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Optimisation and standardisation of extraction and HPLC analysis of rice grain protein

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ABSTRACT

Rice starch composition is considered to be the most important predictor of rice eating quality, however, rice eating quality is not wholly explained by starch since cultivars with very similar starch composition differ in eating quality. Protein constitutes 4%–10% of the milled rice grain and has very diverse properties, suggesting protein composition and not just protein content may contribute to rice grain eating quality. Although many analytical methods have been used to study cereal grain protein, the extraction and analysis of rice grain protein have not been optimised in the context of assessing and improving rice grain quality. In this study, different rice grain protein extraction techniques and high pressure liquid chromatography (HPLC) analysis methods were compared and optimised. The most efficient extraction solvents for prolamins and glutelins were 60% n-propanol and 5 M acetic acid, respectively, and optimised HPLC methods were developed for each of these extracts. These optimised, standardised and reproducible methods distinguish between the proteins of basmati, long, medium and sushi rice grains and quantify differences which might contribute to their different eating qualities.

1. Introduction

Rice grain quality is an important determinant of its commercial value and consistently achieving grain quality benchmarks in new rice cultivars is a significant challenge for rice breeders. Milled rice is composed of starch (~85–95%), protein (~4–10%) and lipids (~1%) (Lasztity, 1995; Liu et al., 2013). Starch is the most abundant component of the rice grain and the genetic control of its structure has been an area of significant research in the context of rice quality. Starch contains amylose and amylopectin which differ by their degree of branching and chain length distribution. Amylose is composed primarily of long, unbranched glucose polymers and constitutes from 0% (waxy rice) to 30% (non-waxy rice) of rice grain starch, while amylopectin differs from amylose by being highly branched (Fitzgerald, 2004). Two genes are important determinants of rice grain starch structure and functionality, the *Waxy/waxy* gene, which codes for GBSSI and controls amylose content (Sano, 1984; Wang et al., 1995), and *Alk/alk* which codes for SSIIa and affects amylopectin structure (Umemoto et al., 2004; Umemoto and Aoki, 2005; Waters et al., 2006). Although these and other starch biosynthesis genes affect rice eating and cooking quality (Kharabian-Masouleh et al., 2013; Tian et al., 2009), starch structure does not explain all variation in rice grain quality parameters in all rice germplasm (Kharabian-Masouleh et al., 2013).

The major rice grain proteins are traditionally classified by their solubility in different solvents following the work of Osborne early last century (Shewry and Casey, 1999). The four classes of endosperm proteins, or "Osborne fractions", and their estimated relative percentages in rice and solubility are: 1) glutelins (80%; dilute acid/alkali soluble); 2) prolamins (~10%; alcohol soluble); 3) globulins

Abbreviations: ACN, acetonitrile; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HMW-GS, high molecular weight glutenin subunits; HPCE, high performance capillary electrophoresis; HPLC, high pressure liquid chromatography; MALDI-TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrophotometry; NaCl, sodium chloride; NaOH, sodium hydroxide; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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(~5%; salt soluble); and 4) albumins (~5%; water soluble) (Lookhart et al., 1987). The modern classification of seed proteins is based on function rather than solubility and following this classification, prolamins, glutelins and globulins provide nitrogen, carbon and sulphur to the embryo during germination while the water soluble albumins are metabolic proteins (Shewry and Casey, 1999).

Total protein (Martin and Fitzgerald, 2002), prolamin (Baxter et al., 2004), albumin (Baxter et al., 2010), glutelin (Baxter et al., 2014) and globulin (Baxter et al., 2014) influence indirect measures of rice grain eating quality. However, the extent to which rice grain protein composition has a quantitative influence on eating quality in different rice varieties is not known. Rice grain proteins can be characterised by a range of techniques including both 1-and 2-D-gel electrophoresis, gel filtration chromatography, high pressure liquid chromatography (HPLC), high performance capillary electrophoresis (HPCE) and mass spectrophotometry (Gao et al., 2010). A direct comparison of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), HPLC, HPCE and matrix assisted laser desorption/ionization time of flight mass spectrophotometry (MALDI-TOF-MS) as tools to differentiate wheat high molecular weight glutenin subunits (HMW-GS) revealed SDS-PAGE is technically simple and inexpensive but tends to overestimate protein molecular mass and provides low resolution (Gao et al., 2010). In contrast, MALDI-TOF-MS provides high resolution and accuracy but has relatively high costs for analysis. HPLC and HPCE were found to be intermediate between SDS-PAGE and MALDI-TOF-MS since both methods demonstrated high resolution and reproducibility for reasonable cost (Gao et al., 2010). Both SDS-PAGE and HPCE separate proteins on the basis of size, while HPLC exploits hydrophobic interactions with the column matrix to separate the protein components on the basis of solubility differences. Differences in solubility may be significant in the context of rice eating and cooking quality which is determined by molecular interactions with an aqueous environment and so HPLC may be the more appropriate platform to investigate protein composition in the context of rice grain quality.

