Food and Bioprocess Technology (2019) 12:1487–1499
https://doi.org/10.1007/s11947-019-02307-w

ORIGINAL PAPER

Biochemical and Techno-Functional Properties of Protein- and Fibre-Rich Hybrid Ingredients Produced by Dry Fractionation from Rice Bran

Pia Silventoinen[1] · Katariina Rommi[1] · Ulla Holopainen-Mantila[1] · Kaisa Poutanen[1] · Emilia Nordlund[1]

Received: 19 December 2018 / Accepted: 19 June 2019 / Published online: 3 July 2019

Abstract

Rice bran is an underutilized side-stream from white rice production, rich in protein and dietary fibre. The aim of the work was to study dry fractionation as a novel approach to enrich protein from non-heated, supercritical carbon dioxide–extracted milled rice bran. One-step air classification allowed protein enrichment from an original 18.5% up to 25.7% in the fine fraction. Alternatively, air classification of the non-milled raw material resulted in a fine fraction (19.7% protein) that was according to microscopy analysis free of pericarp structures, and a coarse fraction containing protein-rich aleurone and germ particles and pericarp structures. Further milling and air classification of the coarse fraction resulted in higher protein enrichment (to 27.4%). All the fine fractions produced by dry fractionation were also enriched in soluble dietary fibre whereas starch content was decreased. Additionally, the fine fractions showed improved protein solubility and colloidal stability and, thus, elevated applicability in food products as compared to the non-fractionated raw material.

Keywords Rice bran · Protein · Supercritical carbon dioxide extraction · Air classification · Dry milling · Functional properties

Introduction

Increasing the use of plant-based proteins and foods in human nutrition is recommended from food security and sustainability perspectives (Aiking 2011). This has raised an interest to valorise protein-rich ingredients from the by-products of the current agro-food industry. Rice bran, accounting for around 10% of the rice grain, is an underutilized side-stream from the white rice production process. Commercial milling of brown rice usually results in a by-product containing outer grain layers, germ, and some endosperm fractions, which are together called as rice bran. Currently, rice bran is mainly utilized for feed or burnt for energy. However, due to the relatively high protein content of 13–19% (Juliano and Bechtel 1985) and dietary fibre (DF) content of 20–30% (Abdul-Hamid and Luan 2000; Juliano 1985; Saunders 1990), rice bran, when further processed, could provide a nutritional plant-based raw material for food products. Rice bran protein can be seen superior to other cereal proteins due to its hypoallergenic (Helm and Burks 1996) and gluten-free properties and high lysine content. The amino acid composition can also be seen competitive to traditional protein sources such as casein or soy protein (Wang et al. 1999). Additionally, high levels of soluble and insoluble dietary fibre suggest possible health effects, when consuming rice bran, due to an inverse relation of fibre with obesity, type 2 diabetes, cancer, and cardiovascular diseases.

The layers included in rice bran are pericarp, as well as endosperm layers aleurone and subaleurone, which is the outermost layer of starchy endosperm. Outer layers of rice grain do not contain starch, but due to polishing efficiency, variable amounts of starchy endosperm are present in the bran increasing the starch content of the bran preparations usually to at least 10–20% (Saunders 1990). Cell wall structures of the aleurone layer are composed of hemicellulose (40%), mainly arabinoxylan, cellulose (30%), lignin (21%), and pectin (10%), whereas the β-glucan content of rice bran can be seen negligible (Shibuya et al. 1985). The dietary fibre (20–30%) of bran is mainly (90.7%) insoluble (IDF) (Abdul-Hamid and Luan 2000). As

✉ Pia Silventoinen
pia.silventoinen@vtt.fi

[1] VTT Technical Research Centre of Finland, Ltd., P.O. Box 1000, FI-02044 VTT Espoo, Finland

reviewed by Juliano (1985), the lipid content of rice bran is high, 17–23%, and it is affected also by the high lipid content of rice germ (19–24%). The germ part of rice grain is also rich in proteins (16–24%) (Juliano and Bechtel 1985).

Due to the limited functionality of bran materials and most plant-based protein sources in food systems, fractionation and further functionalization of the valuable components for improved performance in applications is needed (Day 2013). However, instead of aiming at pure isolates, the studies should focus on the complex food systems and hybrid ingredients enriched in desirable components, as emphasized in the reviews of Van Der Goot et al. (2016) and Wouters et al. (2016). Dry fractionation could provide a useful tool to prepare concentrates and ingredients enriched in valuable components, rather than isolates. Additionally, dry processing has several advantages over wet techniques, such as lower energy consumption and the possibility to better retain the native functionality of the proteins (Schutyser et al. 2015). However, high lipid content of raw materials, valid also for rice bran, is known to impair dry milling resulting in material adhesion to milling equipment (Sibakov et al. 2011). Lipids tend also to oxidize rapidly causing rancidity, which can be prevented by stabilising the bran or removing the fat. Supercritical carbon dioxide (SC-CO_2) has been studied for rice bran oil extraction (Kuk and Dowd, 1998), and application of this gentle extraction procedure prior to air classification ensures that no protein denaturation takes place during the fat extraction process. SC-CO_2 extraction and air classification have been successfully combined to enrich protein from cereal raw materials. Previously, dry processing of SC-CO_2-extracted oat endosperm flour has resulted in protein content of 73%, however, with the mass yield of only 5% (Sibakov et al. 2011). Dry fractionation of rice bran has been studied by several groups, but the highest protein contents reached by air classification, sieving, or electrostatic separation technology have only varied between 17 and 20% (Jayadeep et al. 2009; Noguchi et al. 1981; Park et al. 1993; Wang et al. 2016).

The aim of this study was to evaluate the effects of gentle lipid removal by SC-CO_2 extraction, fine milling, and one- or two-step air classification on rice bran fractionation in terms of protein, dietary fibre, and starch separation and microstructure. Additionally, protein solubility and colloidal stability of the produced fine fractions and rice bran raw material were investigated.

Materials and Methods

Rice Bran Raw Materials

Non-heated rice bran raw material (15.5% protein, 21.8% fat, 19.7% starch, 5.4% soluble dietary fibre, 25.6% insoluble dietary fibre, 8.8% ash, calculated based on mass balances, and median particle size 316 μm) and intact whole rice grains were both of Indica variety (*Oryza sativa* L. ssp. Indica) and were obtained from Südzucker AG rice starch factories (Italy).

Fat Extraction

Prior to dry processing, the rice bran was defatted with supercritical carbon dioxide (SC-CO_2), as described in Sibakov et al. (2011). The extraction process was performed using a Nova Swiss extraction vessel (Nova Werke AG, Effretikon, Switzerland) with a compressor Chematur Ecoplanning (Chematur Engineering Ltd., Pori, Finland). Extraction pressure varied between 29.5 and 30.5 MPa, and the temperature in the extraction container was 40 °C and in the separation container 48 °C, which were all in the supercritical region of carbon dioxide. Five kilograms of carbon dioxide was circulating in the equipment during each extraction.

