Separation and Purification of Rice Oryzenin Subunits by Anion-Exchange and Gel-Permeation Chromatography

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Rice storage protein (oryzenin) was separated in three main subunits (33, 22, and 14 kDa). The low molecular weight (14 kDa) subunit, although it was related immunologically to rice prolamins, was an integral part of oryzenin because it could not be separated from oryzenin (or from rice flour) by H_2O, 5% NaCl, or diluted ethyl or propyl alcohols. The oryzenin subunits were at first partially purified on DEAE Sepharose CL-6B and then further purified on Sephacryl S-200. All three main subunits contained strongly bound glucose or polysaccharide chains based on glucose. Isoelectric focusing of the 33-kDa purified subunit resulted in nine acid spots within the pH range 5–8. Isoelectric focusing of the 22-kDa purified subunit resulted in five basic spots within the pH range 8–11. Isoelectric focusing of the 14-kDa subunit resulted in three basic spots with pH 8.7, 8.8, and 9.0.

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 electrophoresis (Juliano and Boulter, 1976; Villareal and Juliano, 1978; Yamagata et al., 1982; Wen and Luthe, 1985; Robert et al., 1985; Sarker et al., 1986; Krishnan and Okita, 1986; Sugimoto et al., 1986; Snow and Brooks, 1989). The precursor of these oryzenin fractions is a peptide with the molecular weight of 56 000–57 000 (Yamagata et al., 1982; Luthe, 1983; Sarker et al., 1986) and the main fractions after denaturing reduction are at 22 kDa (basic peptides) and 33 kDa (acid peptides). The low molecular weight fraction (14 kDa) was reported by Wen and Luthe, 1985; Krishnan and Okita, 1986; and Snow and Brooks, 1989. These authors have shown that the 22-, 33-, and 14-kDa subunits have different precursors, and the smallest subunit was related immunologically to rice prolamins. In this work we have studied the oryzenin subunits by SDS–PAGE electrophoresis and by isoelectric focusing. The three main fractions of oryzenin (33, 22, and 14 kDa) were separated, purified, and prepared in larger (>0.5 g) quantities by fractionation on DEAE-Sepharose 6B, ultrafiltration, dialysis, and further chromatography on Sephacryl S-200.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were analytical reagents of the highest purity from Sigma Chemical Co., St. Louis, MO, or Pharmacia Biotechnology, Piscataway, NJ. Long-grain rice U.S. variety (Lemont) stored for 6 months at room temperature (25 °C) was used for all experiments.

Grinding. Rice grains were ground in a water-cooled micromill (Technilab Instruments, Pequannock, NL) 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 (300 g) was extracted twice in a closed flask by 1.5 L of ether plus 1.5 L of MeOH for 24 h at 0–5 °C (in an ice–water bath). The extracted flour was filtered, and the extraction was repeated twice. The fat-free flour was extracted twice for 3 h with 3 L of H_2O at room temperature (25 °C) to remove the water-soluble proteins (albumins) and centrifuged at 3000*g* for 15 min. The flour (still wet) was then extracted twice for 3 h with 3 L of 5% NaCl at room temperature (25 °C) (globulin extract) and centrifuged at 3000*g* for 15 min. Finally, the flour was extracted twice for 3 h with 3 L of 60% 1-propanol (prolamin extract) and twice for 3 h with 3 L of H_2O (to wash out the remaining salt and alcohol).

Oryzenin was then extracted twice for 3 h by 3 L 0.025 M NaOH or by 3 L of 1.5% lactic acid at room temperature (25 °C) and centrifuged at 3000*g* for 15 min (Chrastil, 1990; Chrastil and Zarins, 1992). Both extractions were treated separately. The 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 1 L of 70% EtOH and 1 L of water and centrifuged again. The sample was extensively dialyzed against water and lyophilized.

