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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · DECEMBER 2003

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Perfusion Chromatography Purification of a 15 kDa Rice Prolamin

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Prolamin extracted from rice flour using 55% *n*-propanol contained protein impurities. Reverse phase high-performance liquid chromatography (HPLC) on a perfusion column R2/H was used to separate rice prolamin from other proteins in less than 5 min. Prolamin eluted as the major peak. The isolated prolamin migrated as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 4−12% Bis-Tris gel. Matrix-assisted laser desorption ionization mass spectrometry identified the rice prolamin as a 15 013 Da protein. The surface hydrophobicity (S_o) of the HPLC-separated protein fractions was measured using the hydrophobic fluorescent probe PRODAN. A comparison was made with the surface hydrophobicity (S_o) of corn prolamin and bovine serum albumin. Surface hydrophobicity values and solubility in 90% ethanol assisted in rice prolamin identification from other chromatographic peaks. The advantage of perfusion chromatography in purifying rice prolamin from other rice proteins included the reduced separation time, the speed at which the separation was carried, and the ability to regenerate the column in a short period of time and allow for more samples to be purified and separated.

KEYWORDS: Prolamin; zein; perfusion chromatography; hydrophobicity; rice proteins; MALDI-MS

INTRODUCTION

Rice (*Oryza sativa L.*) is the staple food for more than 60% of the world's population, specially in Asia (*1*). The genetic make up of rice cultivars has been completed (*2*). Rice can offer several health-enhancing properties and is appealing to food processors and consumers (*3*). Rice and rice-based ingredients also appeal to both consumers and processors due to their unique combination of taste, nutrition, texture, and physical and functional properties (*4*). In the U.S., rice, fueled by the rising interest in Asian and Latin cuisines, has become the main benefactor of America's new love of grain products (*5*).

Prolamins, which represent up to 25% of rice proteins, are membrane proteins containing 46% hydrophobic, 16% hydrophilic, 29% acidic, and 8% basic amino acid residues (*6*). Rice prolamins are 10−16 kDa in size. The electrophoretic mobility of rice prolamins in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) showed a lot of variability in the size of the proteins (*6*). Rice varieties containing two prolamin subunits as well as one single prolamin band on electrophoretic gels have been reported (*7*, *8*). Unlike prolamins from other cereals such as gluten, rice prolamins do not cause celiac diseases, they differ from other prolamins in their primary amino acid sequence, and incorporation into food is not health threatening (*9*−*11*). Rice prolamins contain a high molecular percentage of glutamine (about 22%) and essential S-containing amino acids cysteine and methionine (about 10% each) (*8*). Nonpolar amino acids such as leucine, alanine, and tryptophan, which are plentiful in rice prolamin and which possess high surrounding hydrophobicity, enhance the hydrophobicity of prolamins. Prolamins showed some antioxidative properties by inhibiting the autoxidation of linoleic, linolenic, and arachidonic acid esters (*12*).

Prolamin is soluble in aqueous alcoholic solution. Solutions of 55% *n*-propanol, 60, 70, or 90% aqueous ethanol are often used to extract prolamins from cereals (*13*, *14*). Ogawa et al. (*6*) suggested that *n*-propanol was a better extraction buffer than ethanol and addition of reducing agent such as β-mercaptoethanol in the extraction solution enhanced prolamin recovery from rice seed. However, the isolated prolamins were contaminated with some water soluble proteins. Further purification is always required in order to obtain a pure prolamin fraction. High-performance liquid chromatography (HPLC) is a widely used analytical technique for prolamin purification. Separation times as long as 50−100 min have been reported (*8*, *9*).

Perfusion chromatography technology was introduced by Afeyan et al. (*15*) and was designed with particles that radically speed access to the interior of the chromatography particles by overcoming the diffusional mass transfer limitations of conventional chromatography. Unlike conventional chromatography particles, POROS perfusion chromatography particles have large throughpores that transect the particles and short diffusive pores that branch off from the throughpores providing a large internal surface area for solute/particle interactions to occur. Because

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10.1021/jf020428c CCC: $25.00
Published on Web 10/18/2003

the length of the diffusive pores is small in comparison to the total particle diameter, the time required for sample molecules to diffuse to and from internal binding sites is very short. The speed advantage of perfusion chromatography is in the order of 10−100 times over that of conventional HPLC columns, bringing separation times for a laboratory scale size column down to 3−5 min.

