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Influence of Storage on Peptide Subunit Composition of Rice Oryzenin

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Peptide subunits of oryzenin preparation from postharvest and stored rice grains of the two typical U.S. rice varieties (long and medium grain) have been studied by SDS–PAGE. Both studied varieties of the postharvest and/or stored rice had similar qualitative peptide subunit compositions. The subunits which occurred in the reduced denatured oryzenin had molecular weights 12 300, 14 200, 22 000–23 000 (double spot), 32 000–37 000 (double spot), 56 000, 79 200, 83 000, 91 300, 104 000, 141 000, 169 000, 181 000, and 202 000. The results revealed differences not only between the rice varieties but also between the postharvest and stored rice grains of the same variety. The low molecular weight peptide subunits decreased and the high molecular weight peptide subunits increased during storage. The average molecular weight of oryzenin increased during storage. This increase was caused not only by the cystine bridges but also by more complex changes in peptide composition.

Oryzenin (rice glutelin) is a major rice storage protein. It is a relatively insoluble protein fraction of rice grain endosperm and accounts for more than 80% of the total protein. After denaturing reduction by SDS, urea, and mercaptoethanol, it dissociates into smaller subunits which can be separated by SDS–PAGE (Juliano and Boulter, 1976; Villareal and Juliano, 1978; Yamagata et al., 1982; Wen and Luthe, 1985; Robert et al., 1985; Sarker et al., 1986; Krishman and Okita, 1986; Sugimoto et al., 1986; Snow and Brooks, 1989). The precursor of these oryzenin fractions is a peptide with molecular weight 56 000–57 000 (Yamagata et al., 1982; Luthe, 1983; Sarker et al., 1986), and the main fractions after denaturing reduction are at 22 000 and 33 000. The chemical and physicochemical properties of oryzenin and starch change during storage of rice (Chrastil, 1990a–c), and these changes influence the functional properties of stored rice grains. This is important for the food-processing industry. Thus, there is a need to know more details about the changes in rice grains during storage.

In this work the protein fractions are named by the classical conventional definition (proteins soluble in water are albumins; in salt, globulins; in diluted alcohol, prolamins; and the rest, which is soluble only in alkali or acids, glutelin or oryzenin). The definition of storage protein is difficult, and usually the major, relatively insoluble protein fraction is defined as storage protein in cereals. This classification by solubility is not always correct because the solubility of the same protein fractions can vary by more or less significant structural changes. Thus here, regardless of these problems, oryzenin (rice storage protein) is defined as the major insoluble protein fraction of rice. In the following paragraphs we report the influence of storage on the electrophoretic patterns of purified oryzenin in two U.S. rice varieties (Lemont, long grain, and Mercury, medium grain).

EXPERIMENTAL PROCEDURES

Materials. All chemicals were analytical grade reagents of the highest purity from Sigma Chemical Co., St. Louis, MO, or J. T. Baker Chemical Co., Phillipsburg, NJ.

Moisture Content. Moisture content was obtained by drying rice grains to constant weight in the ventilated oven with the air flow temperature 110 °C. The accuracy of this method was sufficient for our purpose.

Rice Storage. Highly polished (30% of bran removed) rice grains (less than 1 month after harvest) of the two varieties were stored in triplicates in closed jars at 40 °C. At the beginning of storage and after 12 months, the grains were ground to flour for subsequent extraction of oryzenin.

Grinding. Rice grains were ground in a water-cooled micromill (Technilab Instruments, Pequannock, NJ) to flour (10 g of grains, 3 min of grinding). The flour was sieved, and the fractions with less than 0.01-mm particle size were used for extraction.

