixture of exoprotease and endoprotease, were also measured using CBZ-Gln and BOC-Gln-Pro (Fig. 3). With both substrates the rate of hydrolysis was that of a second order reaction with more ammonia released from CBZ-Gln than BOC-Gln-Pro at all corresponding times. Carbobenzyloxy (CBZ) and N-tert butoxycarbonyl (BOC) is used to protect the amino group of Gln. Protecting groups are stable under a wide range of reaction conditions but it can be removed by selective reactions. Therefore, it is not expected that Flavourzyme would break off CBZ from CBZ-Gln. However, when an endoprotease is incubated with BOC-Gln-Pro, peptide bond hydrolysis of BOC-Gln-Pro is expected, yielding BOC-Gln and proline. Thus, the slower deamidation reaction of BOC-Gln-Pro suggests that Flavourzyme contain C-terminal exopeptidase activity and not a N-terminal one. BOC-Gln-Pro has to be hydrolyzed first before any deamidation can occur, which may account for slowing the deamidation reaction.

We used 25 mg Flavourzyme for 5 g rice bran protein containing 5.00 and 4.35 millimole amides, before and after protein extraction with 88% recovery, respectively. Based on Flavourzyme deamidating activity of 0.115 micromoles ammonia/30 min/0.2 mg enzyme

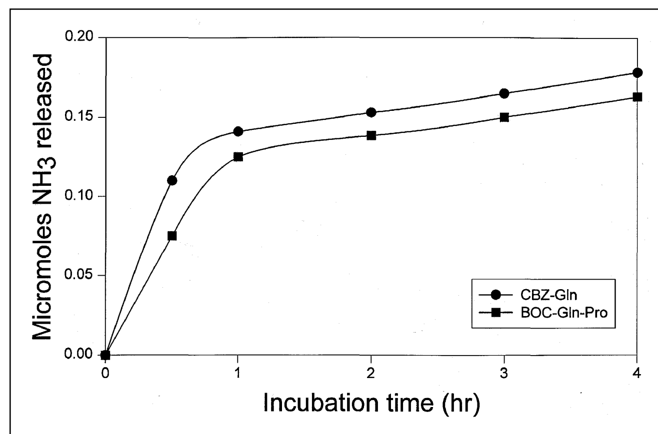


**Fig. 3—Rate of deamidation by Flavourzyme using two synthetic substrates CBZ-L-glutamine and t-BOC-L-glutaminyl-L-proline.**

**Table 3—Solubility of protein hydrolysates at three pH Values[a]**

| Protein hydrolysatee | % Solubility | | |
|---|---|---|---|
| | pH-5 | pH-7 | pH-9 |
| Alcalase | 53a | 61a | 70a |
| Flavourzyme | 56b | 73b | 86b |

[a] Means within a column with no common letter differ significantly.

(Fig. 3), 115 micromoles ammonia should have been released from CBZ-Gln after 4 h of incubation with 25 mg Flavourzyme. Releasing only 115 micromoles of ammonia from protein hydrolysate after 4 h of incubation at 50 °C would have been 2.7% deamidation of protein hydrolysate. This is much less than the actual deamidation occurred during protein hydrolysis using 25 mg Flavourzyme for 5 g protein. The viable explanation for this discrepancy is that a good portion of the C-terminal glutamine or C-terminal asparagine is attacked by exopeptidase component in Flavourzyme immediately after being released by peptide bond hydrolysis before the enzyme starts another cycle. In such a case, the substrate concentration is so small that it will not decrease the velocity of the reaction as it proceeds for long period of times.