Purification of Oryzenin Subunits. The lyophilized oryzenin pellet (2 g) from the last (alkali or lactic acid) extraction was dissolved in starting buffer (see below) with no salt and fractionated on a DEAE-Sepharose CL-6B column (5 × 50 cm). The column was equilibrated with 50 mM Tris buffer, pH 7.5, containing 8 M urea, 0.1 mM EDTA, and 50 mM 2-mercaptoethanol. Oryzenin fractions were eluted by 0–0.5 M NaCl gradient. The eluent was monitored by UV-280 nm monitor.

The peak fractions from the DEAE-Sepharose CL-6B column were concentrated by ultrafiltration on an Amicon YM-10 membrane. Concentrated fractions were further purified on a Sephacryl S-200 column (2.6 × 100 cm) in 0.05 M phosphate buffer, pH 6.5, containing 8 M urea, 0.1 mM EDTA and 50 mM 2-mercaptoethanol. The eluent was monitored at 280 nm, and each peak fraction was checked by SDS–PAGE electrophoresis. The subunit fractions were then concentrated by ultrafiltration on an Amicon YM-10 membrane and purified again on the S-200 Sephacryl column. Only the center of the peak was collected each time, and each peak fraction was checked by SDS–PAGE electrophoresis.

Finally, the purified fractions were dialyzed extensively against water and lyophilized at −20 °C.

Electrophoresis. The electrophoretic purity of the fractions was determined by SDS–PAGE using the Pharmacia Phast Electrophoresis System on 20% homogeneous Phast gels. The conditions used for this automatic electrophoresis and Coomassie Brilliant Blue R-250 staining were those recommended by the Phast System Users Manual.

Oryzenin or oryzenin subunits (0.4–0.5 mg) were heated in 0.2 mL of reducing Tris-buffer (2% SDS, 4.3% 2-mercaptoethanol, and 5 M urea in 0.055 M Tris, pH 6.8) for 5 min at 100 °C. The mixture (0.5 μL) was applied on the Phast gel. 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. Small spots were automatically ignored, and the quantity of peptide subunits was

Table I. SDS-PAGE Electrophoresis of the Column Chromatographic Fractions

fraction[a]	MW	relative %
I	83 000	9.8
	79 000	12.3
	22 000	77.9
II	22 000	100
III	83 000	6.8
	56 000	8.5
	30 000	5.2
	28 000	6.7
	22 000	57.5
	14 000	15.3
IV	22 000	78.8
	14 000	21.2
V	14 000	100
VI	79 000	13.5
	33 000	86.5
VII	33 000	100

[a] Fractions I–VII are described in text.

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; and ferritin, MW = 220 000). From each rice sample three to five 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.

Isoelectric Focusing. The casting solution was prepared from 4.85 g of acrylamide, 0.15 g of *N,N'*-methylenebisacrylamide, 48.48 g of urea, and 2 g of nonidet NP-40 plus distilled water up to 85 mL. The mixture was stirred with 0.8 g of Amberlite MB1 for 1 h. The solution was filtered, 6.25 mL of the appropriate Pharmalyte pH interval was added, and the solution was diluted to 99 mL with distilled water. Then 0.1 mL of TEMED and 1 mL of ammonium persulfate solution (22.8 mg/mL) was added, and the solution was mixed.

The 0.5- × 14-cm tubes were capped at the lower ends with Parafilm. The tubes were inserted in the Pharmacia GRC-16 Casting Stand and straightened. The tubes were the carefully filled by a Pasteur pipet to approximately 4 mm from the top. The gel was overlaid by 0.2 mL of water and allowed to polymerize at room temperature (25 °C) for 2 h.

After polymerization the top of the gel was washed with deionized water and inserted into the Pharmacia GE-2/4 LS electrophoretic apparatus. The tubes were then filled to the top with a solution consisting of a diluted Pharmalyte (1:15), 8 M urea, and 15 mM 2-mercaptoethanol. The top chamber was filled with 200 mL of cathode electrolyte (0.01 M ethylenediamine) and the bottom chamber with 3.1 or 4.3 L of anode electrolyte (0.01 M iminodiacetic acid or HEPES).