Dry Processing by Air Classification and Fine Milling

The protein enrichment protocol was adopted from the procedure previously developed for oat protein concentration (Sibakov et al. 2011). In the one-step air classification (Fig. 1), the SC-CO_2-extracted bran was milled twice with a Hosokawa Alpine 100-UPZ fine impact mill with stainless steel pin disc grinders at a rotor speed of 17,800 rpm (Hosokawa Alpine AG, Augsburg, Germany). Air classification was then performed using a 50-ATP classifier (Hosokawa Alpine, Augsburg, Germany) operated at 21000 rpm, at air flow rate of 50 m^3/h, and feed rate of approximately 0.5 kg/h. In the two-step classification, SC-CO_2-extracted bran as such was used in the first fractionation step, where the material was air classified using a 50-ATP classifier (Hosokawa Alpine, Augsburg, Germany) operated at 10000 rpm, at an air flow rate of 50 m^3/h, and feed rate of approximately 0.5 kg/h. In the second step of the two-step classification, the coarse fraction from the first step was milled twice with the 100-UPZ pin disc mill at a rotor speed of 17,800 rpm followed by air classification (50-ATP classifier, 21,000 rpm, 50 m^3/h, feed rate of approximately 0.5 kg/h). Mass yield (% dm) was calculated as (dry weight of fraction) / (dry weight of raw material) × 100. Protein separation efficiency (PSE % dm) was calculated as (dry weight of fraction × protein content of fraction [% dm]) / (dry weight of raw material × protein content of raw material [% dm]) × 100 (Tyler et al. 1981). Additionally, total PSE (% dm) was calculated as (PSE from previous processing step [% dm]) × (PSE from the fraction in question [% dm]) × 100. All air classification procedures were performed in duplicate.

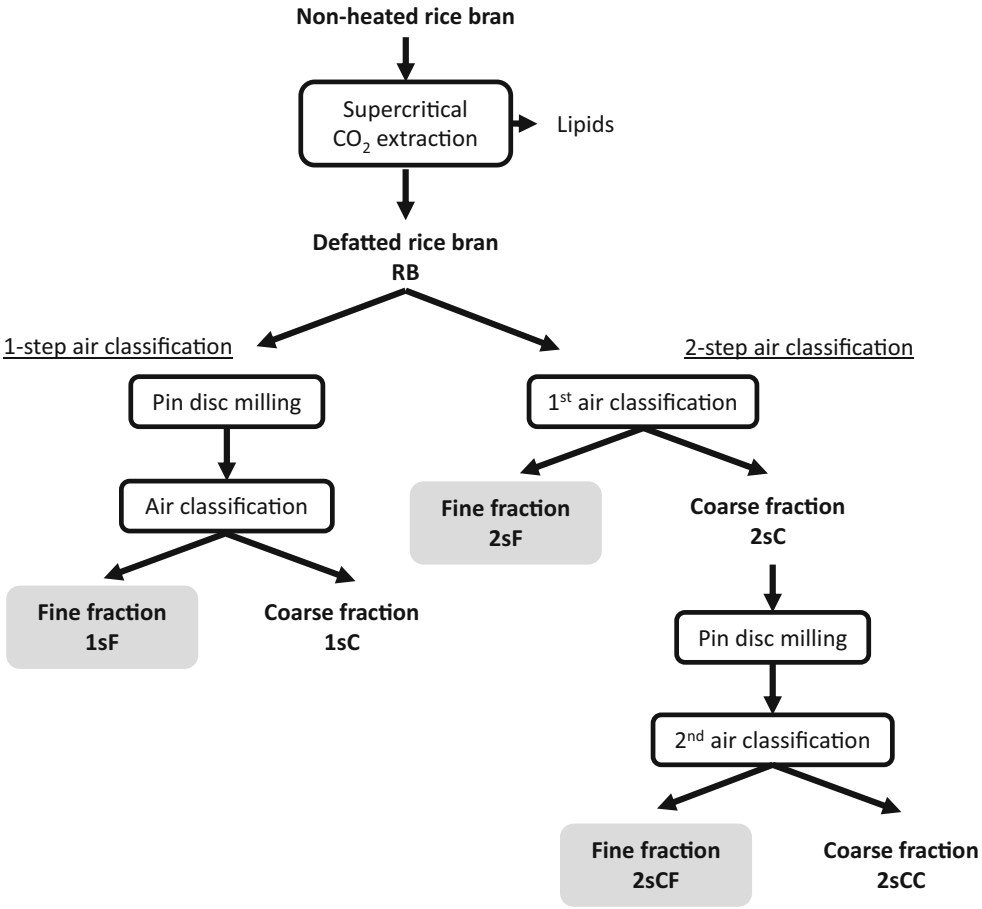

Fig. 1 Schematic presentation of one- and two-step air classification approaches used for rice bran dry fractionation. The fractions enriched in protein are highlighted with grey colour

Biochemical Composition of Rice Bran Samples

Protein content was calculated based on the total nitrogen content quantified using a Kjeldahl autoanalyser (Foss Tecator Ab, Höganäs, Sweden) according to the AOAC method 2001.11 (Thiex et al. 2002). For conversion of the total nitrogen content to crude protein content, the conversion factor of 5.95 (Juliano and Bechtel 1985) was used. Starch content was quantified using Megazyme total starch assay kit according to the AACC method 76–13.01. Soluble (SDF) and insoluble dietary fibre (IDF) were analysed using the enzymatic-gravimetric AOAC method 991.43 (AOAC, 1995). Ash content was quantified gravimetrically after combustion at 550 °C. The total carbohydrate composition was analysed after hydrolysing the samples with sulphuric acid and determining the resulting monosaccharides by high-performance anion-exchange chromatography with pulsed amperometric detection (Dionex ICS-3000 equipped with CarboPac PA1 column) according to the NREL method (Hausalo 1995; Sluiter et al. 2008). The sugar composition of the samples analysed after acid hydrolysis is only indicative, since fructose was not detectable by the method used. The phytic acid content was measured according to the method described by Latta and Eskin (1980) and modified by Vaintraub and Lapteva (1988) using phytic acid dodecasodium salt from corn (P-8810, Sigma) as a standard. In brief, the flour sample was extracted with 2.4% HCl for 2 h at room temperature followed by separation of the supernatant. The phytic acid content was quantified mixing the diluted supernatant with Wade reagent (0.03% $FeCl_3$, 6·H_2O, and 0.3% sulfosalicylic acid) at a ratio of 3:1 and measuring the absorbance by a spectrophotometer at 500 nm. For determination of water extractable pentosan, 0.25 g of the sample was mixed with 8 ml of 4 °C distilled water and shaken with glass pearls for 15 min at 4 °C. After centrifugation, the supernatant was boiled for 15 min, cooled down, and centrifuged again. The amount of pentose sugars in the water extracts was determined by a colorimetric phloroglucinol method using xylose as a standard (Douglas 1981). All biochemical analyses were performed at least in duplicate. Dietary fibre was only analysed once, and the error was estimated based on the deviations from the first step of the analysis, after which the duplicate samples were treated differently, either to analyse ash or protein.