Extraction of Oryzenin. Rice flour (20 g) was extracted by sonication (Tekmar sonic disrupter, used power 20 W, sonication rod 3 cm deep in the middle of the beaker) with 40 mL of

ether plus 40 mL of MeOH for 1 h at 0–5 °C (in ice–water bath). The extracted flour was centrifuged at 3000*g* for 15 min, and the extraction was repeated twice. After the last extraction, the defatted flour was dried in air, extracted by sonication in 100 mL of H_2O for 1 h at 0–5 °C (albumin extract), and centrifuged at 3000*g* for 15 min. This extraction was repeated three times. The flour (still wet) was then extracted by sonication in 100 mL of 5% NaCl at 0–5 °C (globulin extract) and centrifuged at 3000*g* for 15 min. This extraction was also repeated three times. Finally, the flour was extracted three times with 100 mL of 70% EtOH (prolamin extract) and three times with 100 mL of H_2O (to wash out the remaining salt and alcohol).

Oryzenin was then extracted by sonication in 100 mL of 0.025 M NaOH at 0–5 °C and centrifuged at 3000*g* for 15 min. Because of the buffering influence of the protein, the pH of this mixture was 7–7.5. The extraction was repeated three times. Combined supernatants were precipitated by 70% TCA (final TCA concentration was about 5%) and centrifuged at 3000*g* for 15 min. The pellets were washed with water and 70% EtOH and centrifuged again.

Purification of Oryzenin. The pellet from the last extraction (still wet) was dissolved in 500 mL of 0.05 M NaOH and precipitated by $(NH_4)_2SO_4$ (up to 90% saturation). The sample was centrifuged at 3000*g* for 15 min, washed three times with 200 mL of water, and centrifuged each time at 3000*g* for 15 min. Finally, the pellet was washed with 100 mL of acetone and dried in vacuum at room temperature (25 °C).

Protein Content in Oryzenin. Protein was determined in the diluted oryzenin solution (200 mg/L of 0.05 M NaOH) according to the method of Lowry et al. (1951) with 200 mg/L albumin as a standard.

Carbohydrate Content in Oryzenin. Carbohydrate content was determined in the diluted oryzenin solution (0.5 mg/mL oryzenin in 0.05 M NaOH) according to the method of Montgomery (1961). One milliliter of this solution was mixed with 1 mL of 5% phenol and 5 mL of H_2SO_4 (95%). After 15 min, the absorbance was read at 490 nm vs H_2O and compared with the standard curve of glucose (10–100 mg/L).

Cysteine and Cystine Content in Oryzenin. Oryzenin was dissolved in 10% formic acid, and the free –SH and –SS– bonds were determined by the direct method (Chrastil, 1989) without hydrolysis. The results were expressed as an average from triplicate samples. The standard deviation of the mean of the triplicates was <0.002% S.

Hydrolysis of Oryzenin. Ten milligrams of oryzenin was hydrolyzed with 0.5 mL of 4 M trifluoroacetic acid for 4 h at 100 °C under nitrogen. The mixture was evaporated to about 100 μL and analyzed by TLC with the carbohydrate standards (glucose, mannose, galactose, fucose, and glusosamine) with EtOH/MeOH/AcOH (3/1/1) on silica gel G and with BuOH/AcOH/H_2O (4/1/1). Phthalic acid/*p*-anisidine was used as a detection reagent (Stahl, 1969).

Electrophoresis. Oryzenin (0.2 mL) was heated in reducing Tris buffer (2% SDS, 7% glycerol, 4.3% 2-mercaptoethanol, and 5 M urea in 0.055 M Tris, pH 6.8) for 5 min at 100 °C. The mixture (5–10 μL) was applied on the SDS–PAGE gel. The polyacrylamide slab gel (20 × 18 × 0.27 cm) was composed of 2.75% stacking gel (0.1% SDS + 5 M urea, 0.125 M Tris, pH 6.8) and 12% (10% for high molecular weight peptides) separating gel (0.1% SDS, 0.75 M Tris, pH 8.85). The running buffer was 0.1% SDS plus 0.19 M glycine in 0.05 M Tris, pH 8.5.