Lookhart et al. (1987), Hussain et al. (1989) and Huebner et al. (1990) used HPLC for rice variety identification at a time when DNA fingerprinting technology was in its infancy and although Huebner et al. (1990) suggested the speed, sensitivity, resolution and reproducibility of HPLC would be useful for predicting the rice quality for rice breeding programs, no reports of the application of HPLC to rice grain quality in the context of rice breeding have been forth coming. This may be because amylose content was perceived to explain most of the variability in grain quality determined by subjective, non-quantitative measures of rice eating quality which were available at the time. The role of amylose and amylopectin in determining objective quantitative measures of rice grain eating quality is now better understood and this creates a more robust frame of reference in which to investigate the influence of rice grain proteins on rice grain quality. The aim of this research was to optimise and standardise the extraction and HPLC analysis of the major rice grain proteins which would then provide a reliable tool to quantify the effect of protein composition on rice grain quality.

2. Materials and methods

2.1. Sample preparation, grinding and reagents

A sample of commercial store bought sushi-style rice was used to optimise the extraction and HPLC methods. Commercial store bought basmati, long grain, medium grain and sushi-style rice sourced from Australia were analysed to determine if the optimised extraction and HPLC methods could identify differences between rice grain quality classes. Seventeen commercial store bought sushi-style rice sourced from five countries were analysed to determine if the optimised method could identify differences within a single rice quality type. Samples used are summarised in Table 1.

Rice samples were ground to flour using a ball mill (Mixer Mill MM301, Retsch). The grinding jar was filled with 7 g of milled rice sample and shaken five times at 30 r/s for 30 s as previously reported by Liu et al. (2014).

All organic solvents used for extraction and HPLC analysis were HPLC grade and sourced from Scharlau.

2.2. Protein extraction

Extraction methods for the major rice grain proteins glutelin, prolamin, globulin and albumin were compared and optimised based on the published methods of Huebner et al. (1991). Duplicate 250 mg sub-samples of rice flour were transferred to 2 mL microfuge tubes and a range of solvents previously reported in the published literature (Huebner et al., 1990, 1991; Lookhart et al., 1991) were used to extract the major rice grain proteins (Table 2). One mL of solvent was added to each sample and vortexed for 1 min. The samples were mixed with a tube rotator at room temperature for 30 min (for extraction of prolamins, globulins and albumins) or 1.5 h (for extraction of glutelin) then centrifuged at 15,000 *g* for 30 min at 25 °C. The prolamin, globulin or albumin containing supernatant was directly transferred to a 2 mL HPLC vial for HPLC analysis. For glutelins, the supernatant was first transferred to a Corning Costar Spin-X polypropylene centrifuge tube filter (0.45 μm pore size), centrifuged at 12,000 *g* for 5 min at 25 °C, and then the filtrate was transferred to a 2 mL vial for HPLC analysis.

2.3. HPLC method development

HPLC analysis was carried out using an Agilent 1260 HPLC System equipped with a vacuum degasser, quaternary pump, auto-injector, and diode array detector (DAD). The HPLC system was controlled using ChemStation software B.04.03. Column temperature was set at 50 °C and absorbance was monitored at 280 nm. Four columns were compared during method development (Column 1: Phenomenex, Jupiter 5 μm C18 300A, 250 × 4.6 mm; Column 2: Supelco, Discovery BIO Wide Pore C8-5, 250 × 4.6 mm; Column 3: Phenomenex, Jupiter 5 μm C4 300A, 250 × 4.6 mm; Column 4: Phenomenex, Jupiter 5 μm C5 300A, 250 × 4.6 mm). The mobile phase for the HPLC analysis included acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA) and Milli-Q water with 0.1% TFA. Peaks areas were calculated using the ChemStation software B.04.03.

HPLC methods were modified and optimised based on the method of Lookhart et al. (1991) which used Column 1 (C18) and an HPLC gradient that commenced at 25% ACN, increased to 35% at 5 min, 50% at 10 min, 75% at 17 min and reaching a maximum of 85% between 18 and 19 min, returning to 25% between 19 and 25 min. Different columns, elution gradients and injection volumes were compared and optimised to produce chromatograms with the greatest number of discrete peaks and greatest peak heights.

2.4. Protein content

Protein content in the samples was measured by the method of Dumas with reference AOAC 990.03 (4.2.08), 992.15 (39.1.16), 992.23 (32.2.02), 16th edition, 4th revision.