### Protein solubility of extracted rice bran protein hydrolysates

The solubilities of the protein hydrolysates at pH 5, 7, and 9 are presented in Table 3. Solubility of Flavourzyme hydrolysate was greater than solubility of Alcalase hydrolysate at pH values 5, 7, and 9. Early studies of Puski (1975) showed that solubility increased with increasing protein hydrolysis. Nonetheless, increased solubility of the Flavourzyme hydrolysate was surprising since their DH values were close. This difference in solubility of the 2 hydrolysates may reflect different changes in protein conformation due to differences in the mode of actions of the proteases during protein hydrolysis. As indicated above, Flavourzyme hydrolysate contained more of the soluble high-molecular-weight polypeptides and small peptides than Alcalase hydrolysate. Based on difference in amide content between the two hydrolysates, this soluble fraction may have contained substantial amounts of the soluble high-molecular-weight prolamine and glutelin fractions. Adler-Nissen (1986) attributed a considerable decrease in the protein solubility index of a heat-treated 6% DH soy protein hydrolysate to the disappearance of a soluble high-molecular-weight aggregated fractions that consisted of substantial amounts of 2S globulins. Also, the small level of deamidation may have played a role in this regard. Deamidation increases the negative charge of the hydrolysates and disrupts hydrophobic and hydrogen bonds. Motoki and others (1986) and Hamada and Marshall (1989) showed that deamidation of proteins or protein hydrolysates improves solubility at mildly acidic pH such as pH 5.

### Emulsification properties of rice bran protein hydrolysates

Emulsifying properties of the rice bran protein hydrolysates at pH 5, 7 and 9 are presented in Table 4. Emulsifying activity (EA) of hydrolysates was not significantly different at pH 9. Protein hydrolysate prepared by Flavourzyme exhibited greater EA than hydrolysate prepared by Alcalase at pH 5 and pH 7. At pH 5, EA of Flavourzyme hydrolysate was smaller than that of both casein and BSA. However, EA of hydrolysates prepared with Flavourzyme was similar to both casein and BSA at pH 7. Emulsion stability (ES) of protein hydrolysates is also shown in Table 4. As emulsifying activity data show, emulsions obtained from protein

**Table 4—Emulsifying properties of protein hydrolysate and two reference proteins[a]**

| | Emulsifying activity | | | Emulsion stability [b] | | |
|---|---|---|---|---|---|---|
| Sample | pH 5 | pH 7 | pH 9 | pH 5 | pH 7 | pH 9 |
| Alcalase hydrolysate | 0.10a | 0.22a | 0.48a | 0.025b | 0.046c | 0.093d |
| Flavourzyme hydrolysate | 0.20b | 0.43b | 0.51a | 0.016a | 0.041b | 0.069c |
| Casein | 0.28c | 0.38b | 0.97b | 0.015a | 0.041b | 0.054b |
| Bovine serum albumin | 0.37d | 0.45b | 0.55a | 0.043c | 0.029a | 0.042a |

[a] Means within a column with no common letter differe significantly.
[b] Emulsion stability was expressed as decline in absorbance in 20 min.

hydrolysate prepared by Flavourzyme were more stable than emulsions prepared from hydrolysate prepared by Alcalase. ES of Flavourzyme hydrolysate was higher than ES of Alcalase hydrolysate at pH 5, 7, and pH 9. ES of Flavourzyme hydrolysates was comparable to that of casein and much higher than BSA at pH 5. ES of Flavourzyme hydrolysate at pH 7.0 was similar to the ES of casein but was less than ES of BSA at pH 7.

The use of protease here not only was effective in increasing protein solubility, and, hence recovery from bran but it enhanced emulsifying properties of extracted proteins as well. Contrary to emulsification data of rice bran protein hydrolysates, emulsifying properties of protein isolate prepared from rice bran was much lower than those of BSA (Wang and others 1999). Emulsifying activity and stability of BSA using corn oil to prepare emulsions were 3 and 4 times higher than those of the protein isolate. Limited hydrolysis of proteins increases emulsifying capacity and emulsion stability (Puski 1975) but the 2-fold increase in EA of Flavourzyme hydrolysates compared with Alcalase hydrolysates at pH 5 and 7 and similar effect of proteolysis on emulsion stability can only be explained by the small deamidation concurrent with proteolysis. Deamidation improves emulsifying activity and emulsion stability (Kato and others 1987; Hamada and Marshall 1989).