After 16–24 h of developing at 15 °C, the gel was stained with Coomassie Brilliant Blue R-250 (0.25% in 25% MeOH + 10% AcOH + 65% H_2O). The gel was then destained by the same solvent (25% MeOH + 10% AcOH + 65% H_2O). The surface of the gel was carefully dried by a filter paper and used for densitometry.

The electrophoretic spots were measured on a CAMAG TLC Scanner II in refractive mode at 550 nm. In the computerized densitometric analysis very small spots (less than 0.5% total) were automatically ignored (some of them could be albumin, globulin, or prolamin impurities), and the quantity of the remaining peptide subunits was expressed in relative percent of the total. The R_f of the peaks was compared to the standard curve obtained from STD proteins (ribonuclease, MW = 12 640; cytochrome *c*, MW = 13 370; α-lactalbumin, MW = 14 400; myoglobin, MW = 16 890; trypsin inhibitor, MW = 20 100; carbonic anhydrase, MW = 30 000; ovalbumin, MW = 43 000; albumin, MW = 67 000; phosphorylase *b*, MW = 94 000; ferritin, MW = 220 000). From each rice sample 6–20 electrophoretic gels were measured. The results were analyzed statistically and plotted with the automatic baseline corrections by means of a computer program. The average molecular weights and the average relative intensities were calculated with the standard deviations of the mean.

RESULTS AND DISCUSSION

Moisture Content. Moisture content in rice grains used for experiments did not change significantly during storage in closed jars. The average moisture content in all samples was between 12.8 and 13.2%.

Extraction Efficiency. As, for example, in the case of wheat gluten, to solubilize the large molecule of oryzenin, we must partially break the large polymer during extraction into smaller units. When we analyze the protein subunits by SDS–PAGE, we must further break the molecule. In this manner we study the more detailed infrastructure but not the original high molecular structure. This has advantages and disadvantages. To control to some extent these factors, we have checked the extraction efficiency by two methods.

We have measured the total extracted albumin, globulin, prolamin, and oryzenin in the extract (by Lowry's method) and the residual protein after the last extraction of oryzenin (by micro-Kjeldahl nitrogen analysis). We had similar experience with rice oryzenin and other rice proteins as it was found, for example, with wheat proteins by He and Hoseney (1990). The differences in solubility can be measured only with a constant extraction time under constant conditions.

When sonication and a sufficient extraction time were applied, essentially all of the protein is extracted. Although the rate of dissolution decreased after storage, the total amounts of extracted albumin, globulin, prolamin, and/or oryzenin were the same (within ±2%) before and after storage. The residual protein in the rice grains after all extractions was only 1–2% of the total protein. Thus, the possibility that, for example, oryzenin became contaminated after storage by the less soluble albumins, globulins, or prolamins was decreased to a minimum.

Composition of Oryzenin. Purified oryzenin contained 98% protein and 2% carbohydrate. It was impossible to separate this carbohydrate from oryzenin by further repeated purification in 8 M urea plus 2-mercaptoethanol and/or $(NH_4)_2SO_4$ precipitation, but it was possible to liberate it by the 4 M CF_3COOH hydrolysis. The only carbohydrate component found in the hydrolysate was glucose. It was strongly bound to oryzenin, and during the hydrolysis glucose could originate from a bound starch residue. More detailed studies of this problem will be shown elsewhere.

Cysteine and Cystine in Postharvest and Stored Oryzenin. In both medium and long rice grains the number of disulfide bridges (cystine) increased during storage. Oryzenin from the medium-grain rice contained 0.20% S (as –SH) before storage and 0.14% S (as –SH) after storage. On the other hand, the cystine content increased: 0.12% S (as –S–S–) before storage and 0.22% S (as –S–S–) after storage. A similar trend was found in oryzenin from long-grain rice: cysteine content decreased, 0.17% S and 0.14% S (as –SH) before and after storage, but cystine content increased, 0.15% S and 0.18% S (as –S–S–) before and after storage, respectively. The cysteine and cystine content in purified oryzenin subunits (Zarins and Chrastil, unpublished results) was not equally