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) is a pulsed ionization technique, coupled with a TOF mass analyzer that is capable of producing ions of high mass. The analyzer measures the time ions take to traverse the analyzer tube; low mass ions travel faster than high mass ions of the same mass-to-charge ratio. Mass measurement accuracy of 0.01% can be achieved up to moderately high mass (about *m*/*z* 20 000).

Structural analysis of hydrophobic proteins by conventional techniques is difficult because of their limited solubility in aqueous solvents. The same degree of difficulty applies to hydrophobic peptides. The features of MALDI-MS, which include its insensitivity to contaminants, including salts, lipids, and some detergents, and the hydrophobic character of the matrixes employed, make MALDI a viable alternative to other analytical methods for the analysis of water insoluble proteins and peptides.

In this paper, we report on a rapid method for rice prolamin separation by perfusion chromatography on a POROS column and its molecular size determination by MALDI-MS.

MATERIALS AND METHODS

Materials. Rice endosperm was purchased from a local food store. A reverse phase POROS R2/H column (4.6 mm diameter/100 mm length and 1.662 mL of column volume) and the BIOCAD SPRINT were from Perseptive Biosystem (Farmingham, MA). Trifluoroacetic acid (TFA) and *n*-propanol were obtained from Fisher (Fairlawn, NJ). Electrophoretic gels (4−12% Bis-Tris gels, catalog no. NP 0321), lithium dodecyl sulfate sample buffer (catalog no. NP 0007), molecular weight marker (catalog no. LC 5677), running buffer (catalog no. NP 0002), and staining solutions (catalog no. 46-016) were from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was obtained from Pierce Chemical Co. (Rockford, IL). Dialysis membranes (1200 MWCO) and dithiothreitol (DTT) were from Sigma Chemical Co. (St. Louis, MO). Corn prolamin was kindly provided by Dr. Nick Parris (USDA, Peoria, IL). PRODAN (6-propionyl-2-dimethylaminonaphthalene) was a product of Molecular Probes (Eugene, OR). The fluoresecence microplates were obtained from Perkin-Elmer (Shelton, CT). Reagents for the ninhydrin assay were kindly provided by Dr. Barry Starcher at the University of Texas Health Center at Tyler, TX. All other chemicals were of analytical grade.

Extraction of Prolamin from Rice Flour. Rice was ground into a flour using an electric coffee grinder. Rice flour (100 g) was defatted by extraction with 250 mL of petroleum ether at 21 °C overnight. The defatted flour was air-dried, extracted with stirring with 150 mL of 55% *n*-propanol containing 1% (w/v) of DTT for 1 h at 21 °C, and centrifuged at 4000*g* for 10 min at 21 °C. The supernatant was decanted in a container. The precipitate was extracted with another 150 mL of 55% *n*-propanol as described above. The supernatants from the alcoholic extractions were combined, *n*-propanol was removed under vacuum by rotary evaporation, and the protein solution was lyophilized. Protein concentration in the powder was determined by the ninhydrin method (*16*).

Separation of Rice Prolamin by Reverse Phase on a POROS Column. Analytical separation of rice prolamin was performed on a reverse phase POROS R2/H column. Samples of prolamin extract, 100 μL of 20 μg of rice protein mixture in water containing 0.1% TFA, were injected into a POROS R2/H column equilibrated with water containing 0.1% TFA. Solvent A was 0.1% TFA in water. Solvent B was 0.085% TFA in *n*-propanol. Separation was carried out using a BioCAD Sprint. The flow rate was 2.5 mL per min. The UV detector was set at 230 nm. One column volume (CV) was equal to 1.662 mL. Injection of 100 μL of 0.45 μm filtered sample was at 1.5 CV per min flow rate (about 2.5 mL/min). Gradient started at 0 CV and ended at 12 CV. Peaks were collected, dialyzed against distilled water, and lyophilized using a 1200 MWCO membrane, and the protein concentration in the dry powder was determined by the Dumas combustion method using a Perkin-Elmer 2410 Series II Nitrogen Analyzer.