Protein Profile Analysis

Molecular weight distribution of proteins was visualized by SDS-PAGE (Laemmli 1970) under reducing conditions using a Criterion TGX, Stain-Free Precast 4–20% Tris-HCl gradient

gel (Bio-Rad, Hercules, CA, USA) and Precision Plus Unstained Protein standards (Bio-Rad). The water mixtures of the flours were dissolved in sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.02% bromophenol blue in 0.1 M Tris-HCl pH 6.8 buffer) by heating at 98 °C for 5 min, loaded to the gel, and subjected to electrophoresis at a constant voltage of 250 V. The protein bands were visualized using Criterion Stain Free Imager and examined with Image Lab software (Bio-Rad).

Particle Size Measurement

The volume-based particle size distributions of the rice bran samples were analysed by laser light diffraction (750 nm) by Beckman Coulter LS 230 (Beckman Coulter Inc., CA, USA) as described in Silventoinen et al. (2018). The samples were analysed with the liquid module and ethanol as a carrier using refractive indices 1.36 (ethanol) and 1.50 (starch) for media and sample, respectively. Each sample was analysed in duplicate, and median particle sizes of the geometric volume distributions were calculated based on four parallel results.

Microstructure of Rice Bran Samples

Samples were prepared for microscopy as described in Holopainen-Mantila et al. (2013). In brief, samples were embedded in methacrylate resin after dehydration in ethanol. Two-micrometre-thick sections were cut from the sample blocks and stained with Calcofluor and Acid Fuchsin according to Andersson et al. (2011). The stained sections were examined with a Zeiss Axio Imager M.2 microscope (Carl Zeiss GmbH, Göttingen, Germany). Micrographs were obtained using a Zeiss Axiocam 506 CCD colour camera (Zeiss) and the Zen imaging software (Zeiss). In exciting light (excitation 390–420 nm; emission > 450 nm), intact cell wall glucans stained with calcofluor appear blue and proteins stained with acid fuchsin appear red. Due to autofluorescence, pericarp structures can be observed as brownish yellow structures. Starch is unstained and appears black.

Functional Properties of Bran Fractions

Protein solubility (%) analysis was adapted with modifications from Nivala et al. (2017). In brief, the samples were hydrated in water at 2% protein concentration at room temperature and pH was adjusted to 5 or 8. Additionally, analysis at the pH of the water mixture of each flour (6.8 ± 0.2) was performed. The pH was readjusted at 30 and 60 min after which the supernatants were separated by centrifugation (10,000×*g*, 15 min, 20 °C). The amount of protein released in the supernatant was determined by the Kjeldahl method ($N \times 5.95$). Colloidal stability (CS) was analysed by visual observation of the dispersion sedimentation as a function of time using the following equation: CS (% at *X* min) = height of the colloidal dispersion (*X* min) / height of the total mixture (*X* min). All analyses were performed at least in duplicate.

Statistical Analysis

Statistical analysis of protein content and protein solubility was performed using SPSS Statistics software (version 24, IBM, Armonk, NY, USA). Four replicate results from each experiment were analysed by one-way analysis of variance (ANOVA). The level of significance was set at $p < 0.05$ and was assessed by Tukey's post hoc test.

Results

Microstructure of Rice Grains and Rice Bran

The main grain components present in intact rice grains and in the non-defatted bran were visualized by fluorescence microscopy showing proteins (red), glucan containing cell wall structures (blue), and pericarp (yellowish) (Fig. 2a–d). In the whole rice kernel, a pericarp layer was observed as the outermost structure with a cuticle and an aleurone layer composed of cells containing protein underneath (Fig. 2a). The germ was visible on the other end of the grain. In starchy endosperm, the cell walls were thin and had rectangular shapes, and protein appeared brightly stained. Protein was more abundant in the outer layers of the starchy endosperm or subaleurone and in the germ. Protein in the aleurone layer was less brightly stained (Fig. 2b). The microstructure of the non-defatted rice bran showed presence of large particles (> 300 μm) of endosperm and germ in addition to layers composed of pericarp and aleurone structures (Fig. 2c, d). The long (> 500 μm) pericarp structures had some protein-rich aleurone structures attached to them, but only a few intact protein-containing aleurone cells were present. Most of the protein had partially leaked out from the aleurone cell structures.

The SC-CO_2-extracted rice bran (RB) was used as a raw material in dry fractionation. The only difference between the microstructures of the non-defatted (Fig. 2a–d) and SC-CO_2-extracted (Fig. 2g) non-milled rice bran materials was that the aleurone cells in the SC-CO_2-extracted fraction contained some empty black areas that were not visible in the non-defatted sample. Defatting lowered the lipid content of the bran from 21.8 to 3.2%, and the protein content increased from 15.5 to 18.5% (Table 1). The defatted rice bran contained also 23.5% starch, 6.5% SDF, 30.5% IDF, and 10.5% ash.

One-Step Air Classification of Rice Bran

Pin disc milling of the defatted rice bran, RB, containing 18.5% protein decreased the median particle size from 339