### Food applications of rice bran protein hydrolysates

Treatment of rice bran by a mixture of exoprotease and endoprotease allowed the generation of protein hydrolysates that possessed appropriate peptide chain length and functional properties from rice bran. It also caused deamidation that was responsible for dramatic improvement in the solubility and other functional properties of hydrolysates under mildly acidic conditions. Improving solubility and emulsifying properties of protein hydrolysates may enhance their use as functional ingredients in many foods, including beverages, pourable and nonpourable dressings, coffee whiteners, whipped toppings, frozen desserts, confections, baked goods and meat. Furthermore, glutamic acid in peptides is a potent flavor enhancer as has been shown by several investigators (Kuramitsu and others 1997). Therefore, deamidation coupled with debittering of small peptides by Flavourzyme may have dramatic effect on the flavor of hydrolysates. It appears that these protein hydrolysates can be used as value-added ingredients in many foods. Applications in foods not only entail nutritional supplementing but also flavor enhancement of many foods particularly meat products, ready-to-eat meals, soups, sauces, gravies, and other savory items. Accordingly, production of these protein hydrolysates from rice bran can be used as value-added ingredients in many foods. This can lead to new, high value-added products from an underutilized by-product of the rice industry.

## Conclusions

SMALL LEVEL OF DEAMIDATION COUPLED WITH DEBITTERING of small peptides may have a dramatic effect on the flavor of these rice bran protein hydrolysates. We conclude that these high-value protein hydrolysates, produced from rice bran, an underutilized coproduct of the rice milling industry, can be used as value-added ingredients in many foods. They are suitable for a broad range of industrial food applications not only as nutritional supplement and flavor enhancers but also as ingredients for those products requiring potent solubility and emulsification at mildly acidic conditions.

## Materials and Methods

### Materials

CBZ-L-glutamine, blue dextran, protein markers, technical bovine milk casein, and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and t-BOC-L-glutaminyl-L-proline from Peptides International (Louisville, Ky., U.S.A.). Other chemicals were either HPLC reagent grades or the highest purity available. Alcalase 2.4 L and Flavourzyme were obtained from Novo Nordisk Biochem North America, Inc., Franklinton, N.C., U.S.A. Alcalase is from *Bacillus subtilis* and Flavourzyme is a fungal protease preparation produced by *Aspergillus oryzae.* Both are generally recognized as safe (GRAS), according to the manufacturer, Novo Nordisk.

### Protein hydrolysis

Defatted rice bran was obtained from the kernels of Bengal variety by milling and defatting with ethyl ether according to the methods of Hamada (1997). The method of preparing protein hydrolysates from rice bran was previously described (Hamada 1999). Rice bran suspension (5.0 g proteins in 250 mL water) was heated to 50 °C, the pH adjusted to 8.0 and 0.025 g Alcalase 2.4 L or Flavourzyme added for proteolysis. The extent of hydrolysis was controlled to less than 10% degree of hydrolysis (DH) by the pH-stat titration, using an Auto Titrator (Radiometer A/S, Copenhagen). The protease was inactivated by a 10-min heat treatment at 85 °C. After proteolysis, the suspensions of rice brans were homogenized in a 1-L Sorval Omni Mixer jar (Omni Intl., Waterbury, Conn., U.S.A.) using 20-mm sawtooth blade assemblies at 5,000 rpm and 20 ° C for 15 min. Solubilized protein was recovered after three runs of centrifugation at 5,000 *x g* and 20 °C for 20 min. Protein in the bran was washed with 100 mL water during the second and third centrifugation. Combined supernatant solutions were lyophilized and the bran air-dried. The percent protein recovery was determined in replicates and calculated as the ratio of protein extracted from bran to the total protein of bran.

### Chemical analyses of bran and protein hydrolysates

Protein content of the rice brans, before and after protease treatment, and lyophilized hydrolysates was determined by the combustion method using aLeco FP-428 Nitrogen Analyzer (Leco Corporation, St. Joseph, Mich., U.S.A.). The nitrogen conversion factor for proteins and protein hydrolysates was 5.95 (Juliano 1972). The protein contents of protein hydrolysate solutions were determined by the macro-method of Lowry *et al.* (1951) using BCA protein assay reagents (Pierce Chemical Co., Rockford, Ill., U.S.A.). Protein hydrolysates with known protein contents were used as standards. DH values of protein hydrolysates were determined by analyzing free amino groups (Adler-Nissen 1979). Amide contents of protein hydrolysates were measured by determining the ammonia released by the sample after total amide hydrolysis with 2 N HCl at 100 °C, according to the method of Wilcox (1967). Ammonia was measured with an Orion ammonia electrode (model 95-10) connected to an Ion Analyzer model EA 940 (Orion Research Inc., Cambridge, Mass., U.S.A.). In this determination, a correction for free ammonia was applied. Deamidation was determined in replicates and calculated as the ratio of free ammonia to the ammonia released by the total amides of a sample.

