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Extractability and chromatographic separation of rice endosperm proteins

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Abstract

The molecular weight (MW) distribution of proteins extracted with different solvents from defatted rice endosperm was examined by size exclusion-high performance liquid chromatography (SE-HPLC) with 2.0% sodium dodecyl sulfate (SDS) (w/v) as mobile phase. The resulting protein peaks were further characterized by SDS-PAGE. Under the experimental conditions, 2.0% SDS extracted 64% of the proteins. Adding 6.0 M urea resulted in a 15% increase in extractability (up to 79%). With using 20–100 mM NaOH, 70–81% of the proteins were extractable. Maximum extractability was reached with 2.0% SDS, 6.0 M urea and 0.5–1.5% dithiothreitol (DTT). Apparent MW profiles of rice endosperm proteins allowed classification into six fractions of decreasing apparent MW. Fraction VI contained the low MW albumin, globulin, and prolamin protein material. Fractions IV and V originated from α and β glutelin subunits, respectively. The polypeptides of fraction III consisted of an α and a β subunit linked by an intermolecular disulfide bond. The polypeptides of fractions I and II were dimers, trimers or more highly polymerized forms of the (α–β) glutelin subunit dimer in fraction III. While the work confirmed that rice glutelin is composed of polymers of α and β subunits, remarkably, higher MW glutelin aggregates (fractions I–III) only partly dissociated on reduction. Low MW protein material (fraction VI) was entrapped in the aggregated protein network and was released on reduction. The rapid and reproducible SE-HPLC method developed for rice protein separation allows a more quantitative approach than SDS-PAGE.

Keywords: SE-HPLC; Rice protein; Glutelin subunits; Disulfide bonds

1. Introduction

Milled rice or rice endosperm contains 3.8–8.8% albumin, 9.6–10.8% globulin, 2.6–3.3% prolamin and 66–78% glutelin (Cagampang et al., 1966). Rice albumins have a wide range of molecular weights (MWs) (Juliano, 1972), with major components with apparent MWs of 18–20 kDa (Houston and Mohammed, 1970). Rice globulins consist of α-, β-, γ-, and δ-globulins with apparent MWs of 25.5, 15, 200 kDa and higher, respectively (Morita and Yoshida, 1968). Prolamins consist of three polypeptide subunits with apparent MWs of 10, 13 and 16 kDa (Ogawa et al., 1987). The dominating 13 kDa polypeptide is readily solubilised in alcoholic solutions, while the 10 and 16 kDa polypeptides with a high level of sulfur containing amino acids require a reducing agent for solubilization in alcoholic solutions (Hibino et al., 1989; Ogawa et al., 1987). The major storage protein of rice is glutelin. Native rice glutelin is extremely insoluble in water because of hydrophobic, hydrogen and disulfide bonding (Hamada, 1996; Juliano, 1985). It has a high MW, and is heterogeneous (Juliano, 1985). Glutelin is largely soluble in acidic (pH below 3.0) or alkaline solutions (pH above 10.0) (Shih, 2004). Rice glutelin is composed of two major polypeptide subunits classified as α, or acidic, and β, or basic subunits with apparent MWs of 30–39 and 19–25 kDa, respectively (Juliano, 1985; Kagawa et al., 1988; Kishimoto et al., 1999; Shih, 2004). Glutelin is proteolytically processed by cleavage of a 57 kDa polypeptide precursor (Sarker et al., 1986) into the α and β polypeptides, which are covalently linked to each other by an intermolecular disulfide bond (Katsube et al., 1999; Katsube-Tanaka et al., 2004a; Utsumi, 1992; Yamagata et

Abbreviations: DTT, dithiothreitol; HMW, higher molecular weight; LMW, lower molecular weight; 2-ME, 2-mercaptoethanol; MW(s), molecular weight(s); NaOH, sodium hydroxide; SDS, sodium dodecyl sulfate; SE-HPLC, size exclusion-high performance liquid chromatography.

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al., 1982), resulting in glutelin molecules with MWs ranging from 64 to 500 kDa (Sugimoto et al., 1986). Rice glutelin polymerizes by disulfide bonding and hydrophobic interactions to form very large macromolecular complexes (Utsumi, 1992). The formation of higher-order structures may explain the lack of functional properties of rice glutelin (Katsube-Tanaka et al., 2004a). So far, six glutelin genes have been identified and are classified into two subfamilies, i.e. A type and B type glutelin, according to the degree of nucleotide sequence similarity (Katsube-Tanaka et al., 2004b).