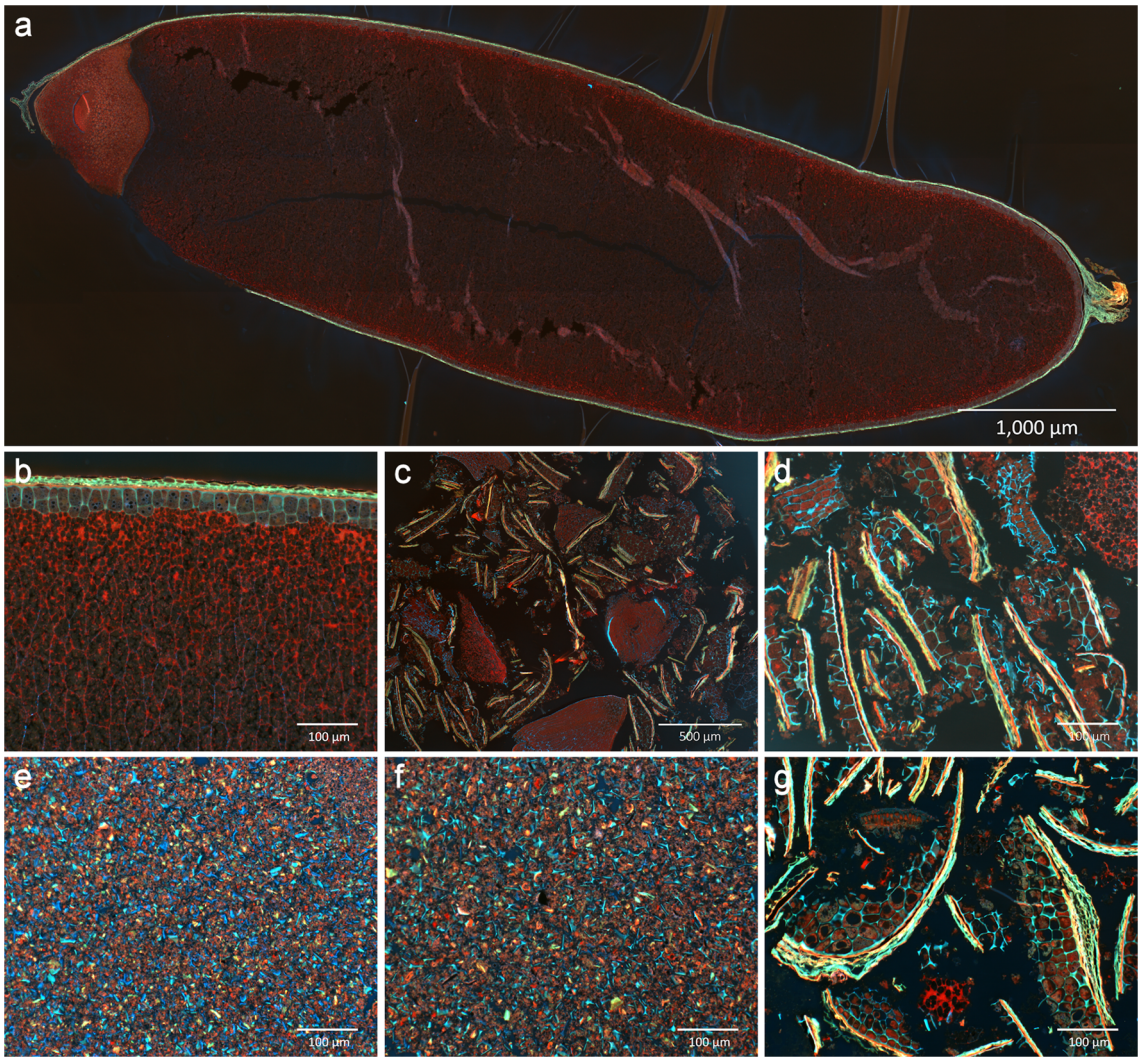

Fig. 2 Microstructure of **a** rice grain, **b** rice grain outer layers, **c**, **d** fresh, non-defatted, and non-milled rice bran, **e** fine fraction 1sF from one-step air classification of SC-CO_2-extracted, milled, rice bran (50 ATP, 21,000 rpm, 50 m^3/h), **f** fine fraction 2sF, and **g** coarse fraction 2sC from air classification of SC-CO_2-extracted, non-milled, rice bran (50 ATP, 10,000 rpm, 50 m^3/h), after staining with calcofluor white and acid fuchsin, showing cell walls blue and proteins red, respectively. Pericarp structures appear yellowish due to autofluorescence. Starch is unstained and appears black

to 62 µm (data not shown). One-step air classification of the milled bran (Fig. 1) resulted in a fine fraction (1sF) significantly ($p < 0.05$) enriched in protein (25.7%) (Table 1) with a median particle size of 5.1 µm (Table 2). The mass yield of the fine fraction 1sF was 27.2%, and protein separation efficiency (PSE) from the raw material was 38.0% (Table 2). Furthermore, efficient protein fractionation between the fine (1sF, 25.7% protein) and coarse (1sC, 15.4% protein) fractions was observed. Finer milling of bran or air classification with higher air classifier wheel speeds and lower air flow values, resulting in smaller fine fraction mass yields, did not result in any higher protein content when the aim was also to retain the protein separation efficiency at higher level than one-third of the raw material protein (data not shown).

Fractionation of starch was more intense compared to protein. The fine fraction 1sF was clearly depleted in starch (7.9%) whereas the coarse fraction 1sC had higher content of starch (30.5%) compared to the raw material (RB, 23.5%) (Table 1). Fractionation of DF was also notable. Raw material contained 37.0% of DF and the 1sF fraction 21.3% of DF. For 1sF, SDF accounted for 33.3% of the total DF whereas for the raw material SDF only accounted for 17.6% of the total DF. Ash was enriched in the fine fraction (25.5%) as compared to the raw material (10.5%). Content of phytic acid was almost three times higher in the 1sF fraction (21.6%) than in the raw material (8.7%). According to the monosaccharide profile analysed after acid hydrolysis, glucose was the most abundant sugar in both raw material and fine fraction. However, glucose content of 1sF (17.0%) was approximately half of the amount present in the raw material (33.0%). The contents of arabinose, galactose, and xylose were also lower in the fine fraction than in the raw material. The amount of water-extractable

Table 1 Composition of SC-CO_2-extracted rice bran raw material and air-classified fractions

	Rice bran, SC-CO_2-extracted	One-step air classification		Two-step air classification, fractions from the first air classification step		Two-step air classification, fractions from the second air classification step	
	Raw material	Fine 1sF	Coarse 1sC	Fine 2sF	Coarse 2sC	Fine 2sCF	Coarse 2sCC
Protein (% dm)[a]	18.5 ± 0.5	25.7 ± 0.5	15.4 ± 0.6	19.7 ± 0.4	18.3 ± 0.3	27.4 ± 0.2	15.9 ± 0.4
Starch (% dm)[b]	23.5 ± 0.2	7.9 ± 0.2	30.5 *	12.9 ± 0.2	23.4 ± 2.2	6.8 ± 0.2	27.8 ± 0.2
Dietary fibre (% dm)	37.0	21.3	43.5 [c]	20.9	32.2	27.2	33.3 [c]
Soluble dietary fibre (% dm)[b]	6.5 ± 0.2	7.1 ± 0.2	6.2 [c]	7.7 ± 0.0	1.8 ± 0.0	6.8 ± 0.2	0.6 [c]
Insoluble dietary fibre (% dm)[b]	30.5 ± 0.3	14.2 ± 0.3	37.3 [c]	13.2 ± 0.2	30.4 ± 0.4	20.5 ± 0.9	32.6 [c]
Ash (% dm)[b]	10.5 ± 0.0	25.5 ± 0.1	n.a.	25.9 ± 0.0	8.4 ± 0.0	21.1 ± 0.3	n.a.
Phytic acid (% dm)[b]	8.7 ± 0.5	21.6 ± 0.0	n.a.	24.5 ± 0.2	n.a.	16.5 ± 0.3	n.a.
Monosaccharide composition after acid hydrolysis (% dm)	47.2	27.2	n.a.	31.2	51.6	30.2	n.a.
Glucose (% dm)[b]	33.4 ± 0.6	16.9 ± 0.6	n.a.	21.3 ± 0.3	36.4 ± 0.3	18.6 ± 0.4	n.a.
Arabinose (% dm)[b]	4.3 ± 0.1	2.4 ± 0.0	n.a.	2.9 ± 0.0	4.8 ± 0.1	3.3 ± 0.1	n.a.
Galactose (% dm)[b]	1.3 ± 0.0	0.9 ± 0.0	n.a.	0.8 ± 0.0	1.4 ± 0.0	1.2 ± 0.0	n.a.
Xylose (% dm)[b]	4,6 ± 0.0	1.8 ± 0.0	n.a.	2.6 ± 0.0	5.2 ± 0.1	1.8 ± 0.0	n.a.
Mannose (% dm)[b]	0.2 ± 0.0	0.2 ± 0.0	n.a.	0.2 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	n.a.
Rhamnose (% dm)[b]	0.1 ± 0.0	0.1 ± 0.0	n.a.	0.1 ± 0,0	0.2 ± 0.0	0.0 ± 0.0	n.a.
Fructose[b]	3.1 ± 0.1	4.8 ± 0.0	n.a	3.3 ± 0.0	3.5 ± 0.1	5.2 ± 0.0	n.a.
Water-extractable arabinoxylans (% dm)[b]	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	n.a.