Table I. Peptide Subunits in Oryzenin from Postharvest and Stored Rice[a]

subunit MW	M (P), %	M (S), %	P	L (P), %	L (S), %	P
12 300 ± 200	<1	<1		<1	<1	
14 200 ± 200	9 ± 0.4	8 ± 0.3	0.98	8 ± 0.3	7 ± 0.2	0.99
22 000–23 000	34 ± 1.0	31 ± 1.0	0.99	33 ± 1.0	30 ± 0.8	0.99
32 000–37 000	37 ± 0.5	35 ± 0.6	1.00	36 ± 0.2	35 ± 0.3	0.98
56 000 ± 300	3 ± 0.3	2 ± 0.2	0.99	3 ± 0.2	2 ± 0.3	0.98
79 200 ± 500	5 ± 0.3	6 ± 0.3	0.97	5 ± 0.1	6 ± 0.3	0.99
83 000 ± 400	4 ± 0.2	4 ± 0.2	0.25	3 ± 0.3	4 ± 0.2	0.98
91 300 ± 400	2 ± 0.2	3 ± 0.2	1.00	3 ± 0.3	5 ± 0.6	0.99
104 000 ± 500	1 ± 0.2	3 ± 0.3	1.00	1 ± 0.2	2 ± 0.2	1.00
141 000 ± 2000	<1	<1		<1	<1	
168 000 ± 2000	<1	<1		<1	<1	
181 000 ± 5000	<1	<1		<1	<1	
202 000 ± 5000	5 ± 0.6	8 ± 1	0.99	7 ± 0.5	9 ± 0.5	1.00

[a] M (P) and M (S), medium-grain postharvest (P) and stored (S) rice (Mercury); L (P) and L (S), long-grain postharvest (P) and stored (S) rice (Lemont). Molecular weights are means and standard deviations from 40–60 samples. Relative percentages are means and standard deviations from 6–20 samples. *P* values are the *P* statistics probability values representing the statistical difference between postharvest and stored rice.

distributed. The 33-kDa oryzenin subunit contained 0.48 ± 0.01% cysteine plus cystine sulfur (0.38% S in -SH groups and 0.10% S in -S-S- groups), the 22-kDa subunit contained 0.29 ± 0.01% cysteine plus cystine sulfur (0.28% S in -SH groups and 0.01% S in -S-S- groups) which agreed with the structure of these two subunits obtained by DNA cloning (Yamagata et al., 1982; Sugimoto et al., 1986; Takaiwa et al., 1987; Takaiwa and Oono, 1990). The 14-kDa subunit contained 0.90 ± 0.01% cysteine plus cystine sulfur (0.45% S in -SH groups and 0.45% S in -S-S- groups). The cystine increase during storage could be both, intramolecular and/or intermolecular, where only the last one was directly involved in the molecular weight increase.

Peptide Subunits in Postharvest and Stored Oryzenin. The electrophoretic patterns of reduced denatured oryzenin from the two postharvest or stored rice varieties were similar and always contained peptide subunits with molecular weights 12 000, 14 000, 22 000–23 000 (often double spot), 32 000–37 000 (often double spot), 56 000, 79 000, 83 000, 91 000, 104 000, 141 000, 168 000, 181 000, and 202 000. Similar peptide subunits were found also in acid oryzenin preparations, for example, with 1% formic or lactic acids (not shown here). This avoided the possibility of some disulfide bond interchanges during the alkali extraction. The peptide subunits were not albumin, globulin, or prolamin impurities because they did not decrease or disappear by repeated intensive washing of oryzenin with water, 5% NaCl, and/or 70% ethyl alcohol or 50% 1-propyl alcohol. About two-thirds of the oryzenin preparations were the main subunits (22 000–23 000 and 32 000–37 000), and the rest of the rice insoluble fraction was the mixture of other subunits present in smaller quantities.