The extractability of rice proteins has been exhaustively studied. NaOH solutions are efficient solvents for rice proteins (Cagampang et al., 1966; Juliano and Boulter, 1976; Tecson et al., 1971), however, they cause degradation (Shewry and Miflin, 1985). A more effective extractant is a combination of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2-ME) in water (Juliano and Boulter, 1976, Tecson et al., 1971) with 2.0% SDS + 5.0 M urea + 0.5% 2-ME reported to be the most effective glutelin extractant (Udaka et al., 2000). For rice bran proteins, a strongly dissociating solution containing 0.1 M acetic acid, 3.0 M urea and 0.01 M cetyltrimethylammonium bromide (AUC) extracted most proteins (67–94%).

The molecular weight distribution of the major rice protein, glutelin, was determined by Sephadex G-200 chromatography. (Tecson et al., 1971). Reduction and alkylation resulted in partial dissociation of glutelin into its subunits. A fraction with a MW 6.5×10^4, i.e. one-tenth that of the native glutelin, was obtained. Villareal and Juliano (1978) separated glutelin on an Ultrogel 44 column but the separation was poor and homogeneous subunits were obtained only after re-chromatography on a Ultragel 34. Snow and Brooks (1989) using Sepharose CL-6B gel filtration chromatography separated crude rice protein extracts into four broad and incompletely resolved fractions that eluted over a wide MW range. Using a combination of anion exchange and gel chromatography, Zarins and Chrastil (1992) separated 33, 22, and 14 kDa glutelin subunits. Hamada et al. (1998) described a reproducible chromatographic separation of rice bran proteins on Shodex Protein WS-2003 that allowed determination of their apparent molecular mass distribution and further fractionation and characterization of the individual polypeptides. However, no well-resolved peaks were obtained. Katsube-Tanaka and co-workers (2004a) separated polymeric and lower MW fractions of glutelin using Sephacryl S-300HR chromatography. Notwithstanding these attempts to fractionate glutelin polypeptides by gel filtration chromatography, success has been limited and so far no effective separation of α and β subunits has been achieved.

The insolubility and high molecular weight of rice proteins complicates the extraction and separation of glutelin polypeptides into well-resolved size classes. Here, we report the use of dissociating and reducing agents to extract rice endosperm proteins and size-exclusion high performance liquid chromatography (SE-HPLC) with 2.0% SDS (w/v) as mobile phase for their fractionation and SDS-PAGE for their further characterization.

2. Materials and methods

2.1. Materials

Brown *Japonica* rice was obtained from Remy Industries N.V. (Wijgmaal, Belgium). All electrophoretic media, MW markers, and chromatographic media were from Pharmacia Biotech (Uppsala, Sweden). MW markers were phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). For SE-HPLC, two additional markers were used, i.e. chymotrypsinogen A (25 kDa) and insulin B chain (3.5 kDa). All other chemicals and reagents were from Sigma–Aldrich (Bornem, Belgium) and were of analytical grade.

2.2. Milling and grinding

White *Japonica* rice was obtained by milling brown *Japonica* rice with a Satake Batch debranner Type TM05 (Satake, Tokyo, Japan) in batches of 200 g for 60 s. The white *Japonica* rice was ground with a laboratory mill (M20 Universal Mill, Ika, Wilmington, NC, USA) to obtain flour.

2.3. Defatting

Flour was defatted with hexane (1:5, w/v) for 1 h at room temperature with continuous stirring. The flour was recovered using a Buchner funnel, air dried under a hood and passed through a 250 μm sieve.

2.4. Composition of rice flour

The moisture content was determined according to AACC-methods 44-15a (AACC, 2000). Protein content was determined by the Dumas method ($N \times 5.95$), an adaptation of the AOAC Official Method (1995) using an automated Dumas protein analysis system (EAS VarioMax N/CN, Elt, Gouda, The Netherlands).

The defatted endosperm contained 6.2% protein on d.m. flour basis.

2.5. Protein extraction

Rice proteins were extracted from defatted rice flour (90.7 mg flour equal to 5.0 mg proteins on d.m. flour basis) with (i) 2.0% (w/v) SDS, (ii) 2.0% SDS/6.0 M urea/0–1.5% (w/v) dithiothreitol (DTT), (iii) 2.0% SDS/6.0 M urea/1.0% 2-ME, and (iv) 20–100 mM NaOH (5.0 ml). The suspensions were shaken (1 h, 150 rpm, ambient temperature) and the solubilised protein recovered by centrifugation (2800*g*, 15 min).

2.6. Size-exclusion high performance liquid chromatography (SE-HPLC)

The protein extracts (Section 2.5) were filtered (0.45 μm filter) and loaded on a Shodex Protein KW-803 steel column