Solubility of Protein Fractions. The solubility of the protein fractions separated by perfusion chromatography was determined by comparing the solubility of the protein fractions in 90% ethanol with some modifications (*17*). Lyophilized fractions from perfusion chromatography (10 mg protein by Dumas combustion method) separation were suspended in 1 mL of 90% aqueous ethanol and mixed for 1 min. The protein mixture was filtered through 0.22 μm, and the filtrate was separated by electrophoresis using 4−12% Bis Tris gel as described above.

Electrophoresis of Rice Protein Mixture. SDS−PAGE electrophoresis was carried out as follows. Lyophilized rice protein extract powder at 1 mg/mL was dissolved in sample buffer. Ten microliters of the protein sample was added to 25 μL of sample buffer and 65 μL of deionized distilled water following instructions from the gel's manufacturer. Electrophoretic separation was carried out using a Mini-VE electrophoresis unit (Amersham Pharmacia Biotech, Piscataway, NJ). The gel was stained using Novex Colloidal Blue.

Determination of Protein Fractions Surface Hydrophobicity. The surface hydrophobicity (S_o) of each separated protein peak was measured using the fluorescence probe PRODAN as described by Alizadeh-Pasdar and Li-Chan (*18*) with some modifications. PRODAN at a concentration of 1 mM was dissolved in absolute ethanol. Rice protein fractions at a concentration of 0.2% were dissolved in 70% ethanol. Corn prolamin (zein) at a concentration of 0.2% was also dissolved in 70% ethanol. BSA at a concentration of 0.2% was dissolved in water. PRODAN (10 μL of 1 mM) was added to 4 mL of 0.2% protein solution. The mixture was incubated for 15 min at room temperature in the dark. Two hundred fifty microliters of the mixture was transferred to a fluorescence microplate. The fluorescence of the solution was read on a Perkin-Elmer LS 50B Luminescence Spectrometer. The excitation and emission slits were at 5 nm each. The excitation wavelength of the spectrofluorometer was set at 365 nm, and the emission was at 465 nm. To obtain the net relative fluorescence index (RFI), the difference in relative fluorescence between the sample with probe and the sample without probe was computed. Hydrophobicity and 90% aqueous ethanol solubility values of rice protein fractions were compared to hydrophobicity and ethanol solubility values of corn prolamin and BSA. The protein fraction with a high hydrophobicity value and a high ethanol solubility comparable to corn zein hydrophobicity was selected for further analysis by MALDI-MS.

MALDI-TOF-MS of Rice Prolamin. The molecular size of purified prolamin was determined by MALDI-MS as follows. Purified rice prolamin with the highest hydrophobicity value and water insolubility was analyzed for size determination by MALDI-MS. The prolamin sample, at about 45 pmol (arbitrary calculated using an average molecular mass of 14 000 Da for prolamin) was dissolved in formic acid/water/2-propanol (1:3:2 v/v/v) (*19*). The matrix solution was formic acid/water/2-propanol saturated with 4-hydroxy-α-cyano-cinnamic acid (*20*). A 0.5 μL of the prolamin−matrix solution was deposited onto the MALDI sample probe and allowed to air-dry. MALDI spectra were obtained on a linear mode using a Finnigan MAT operating at 20 kV accelerating voltage and using 337 nm radiation from a nitrogen laser. Data were obtained by averaging 34 shots acquired by rastering across the sample surface. External mass calibration was provided by the $[M + H]^+$ ions of insulin and cytochrome *c*.

RESULTS AND DISCUSSION

Prolamin Concentration in Rice Flour. From 100 g of rice flour, an average of 1700 mg of prolamin was obtained. Results obtained using ninhydrin were compared to data obtained for

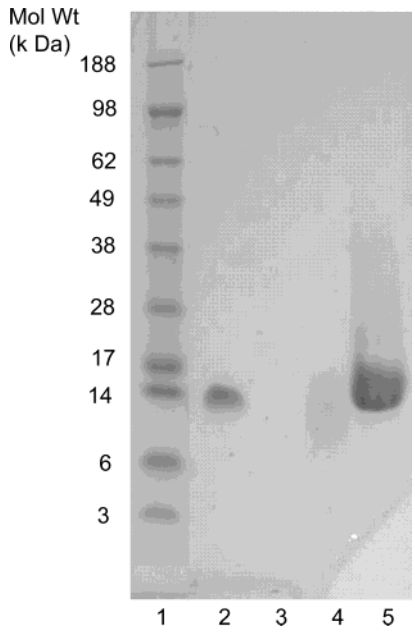

Figure 1. Analytical separation of rice protein mixture by reverse phase on a POROS R2/H column. One hundred microliters of rice protein mixture in water containing 0.1% TFA was injected into the POROS R2/H column. The flow rate was 2.5 mL min^{-1}. The gradient started at 0 CV and ended at 12 CV (0.085% TFA in *n*-propanol).