The dietary fibre loss during air classification has been taken into account in the calculations by assuming that the mass lost during air classification contains the same amount of dietary fibre as the raw material

n.a. not analysed

[a] ± Standard deviation

[b] ± Average deviation

[c] Values have been calculated based on the contents analysed in the raw materials of those fractions and corresponding fine fractions

arabinoxylans was low, 0.2%, in both fine fraction and raw material.

Analysis of the 1sF fraction by fluorescence microscopy (Fig. 2e) revealed that, in addition to proteins, the fraction also contained a notable amount of broken cell wall structures. Only a few pericarp structures and black and unstained starch areas were present. Protein in the fine fraction was stained with two different shades of red colour.

Two-Step Air Classification of Rice Bran

Air classification of the non-milled, SC-CO_2-extracted rice bran was optimized by changing the speed of the air classifier wheel (data not shown) to result in fine (2sF, mass yield 18.0%) and coarse (2sC, mass yield 78.1%) fractions (Fig. 1) showing visible differences for example in colour and particle size (6.5 and 327.3 μm for 2sF and 2sC, respectively) (Table 2). Air classification of the non-milled material resulted in only a very slight protein fractionation (19.7 and 18.3% for 2sF and 2sC, respectively) (Table 1). Starch fractionation was more intense resulting in contents of 12.9 and 23.4% for 2sF and 2sC, respectively. SDF accounted for 36.8% of the total DF in 2sF whereas in the coarse fraction 2sC, it only accounted for 14.3%. Ash was more abundant in 2sF (25.9%) than in 2sC (8.4%), and also phytic acid showed clear enrichment into the fine fraction 2sF. Monosaccharide profiles analysed after acid hydrolysis revealed that the coarse fraction was enriched in glucose, arabinose, galactose, and xylose whereas the fine fraction was depleted in those monosaccharides. Water-extractable arabinoxylans were evenly distributed among the fractions (0.2%).

Based on microscopy, the fine fraction 2sF (Fig. 2f) was mainly composed of loose proteins and broken aleurone cell wall structures, whereas the coarse fraction (Fig. 2g) contained also large, partly intact components of pericarp, germ, and endosperm. Furthermore, remarkably few pericarp structures were found in the fraction 2sF. The fraction 2sC had more protein-containing intact aleurone cells still attached to the

Table 2 Total mass yield, total protein separation efficiency, and particle size of the fractions produced from the SC-CO_2-extracted rice bran raw material using one- and two-step air classification approaches

	One-step air classification		Two-step air classification, fractions from the first air classification step		Two-step air classification, fractions from the second air classification step	
	Fine 1sF	Coarse 1sC	Fine 2sF	Coarse 2sC	Fine 2sCF	Coarse 2sCC
Total mass yield (% dm)[a]	27.2 ± 0.6	65.4 ± 1.0	18.5 ± 1.3	77.8 ± 1.5	13.9 ± 0.1	59.6 ± 1.9
Total protein separation efficiency (% dm)[b]	38.0 ± 0.9	54.6 ± 2.6	19.7 ± 1.7	76.7 ± 2.5	20.2 ± 0.4	50.3 ± 3.3
Particle size median (μm)[b]	5.1 ± 0.1	87.3 ± 3.4	6.5 ± 0.1	327.3 ± 4.7 [c] 84.3 ± 1.6 [d]	5.6 ± 0.2	107.7 ± 1.8

[a] ± Average deviation

[b] ± Standard deviation

[c] Non-milled material

[d] Milled material

pericarp layer (Fig. 2g) than what was observed for the 2sF (Fig. 2f).

Due to the high mass yield of the coarse fraction 2sC, most of the bran proteins (PSE 76.7%) were transferred to this fraction during air classification of the non-milled raw material (Table 2). Grinding of the 2sC was applied to liberate protein from the aleurone layer and germ particles. Air classification of the pin disc–milled 2sC resulted in fine fraction (2sCF) having the highest protein content of 27.4% obtained in this work (Table 1) at a feasible total PSE (20.2%) (Table 2). Additionally, starch was efficiently removed from the fine fraction during this step resulting in a content of 6.8%. An intensive fractionation took place in terms of soluble and insoluble dietary fibre contents. SDF accounted for 25.0% of the total DF in the fine fraction 2sCF but only for 1.8% in the coarse fraction 2sCC. Again, ash and phytic acid showed enrichment into the fine fraction 2sCF, and contents of all monosaccharides were lower in the fine fraction 2sCF than in the raw material of this fractionation step (2sC) (Table 1).

Functional Properties and Protein Profiles of Rice Bran Fractions

Protein solubility and colloidal stability were studied for all the produced fine fractions (1sF, 2sF, and 2sCF) and SC-CO_2-extracted, milled raw material rice bran at three different pH values, namely pH 5, native pH (6.8 ± 0.2), and pH 8. Protein solubility was significantly ($p < 0.05$) higher at alkaline pH values for all samples, and the air-classified fractions had higher solubility at all the studied pH values as compared to the raw material (Fig. 3). At pH 5, the solubility varied between 30 and 46%, and at pH 8, between 67 and 83%. Colloidal stability data showed that stability was clearly better for the fine fractions than for the raw material (Fig. 4). Further, improved stability was observed at pH 6.8 and pH 8, compared to that at pH 5.