The relative quantities of the polypeptides in the oryzenin fraction varied slightly but significantly, not only in different varieties of rice but also before and after storage of rice grains as well (Table I; Figures 1 and 2).

During storage the lower molecular weight peptides decreased and the higher molecular weight peptides increased in both studied varities. These relative changes in the distribution of peptide subunits in the oryzenin fraction caused by storage were smaller than the changes of the average molecular weight of the whole oryzenin molecule, which almost doubled during storage (Chrastil, 1990b).

Nevertheless, they were significant enough (the *P* statistics, which expressed the probability that the sets of values before and after storage are statistically different, was always higher than 0.97) to show that the physicochemical and chemical changes of oryzenin during storage

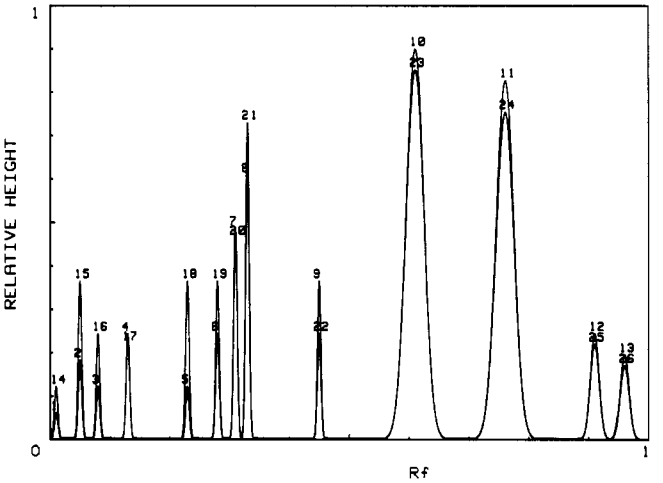

Figure 1. Densitometric analysis of the peptide subunits from medium rice grains. Peaks and valleys were automatically transformed to the same baseline and the noise or small peaks were cut off. The molecular weight decreases from left to right. 1–13, peptides from postharvest rice; 14–26, peptides from stored rice.

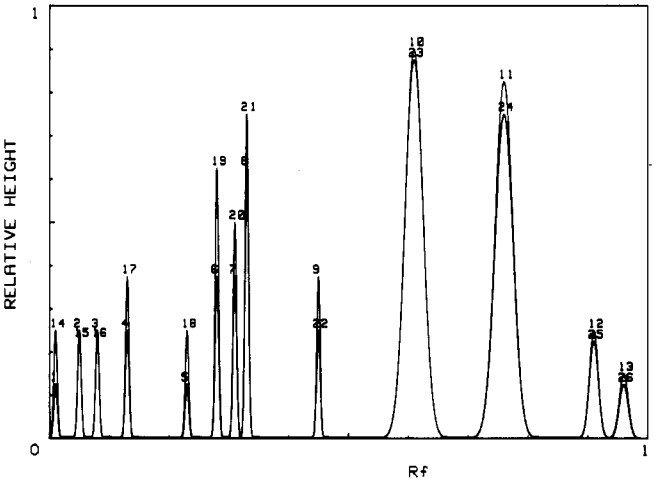

Figure 2. Densitometric analysis of the peptide subunits from long rice grains. See caption to Figure 1.

were more complex. Thus, changes in the cysteine–cystine bridges were evidently accompanied by apparent changes in the peptide subunit distribution. These changes in peptide subunit composition (even when small) could influence the association forces and thus play an important part in the increase of the apparent molecular

weight of the whole (undissociated) oryzenin fraction during storage.

Because similar trends in peptide subunits have also been observed during the ripening of rice grains (Yamagata, 1982), it seems probable that the biochemical ripening process, although it slows down during storage at a relatively low moisture content, is not completely obliterated and continues in a modified form during storage.

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