nitrogen analysis by the Dumas combustion method and were not significantly different ($P < 0.05$). Ogawa et al. (*6*) indicated that 5% β-mercaptoethanol in the extraction buffer significantly improved rice prolamin recovery, and the isolated prolamin represented 25% of total seed proteins. Li and Okita (*21*), using immunoblot coated with antibodies to rice prolamin, reported prolamin to be 18−20% of total seed protein. Unlike previous estimations, data from Ogawa et al. (*6*) and Li and Okita (*21*) indicated that rice was a good source of prolamins because on average 2 g of prolamin may be obtained from 100 g of flour.

Reverse Phase Perfusion Chromatography and Electrophoretic Separation of Rice Prolamin. Analytical separation of rice protein extract was accomplished using a reverse phase POROS R2/H column. One major peak and three small peaks were identified following separation (**Figure 1**). The uniqueness of perfusion chromatography columns lies in their ability to perform bioseparation 10−100 times faster than regular HPLC columns without loss of column resolution or column capacity. As a result, separation can be achieved in less than 5 min (**Figure 1**). The absorbance reading was carried at 230 nm because *n*-propanol has a UV cut off at 210 nm (*22*). At a flow rate of 5 mL per min, which is a normal flow rate for a perfusion chromatography column, the high viscosity of *n*-propanol at room temperature (1.72 centipoise at 30 °C) as compared to viscosity values of 0.54 or 0.99 centipoise for methanol and ethanol, respectively, generated a pressure that easily exceeded the maximum pressure drop of the column. Elution was therefore accomplished at a flow rate of 2.5 mL per min. The flow rate of 2.5 mL per min was still two and half times faster than the normal flow rate, i.e., 1 mL per min, commonly used in normal conventional chromatography. Following analytical separation by HPLC, the peaks were lyophilized following removal of the elution solvents and analyzed for solubility, electrophoresis, and S_o.

Solubility, Hydrophobicity, and Identification of Rice Prolamin. Solubility and S_o tests allowed selection of peak 4

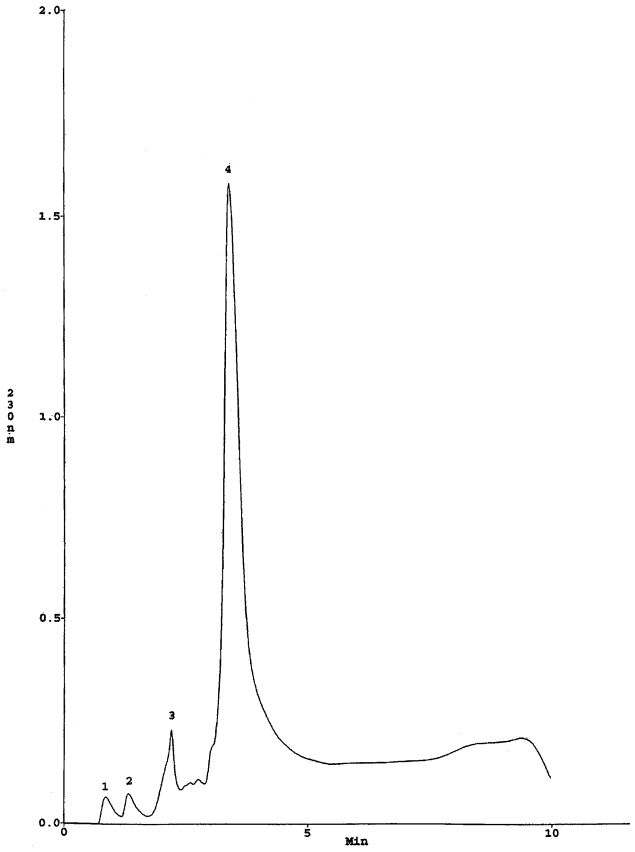

Figure 2. SDS–PAGE of the rice protein mixture. The rice protein mixture was extracted with 55% *n*-propanol, the solvent was removed by vacuum evaporation, and the protein mixture was lyophilized. Lane 1 represents the molecular weight marker. Lane 2 represents the 15 kDa rice prolamin obtained as peak 4 of the perfusion chromatography. Lane 3 represents peak 1 of the chromatographic separation; lane 4 represents fraction 3 from the chromatogram profile; and lane 5 is a representative of the 55% *n*-propanol extract.