### HPLC analyses

Delta Prep 3000 (Waters Corp., Milford, Mass., U.S.A.) was used for anion exchange fractionation and size exclusion analysis. Samples were filtered through a 0.45 µm Millex-HV Filter (Millipore, Bedford, Mass., U.S.A.) before injection. Elution was monitored at 280 nm by a Model 481 Lambda-Max spectrophotometer detector. Data analysis was obtained with PC-based Maxima 810 chromatography software. A 20-mm ID x 30 cm Shodex "Protein WS-2003" steel column packed with a bonded diol-coated silica gel (Waters Corp.) was used for size exclusion HPLC analysis of the protein hydrolysates. Fractionation range of this column was 150-1 kDa. Two mg protein was injected and the flow rate of eluent, Tris-HCl buffer (pH 8.5), was 2.0 mL/min. Blue dextran and protein markers were used to calibrate the HPLC column. Molecular weights of standard proteins were 6.5-150 kDa. Ion exchange separation of protein hydrolysates was done on a steel column (25 mm ID x 30 cm) packed with a quaternary methylamine (QM) anion exchange resin (Accell Plus™ QMA, Waters Corp.). The protein load was 25 mg proteins in 1.0 mL 0.02 M Tris-HCl buffer, pH 8.0. Elution by 0.1 M Tris-HCl buffer, pH 8.0 and 0-0.25 M KCl (from 20 to 120 min) was completed in < 3 hr at a flow rate of 6 mL/min.

### Measurement of protease deamidation using synthetic substrates

A 0.1-mL aliquot of the protease solution containing 0.2 mg of enzyme was added to 2 mL of substrate (0.015 mg CBZ-L-glutamine or t-BOC-L-glutaminyl-L-proline) in 0.05 M phosphate buffer, pH 8.0. Reaction mixtures were incubated at 50 °C for 0.5, 1, 2, 3 and 4 hr. The ammonia contents of the reaction mixtures and controls were measured as described above with the Orion Ion Analyzer. The extent of deamidation was expressed as the ammonia released enzymatically in time specified.

### Protein solubility

Protein hydrolysates (1.2%, w/v) were homogenized in 0.2 M NaCl using an Omni mixer (Ivan Sorvall Inc., Norwalk, CT) at 4,000 rpm for 1 min at 25 °C. The pH of a 5-mL aliquot was adjusted to 5, 7 or 9 with either 0.2 M NaOH or 0.2 M HCl. Then 0.2 M NaCl was added to a volume of 6 mL. Protein hydrolysate solutions or dispersions were stirred for 45 min and centrifuged at 2000 x g for 10 min at 25 °C. Protein concentration in both the supernatant and the precipitate (dissolved in 0.01 M NaOH) was determined using the BCA reagent according to Hamada and Marshall (1989).

### Emulsifying properties

Emulsifying activity was determined by the method of Pearce and Kinsella (1978). Emulsions were prepared by homogenizing 5 mL of 0.5% protein hydrolysate dispersions in

0.1 M NaCl, pH 5, 7 or 9, and 5 mL corn oil aliquot in an Omni mixer at 15,000 rpm for 30 sec at 20 °C. Emulsions (0.2 mL) were diluted to 1/1000 using 0.1% sodium dodecyl sulfate (SDS) and the absorbance at 500 nm was determined. Emulsion stability was determined at pH 5, 7 and 9 by the method of Pearce and Kinsella (1978). Emulsion samples taken initially, diluted and read were considered the 0 time samples. More samples were taken from the bottom of the container (0.2 mL) 5 and 10 min after emulsion formation and in three 10-min intervals, diluted to 1/1000 with 0.1% SDS and the absorbance was determined at 500 nm.