The molecular weight profiles of proteins in rice bran fractions analysed by SDS-PAGE in reducing conditions revealed that mostly the same proteins were enriched in all of the fine fractions (Fig. 5). Proteins sizing from 30 to 35 kDa as well as the proteins at 18–20 and 55 kDa were more abundant in all of the fine fractions. On the other hand, raw material and the coarse fractions contained more proteins at 10, 16, 22–25, 50, and 53 kDa.

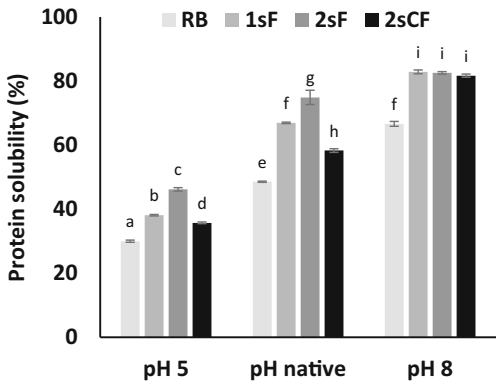

Fig. 3 Protein solubility of the raw material rice bran and all the air-classified fine fractions. RB: SC-CO_2-extracted and milled rice bran raw material; 1sF: fine fraction from the one-step air classification; 2sF: fine fraction from the first step of the two-step air classification; 2sCF: fine fraction from the reclassification of the milled coarse fraction (2sC) from the first step of the two-step air classification. Error bars represent standard deviations of two parallel protein analysis performed after each of the two replicate extractions ($N = 4$). The solubility values were categorized into subsets (**a**–**i**) on the basis of Tukey's test ($p < 0.05$)

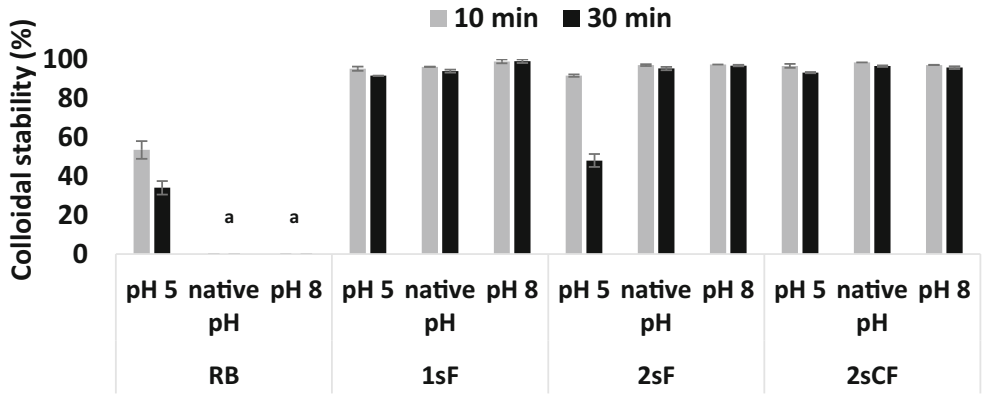

Fig. 4 Colloidal stability of the raw material rice bran and all the air-classified fine fractions at 10- and 30-min time points. RB: SC-CO_2-extracted and milled rice bran raw material; 1sF: fine fraction from the one-step air classification; 2sF: fine fraction from the first step of the two-step air classification; 2sCF: fine fraction from the reclassification of the milled coarse fraction (2sC) from the first step of the two-step air classification. Error bars represent average deviations of duplicate analysis. [a]For the raw material (RB), a clear sedimentation of the large particles at native pH and pH 8 was observed both after 10 and 30 min, but some flour, however, remained in colloidal dispersion

Discussion

Microstructure and Composition of the Rice Bran

Whole rice grains and rice bran were analysed by microscopy to study component distribution within rice kernel. Generally, removal of bran from the starchy endosperm in industrial milling of rice results in a mixture of outer layers of grain, germ, and some endosperm particles (Saunders 1990), and this was true in the current study as well. As expected, based on various earlier milling studies (Juliano 1985; Marshall and Wadsworth 1993; Mitsuda et al. 1967), more protein was located in the outer endosperm layers and starch was enriched in the inner regions of the grain. The proteins in the aleurone layer and starchy endosperm acquired different shades of red when stained with Acid Fuchsin (Fig. 2b) most probably indicating differences in protein classes between the two layers. Bran protein content of 18.5% was consistent with the values of 15.9–23.2% reviewed in literature for defatted rice bran (Juliano and Bechtel 1985). Additionally, starch content (23.5%) and amount of dietary fibre (37.0%) in the bran were in line with the values of 10–20% and 20–30% reported in literature, respectively (Abdul-Hamid and Luan 2000; Juliano and Bechtel 1985; Saunders 1990). Starch indicates the presence of endosperm particles in the bran (Marshall and Wadsworth 1993; Saunders 1990). The same phytic acid content of 8.7% observed in the current bran has also been reported in literature (Lehrfeld 1994).

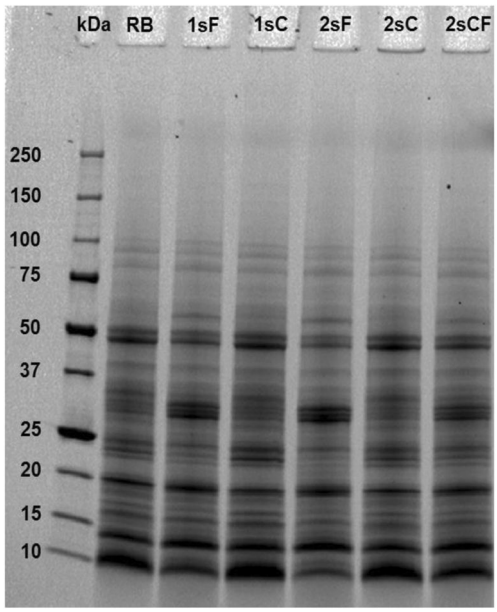

Fig. 5 SDS-PAGE of rice fractions under reducing conditions. RB: raw material rice bran; 1sF: fine fraction from the one-step air classification; 1sC: coarse fraction from the one-step air classification; 2sF: fine fraction from the first classification step of the two-step air classification; 2sC: coarse fraction from the first classification step of the two-step air classification; 2sCF: fine fraction from the second classification step of the two-step air classification