The protein in the 1:15 Pharmalyte solution was carefully layered on the top of the gel (2–5 μg of protein per band), and the gel was focused at 1–2 mA/gel rod (constant current) and 500 V. After 14–16 h the gel was removed and fixed by 150 mL of MeOH and 350 mL of H_2O with 17.25 g of sulfosalicylic acid and 57.5 g of MeOH and 350 mL of H_2O with 17.25 g of sulfosalicylic acid and 57.5 g of TCA. Finally, the gel rods were stained with Coomassie Brilliant Blue R-250 (1.12 g/L water) overnight and destained with EtOH + acetic acid in H_2O (250 mL + 80 mL per liter) until a clear background was obtained.

Protein Content in Oryzenin. Protein was determined in the diluted solution of oryzenin subunits (200 mg/L of 0.05 M NaOH) by 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 solution of oryzenin subunits (0.5 mg/mL oryzenin in 0.05 M NaOH) by 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 sample was read at 490 nm vs H_2O and compared with the standard curve of glucose (10–100 mg/L).

RESULTS AND DISCUSSION

Composition of Oryzenin Subunits. Purified oryzenin subunits were analyzed for protein and carbohydrate content. The 33-kDa oryzenin subunit contained 98.1 ± 0.2% protein and 2.2 ± 0.1% carbohydrate, the 22-kDa subunit contained 99.3 ± 0.2% protein and 0.80 ± 0.1% carbohydrate, and the 14-kDa subunit contained 99.2 ± 0.2% protein and 0.83 ± 0.1% carbohydrate. The only carbohydrate bound covalently to oryzenin was glucose (Chrastil and Zarins, 1992).

Purification of Oryzenin Subunits. Oryzenin was separated on the DEAE-Sepharose CL-6B column in five fractions. The first fraction (I) was separated on Sephacryl S-200 column into two fractions. The second fraction from Sephacryl S-200 was almost pure 22-kDa oryzenin subunit (II). The second fraction from the Sepharose CL-6B column (III) was separated on Sephacryl S-200 into two fractions. The second fraction from this column (IV) was separated again on Sephacryl S-200 column into two fractions. The second fraction from this column was almost pure 14-kDa oryzenin subunit (V). The purified 14-kDa subunit was insoluble in aqueous alcohols. Thus by definition it should not be called prolamin (as it was sometimes named studies because of its electrophoretic and immunologic behavior and different precursor than the 22- and 33-kDa subunits). The fourth fraction from the Sepharose CL-6B column (VI) was separated on Sephacryl S-200 column into three fractions. The second fraction from this column was almost pure 33-kDa oryzenin subunit (VII).

Isoelectric Focusing of Oryzenin Subunits. By isoelectric focusing we were able to further separate the 33-kDa pure oryzenin subunit (often called an acid subunit) into nine acid spots within the pH range 5–8. The pure 22-kDa oryzenin subunit (often called a basic subunit) was separated by isoelectric focusing into seven basic spots within the pH range 8–10. The low molecular weight pure oryzenin subunit (14 kDa) (often called a prolamin subunit) was separated by isoelectric focusing into three basic spots with pH 8.7, 8.8, and 9.0, respectively.

The method described here effectively separated larger quantities (up to 1 g) of the main oryzenin subunits by the combination of the DEAE-Sepharose CL-6B and Sephacryl S-200 column chromatography into its subunits. These subunits were electrophoretically homogeneous, but isoelectric focusing revealed several spots from each subunit. The small molecular weight oryzenin subunit (14 kDa) was always an integral part of oryzenin. It belonged there by its solubility but not by its genetic origin. All studied oryzenin subunits contained 0.5–2.5% of inseparable carbohydrate. The strongly bound carbohydrate residues were hydrolyzable to glucose and could be longer or shorter chains of a glucose polysaccharide. This question will be studied in future experiments.

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