Table 1. Surface Hydrophobicity (S_o) Value of Rice Protein Extracted with 55% *n*-Propanol and Separated by Reverse Phase Chromatography on a POROS R2/H Perfusion Column[a]

protein fraction	surface hydrophobicity (S_o)
fraction 1	326
fraction 2	484
fraction 3	626
fraction 4	1539
BSA	388
zein	1926

[a] Fractions 1–4 represent peaks 1–4 from the HPLC separation in **Figure 2**.

as the most probable fraction associated with prolamin. Peaks 1−3 were completely insoluble in 90% ethanol whereas peak 4 was completely soluble. As a result, electrophoretic separation of the 90% aqueous ethanol extract of peak 4 showed only one band at about 15 kDa while other fractions showed no peaks (**Figure 2**, lanes 2−4, respectively). The electrophoretic separation of peak 2 also showed no protein band (data not shown). This test served to indicate that peak 4 was probably associated with prolamin.

Surface hydrophobicity (S_o) values of rice protein fractions as determined by fluorescence are given in **Table 1**. Comparison

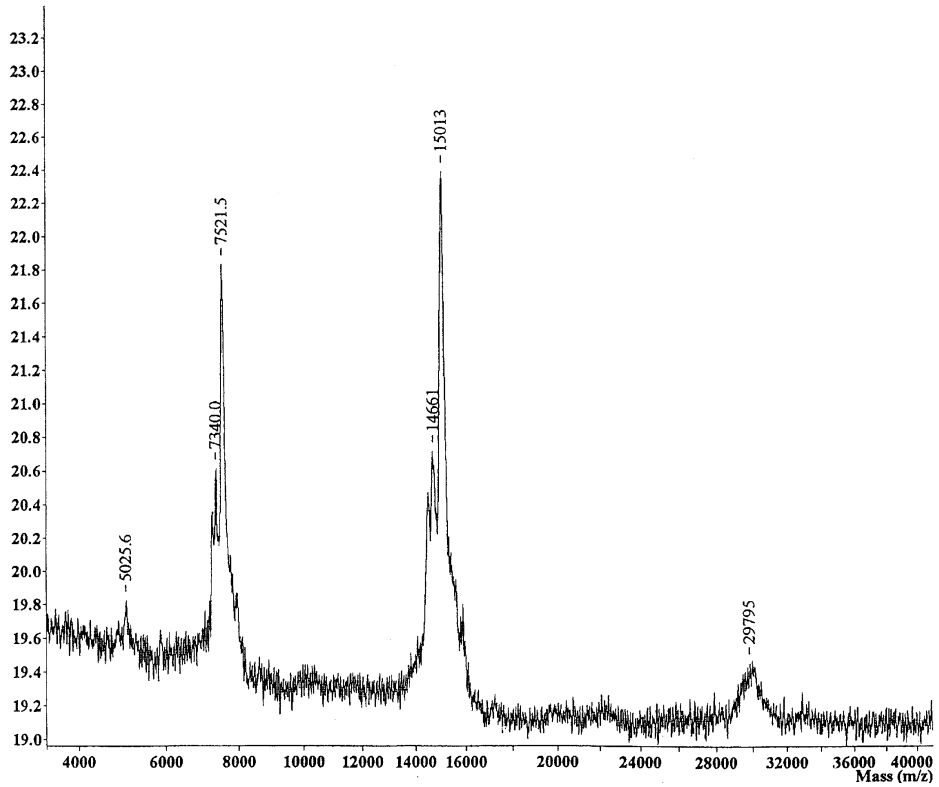

Figure 3. MALDI-MS of purified rice prolamin. Perfusion chromatography-purified rice prolamin was dissolved in formic acid/water/2-propanol (1:3:2 v/v/v). The matrix solution was formic acid/water/2-propanol saturated with 4-hydroxy-α-cyano-cinnamic acid. The sample in the matrix solution was loaded at 0.5 μL onto the MALDI probe and allowed to dry. Thirty-four shots were obtained and averaged.