In the current study, a fresh, non-heat stabilising, and defatted rice bran was used as a raw material in dry fractionation trials, since defatting is a prerequisite for milling which is needed in efficient dry fractionation (Schutyser et al. 2015; Sibakov et al. 2011). SC-CO_2 extraction was used for defatting to avoid denaturation of the bran proteins, thus making use of native proteins in the produced fractions possible. The former studies reporting dry fractionation of rice bran have used hexane for defatting the bran (Jayadeep et al. 2009; Saio and Noguchi 1983) or have not specified the fat extraction method used (Park et al. 1993). Solvent extraction of fat applying heating has a negative impact on protein separation, mostly due to the heat-induced aggregation of proteins or complexation of proteins to carbohydrates or other components (Tang et al. 2002). Moreover, solvent extraction requires drying of the product, which may also alter interactions between the components. In addition to solvent

extraction, heat-induced protein aggregation can also take place during the parboiling process applied in commercial rice processing. In the study of Jayadeep et al. (2009), half of the initial rice bran raw material had been parboiled, most probably causing protein denaturation and thus limiting efficient protein separation during dry fractionation. The native structural state of the proteins in the bran used in the present study was supported by the microscopy analysis which showed non-aggregated proteins (Fig. 2c, d). Based on microscopy analysis of a heat-stabilized rice bran, the denatured proteins had a more clustered appearance and formed tighter structural arrangements (data not shown). In analogy to bran raw material, no protein aggregation was observed in microstructure of the SC-CO_2-extracted rice bran (Fig. 2e–g) confirming that SC-CO_2 extraction did not cause protein denaturation in the conditions studied. The only difference observed between the non-defatted (Fig. 2c, d) and SC-CO_2-extracted (Fig. 2e–g) non-milled rice bran was that the protein-rich aleurone cells in the SC-CO_2-extracted sample contained some empty unstained areas that were not visible in the non-defatted sample. Aleurone layer is known to contain most of the bran lipids in a form of lipid bodies (Bechtel and Pomeranz 1977), and the unstained areas visible in the de-fatted samples could be associated with the lipid bodies removed during SC-CO_2 extraction.

Effect of Air Classification on the Composition and Microstructure of Rice Bran Fractions

Dry fractionation of the defatted rice bran was carried out in either one or two steps. In two-step fractionation, air classification was used as a pre-fractionation step to produce two fractions, 2sF and 2sC, from the non-milled SC-CO_2-extracted rice bran raw material. This approach has been successfully applied for example with chia seeds where separation of embryo and perisperm components was obtained after mild milling followed by air classification (Avila Ruiz et al. 2016). Even though air classification of the non-milled rice bran material in the present study did not result in a protein-enriched fraction, microstructural observations and chemical composition revealed an interesting hybrid ingredient fine fraction (2sF) containing protein and DF originating from the aleurone cell wall structures and being free from pericarp particles. In this fraction, the proteins were not entrapped inside the aleurone cells due to the fact that only the aleuronic structures that were broken down already in the raw material bran were transferred to 2sF.

Air classification of the milled raw material directly, or milling and classification of the coarse fraction 2sC (Fig. 1), resulted in protein enrichment from 18.5 to 25.7 (1sF) and 27.4% (2sCF), respectively. The notable particle size reduction as a result of milling indicates disintegration of the bran matrix structure and liberation of proteins from intact aleurone cells and germ particles so that size- and density-based fractionation of the free components by air classification could be efficiently achieved. Native rice proteins are situated in protein bodies sizing from 0.5 to 3.5 μm (Juliano and Bechtel 1985) and are therefore expected to be enriched in the fine fraction during air classification. The higher protein contents compared to those earlier reported in literature (17–20%) most probably resulted from the differences in the pre-treatment steps, such as fat extraction, and from exclusion of the heat stabilization step that is typically applied for rice bran, i.e. the gentle SC-CO_2 extraction seemed to aid in protein separation. Heat-induced protein aggregation that can occur during heat stabilization, parboiling, and solvent extraction, on the other hand, would hinder size-based protein fractionation if the proteins cluster with each other or other components like starch and fibres forming larger particles.

Reduction of the particle size is not always beneficial for separation of components in air classification (Pelgrom et al. 2013). For example, rice starch, when located inside intact amyloplasts, has a particle size diameter of 7–39 μm whereas individual starch granules can be as small as 3–9 μm in diameter (Juliano 1985; Saio and Noguchi 1983). Thus, too efficient grinding of starch can negatively affect the separation efficacy of starch from fine particles such as loose proteins in air classification. In literature, the most successful separation of protein and starch has been obtained for legumes that have a clear difference in sizes of starch granules and protein bodies (Pelgrom et al. 2015), whereas applicability of air classification for cereal protein enrichment remains challenging. Nevertheless, in the present experiment, starch tended to enrich in the coarse fractions suggesting that milling did not result in fragmentation of starch granules as has been reported to be the problem with dry fractionation of legumes (Pelgrom et al. 2013). However, based on microscopy, particle size reduction resulted partly in a too efficient pulverization of cell wall structures and broken cell walls present originally in the raw material, causing their enrichment to fine fraction 1sF or 2sF. The protein separation efficiencies of the fine fraction 1sF and sum of 2sF and 2sCF remained at 38.0 and 39.9%, respectively, showing that more than half of the raw material protein remained in the coarser fractions. This indicates that some interactions are still present between proteins and other bran particles after pin disc milling and air classification. These interactions can be either due to mechanical barriers or chemical interactions and should be studied in more detail. One further consideration in terms of the limited protein enrichment includes the presence of certain amount of protein that is chemically bound to rice bran cell wall hemicellulose (Mod et al. 1978).

In addition to the protein separation, fractionation of carbohydrates was monitored during the air classification. All the produced fine fractions had lower total carbohydrate contents (analysed as monosaccharide contents after acid hydrolysis)

than the bran raw material. The fine fractions contained less total glucose, indicating that the reduction in total carbohydrate content most probably resulted from removal of starch and cellulose from the fine fractions during air classification. Presence of lower amounts of IDF in the fine fractions most probably resulted, in addition to the removal of cellulose, from the removal of lignin. Lignin is known to be present in the pericarp structures that were, based on microscopy, absent in the fine fractions. Since β-glucan content of rice bran is reported to be zero (Juliano and Bechtel 1985) and as water-extractable arabinoxylan contents were low (max 0.3%) in all the studied fractions, it is suggested that the soluble dietary fibre was mainly originating from pectic substances or xyloglucan (Shibuya et al. 1985). As arabinose and xylose contents were higher in the raw material than in the fine fractions, it is clear that water un-extractable arabinoxylans also contributed to the amount of insoluble dietary fibre. Higher arabinose-to-xylose ratios were obtained for the fine fractions than raw material or coarse fraction. Rice endosperm pectin and hemicellulose are reported to have a lower arabinose-to-xylose ratio than bran (Shibuya et al. 1985), giving evidence for the enrichment of aleurone components to the fine fractions in the current work. Interestingly, it was also observed that SDF accounted only for 1.8% in the coarse fraction 2sCC showing that almost all of the soluble rice bran fibre could be recovered in the two fine fractions produced during the developed two-step air classification protocol.