### Statistical analysis

Multifactor analysis of variance of variables, determined in duplicate, was performed using Statgraphics Plus, a software package from Statgraphics Corp. (Rockville, Md., U.S.A.).

## References


Adler-Nissen J. 1979. Determination of the degree of hydrolysis of food proteins by trinitrobenzenesulfonic acid. J. Agric. Food Chem. 27: 1258-1262.

Adler-Nissen J. (Ed.). 1986. Enzymatic Hydrolysis of Food Proteins, London and New York: Elsevier Applied Science Publishers. p. 110-186.

Adler-Nissen J, Olsen HS. 1979. The influence of peptide chain length on taste and functional properties of enzymatically modified soy protein. ACS Symp. Ser. 92: 125-146.

Giese J. 1994. Proteins as ingredients: Types, functions, applications. Food Technol. 48(10): 50-60.

Hamada JS. 1991. Peptidoglutaminase deamidation of proteins and protein hydrolysates for improved food use. J. Am. Oil Chem. Soc. 68:459-462.

Hamada JS. 1997. Characterization of protein fractions of rice bran to devise effective methods of protein solubilization. Cereal Chem. 74: 662-668.

Hamada JS. 1999. Use of proteases to enhance solubilization of rice bran proteins. J. Food Biochem. 23: 307-321.

Hamada JS, Marshall WE. 1989. Preparation and functional properties of enzymatically deamidated soy proteins. J. Food Sci. 54:598-591.

Hamada JS, Spanier AM, Bland JM, Diack M. 1998. Preparative separation of value-added peptides from rice bran proteins by high performance liquid chromatography. J. Chromatogr. A. 827: 319-327.

Juliano B. 1972. The rice caryopsis and its composition. In: Hudson DF, editor. Rice Chemistry and Technology. St. Paul, Minn.: American Association of Cereal Chemists. p 16.

Kato A, Tanaka A, Lee Y, Matsudomi N, Kobayashi K. 1987. Effects of deamidation with chymotrypsin at pH 10 on the functional properties of proteins. J. Agric. Food Chem. 35: 285-288.

Kuramitsu R, Takata T, Kamasaka K, Funkuma M, Nakamura H, Okai H. 1997. Role of ionic tastes in food materials. In: Spanier AM, Tamura M, Okai H, Mills O, editors. Chemistry of Novel Foods. Carol Stream, Ill.: Allured Publishing. p 19.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 267-275.

Motoki M, Seguro K, Nio N, Takinami K. 1986. Glutamine-specific deamidation of á$_{s1}$-casein by transglutaminase. Agric. Biol. Chem. 50: 3025-3030.

Pearce KN, Kinsella JE. 1978. Emulsifying properties of proteins: Evaluation of a turbidimetric technique. J. Agric. Food Chem. 26: 716-723.

Pommer K. 1995. New proteolytic enzymes for the production of savory ingredients. Cereal Foods World. 40: 745-748.

Puski G. 1975. Modification of functional properties of soy proteins by proteolytic enzyme treatment. Cereal Chem. 52: 655.

Saunders RM. 1990. The properties of rice bran as a foodstuff. Cereal Foods World. 35: 632-636.

Wang M, Hettiarachchy NS, Qi M, Burks W, Siebenmogen T. 1999. Preparation and functional properties of rice protein isolate. J. Agric. Food Chem. 47: 411-416.

Wilcox PE. 1967. Determination of amide residues by chemical methods. Methods Enzymol. 11: 63-76.


This paper was presented in part at the 1998 IFT Annual Meeting (June 20-24, 1998), Atlanta, Ga., U.S.A. Commercial firms are mentioned in this publication solely for providing specific information. Mention of a company is not a guarantee or warranty of its products by the U.S. Department of Agriculture nor an endorsement by the Department over products of other companies not mentioned.

*Author is with the USDA-ARS, Southern Regional Research Center, P.O. Box 19687, New Orleans, LA 70179 (E-mail: jhamada@nola.srrc.usda.gov).*