was made with S_o values of corn prolamin and BSA. BSA had the lowest RFI value. Fractions 1−3 separated by reverse phase HPLC also had S_o values very low as compared to peak 4 from the same separation and corn prolamin. The S_o of prolamin (fraction 4) was close to the S_o of zein (**Table 1**). PRODAN is a very hydrophobic and electrically neutral probe. PRODAN is very sensitive to change in environment polarity. PRODAN was dissolved in ethanol and was easily transferred to protein fractions separated by chromatography.

The presence of a single prolamin subunit in rice cultivars has been demonstrated by different investigators. A single band of 16.6 kDa prolamin in rice seeds was reported by Mitsukawa et al. (*8*). Udaka et al. (*23*) isolated a 15 kDa prolamin from a wild rice cultivar using 55% *n*-propanol and 21% SDS. Krishnan et al. (*7*) reported a single prolamin band at 14 kDa in rice line M201. Prolamin with two subunits of size between 10 and 16 kDa were reported by Ogawa et al. (*6*). The significance of the different prolamin subunits is not well-known.

The advantages of perfusion chromatography over conventional chromatography include the flow of solution through and around the macroporous resins. As a result, the mass transfer resistance is significantly reduced, separation time is significantly reduced, and the regeneration time is shortened without compromising resolution and loading capacity (*24*, *25*). An additional advantage of perfusion chromatography is that the short separation time reduces the probability of inactivation of sensitive biomolecules. The cycle adsorption−washing−desorption−regeneration is reduced and more economical.

Rice prolamin contains 46% of hydrophobic amino acid residues. However, unlike other cereals, rice prolamins lack the typical tandem repeats of conserved proline-rich peptides common to other cereals. The proline content of rice prolamins is also lower than the proline content of other cereals such as maize, sorghum, *Coix*, and millet (*26*).

MALDI-MS of Rice Prolamin. The molecular size of the most hydrophobic protein peak, presumed to be prolamin because of its hydrophobicity and solubility, was determined to be 15 013 by MALDI-MS using a Finnigan MAT MALDI-MS unit (**Figure 3**). From **Figure 3**, the peak at 29 795 Da, which appears to be the dimer of the peak at 15 013, is very minute and cannot be considered as another protein in the protein solution. The peak at 14 661 could have been a subunit of prolamin, but it was not resolved by HPLC. Peaks at 7521 and 7339 are laser-induced fragments of the peaks at 15 013 and 14 661 Da, respectively. We have previously obtained values of 15 041 for the same rice sample using a Triflex MALDI-MS unit (graph not shown). These close MALDI values for rice prolamin size confirmed the accuracy of MALDI. The accuracy of MALDI-MS is 0.01%. Analysis of other rice samples, obtained from a local market, by MALDI has indicated the presence of two subunits at 14 947 and 10 658 Da, respectively (unpublished observation). Hirokoshi et al. (*9*) reported a major prolamin containing 131 amino acid residues long and at size of 14 930 Da. The difference between 14 930 and 15 013 Da is 83 Da and is statistically insignificant. Prolamin may exist in rice seeds as a single protein or a mixture of two protein subunits (*27*). Rice seeds with a single prolamin band at 16 kDa were reported by Mitsukawa et al. (*8*). Krishnan et al. (*7*) have also documented the existence of rice seeds with one single prolamin band at 14 kDa. Rice prolamins are rich in Glu, Leu, Ala, Val, Phe, Pro, and Ile. Glu would enhance rice prolamin nutritional value if the protein is properly digested. Work is in progress, in our laboratory, to improve the digestibility of rice prolamin by in vitro proteolysis so that the benefits of consuming rice can be fully obtained.

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Received for review April 11, 2002. Revised manuscript received August 20, 2003. Accepted August 25, 2003. The financial support of the Louisiana Rice Research Board is fully appreciated. Paper 02-21-0257 of the Louisiana Agricultural Experiment Station.

JF020428C