In addition to protein and soluble fibre enrichment, an increase in the ash and phytic acid content in the fine fractions was detected. Enrichment of ash to the smallest particle size fraction, which was also enriched in protein, has also been observed by Jayadeep et al. (2009) who studied the effect of sieving and air classification procedures on the protein fractionation from hexane-defatted rice bran. During the present study, 67.5 and 78.5% of the whole phytic acid present in the raw material bran was transferred to the fine fractions 1sF and sum of 2sF and 2sCF, respectively. In rice grain, phytic acid (*myo*-inositol hexakisphosphate) is known to be situated in the aleurone layer, more specifically in protein bodies (aleurone grains) containing several phytic acid–rich electron dense globoids (Tanaka et al. 1973; Wada and Lott 1997). Thus, elevated amounts of phytic acid in the fine fractions indicate presence of particles originating from aleurone and prove successful separation of aleurone from other grain constituents. Protein bodies in the aleurone layer of rice have been reported to contain only 14% protein based on dry matter, whereas the phytic acid content is 56% (Tanaka et al. 1973). In comparison, protein content of rice endosperm protein bodies is 60% (Mitsuda et al. 1967). Thus, the limited protein enrichment achieved in this study might be attributed to the presence of non-proteinaceous components in the aleurone protein bodies. In addition to the impact of phytic acid on protein fractionation, the effect of phytic acid on the nutritional quality of the fractions should be considered. It is generally known that phytic acid binds minerals and proteins, which reduces their bioavailability and should therefore be examined in the further research (Selle et al. 2000; Kies et al. 2006). Moreover, the possible presence and enrichment of contaminants, such as heavy metals or toxins, that may hinder the applicability of the rice bran fractions for food, should be considered in the further studies.

Effect of Air Classification on the Functional Properties and Protein Profiles of the Rice Bran Fractions

Protein solubility exhibited interestingly high values also at acidic pH where rice bran protein isolates and concentrates have reported to have lowered solubility due to the isoelectric point of the proteins (Zhu et al. 2017; Gnanasambandam and Hettiarachchy 1995). The differences in protein solubility reported in literature for rice bran protein isolates and concentrates compared to the ingredient in the present study most probably resulted from the different protein composition obtained by wet and dry fractionation procedures as well as from the differences in the degree of protein denaturation. In the current study, protein solubility was substantially higher in the fine fractions 1sF, 2sF, and 2sCF than in the non-fractionated bran. Approximately one-third of rice bran proteins are water-soluble albumins (Hamada 1997), and protein solubility of the aleurone protein bodies rich in phytate is reported to be 70% (Tanaka et al. 1973). Additionally, some salt-soluble globulins may be extracted in water due to dissolving of minerals present in rice bran (Villareal and Juliano 1981). In this work, at the native pH of the flour (6.8), the protein solubility of the fine fractions ranged between 58 and 75% whereas it was only 49% for the raw material suggesting somewhat selective enrichment of water-soluble proteins to the fine fractions. Improved solubility of protein in the fine fractions was observed at all the studied pH values. Differences between the solubility values of the fine fractions derived possibly from differences in the composition of the studied fractions. For example, milling and air classification of the coarse fraction (2sC) from the air classification of the non-milled bran most probably resulted in the pulverization of protein-rich endosperm particles that were intact in the non-milled coarse fraction, and during the second air classification, these pulverized particles may have ended up in the fine fraction 2sCF. Rice endosperm protein is mainly glutelin, which has limited solubility in water (Juliano 1985; Zhao et al. 2012). Therefore, the protein-enriched fraction (2sCF) from this coarse fraction (2sC) could have slightly lower protein solubility at all the studied pH values than the fine fraction 2sF. Similar lowered solubility observed for the 2sCF applies even more distinguishably for the raw material containing all the possible proteinaceous structures present in the rice bran, including both

aleurone and endosperm protein bodies. Additionally, small differences in protein solubility could derive from the distinct particle size distribution of the samples. The raw material contained larger particles, and it is possible that the proteins were not liberated from the intact cell wall structures during protein solubility assessment. In the case of the fractions, particle size was remarkably smaller and as seen from the microscopy, no protein structures seemed to be physically entrapped.

Colloidal stabilities of the fine fractions when dispersed in water were superior to the raw material. In the case of colloidal stability, the particle size of the dispersed flours most probably had significant influence as lighter and smaller particles in fine fractions (median particle sizes 5.1–6.5 μm) remained better in dispersion than the large raw material particles (median particle size 61.6 μm). Similarly, for wheat bran samples, the effect of particle size reduction by wet grinding has resulted in improved stability of particles since the gravitational sedimentation has a smaller effect on the disintegrated bran (Rosa-Sibakov et al. 2015).

The protein profiles of the fractions showed some differences which could partly explain differences in protein solubility. Rice flour globulin contains, based on literature, a clear polypeptide of around 55 kDa, which was enriched in the fine fractions in the present study. Due to the fact that rice albumin has been reported to be a heterogeneous protein with molecular weight ranging from 13 to 110 kDa (Amagliani et al. 2017), it is not easy to conclude based on the protein profile whether it was mainly the albumin proteins that were enriched in the fine fraction, thus causing elevated protein solubility. The polypeptides having size of 19–20 kDa were more intensively found in the fine fractions and can derive either from albumin, globulin, or even glutelin fractions of rice protein (Amagliani et al. 2017). Rice glutelins, which are the main endosperm proteins, are reported to be composed of alpha and beta subunits that have sizes of 19–25 and 30–39 kDa, respectively. Some of those components even showed slight enrichment to the fines, thus restricting the total solubility. Rice prolamins have been reported to be composed of subunits sizing 10, 13, and 16 kDa and at least those of 10 and 16 kDa were somewhat enriched in the raw material and coarse fractions. Differences between the profiles of fine fractions most probably derived from the different amounts of endosperm protein present in the fractions.

Conclusions

In the present work, dry fractionation of non-heated, gently SC-CO_2-extracted rice bran allowed production of protein-enriched or pericarp-free ingredients either in one- or two-step approaches. All the studied dry fractionation approaches produced fine hybrid ingredient fractions composing mainly of protein and dietary fibre, and the fractions could therefore have interesting nutritional properties. Higher protein solubility and colloidal stability of the fine fractions compared to the raw material suggest potential use of these fractions in various food systems. To reveal the full potential of the fractions for food applications, they should be studied in more detail in terms of functional properties and nutritional quality. For example, concentration of phytic acid in the protein-enriched fractions should be considered in the further experiments as it may have a negative impact on the protein digestibility in the human digestive tract due to binding of proteins. However, the results are promising for the use of dry fractionation in production of functional and nutritionally interesting rice bran ingredients for food applications.

Acknowledgements Open access funding provided by Technical Research Centre of Finland (VTT). Ilkka Kajala, Atte Mikkelson, Leila Kostamo, Liisa Änäkäinen, Ritva Heinonen, Anna-Liisa Ruskeepää, Lauri Kuutti, and Juha Tähtiharju at VTT are acknowledged for their skilful assistance in the experimental part of the work.

Funding Information The research leading to these results has received funding from the Bio-based Industries Joint Undertaking under the European Union's Horizon 2020 research and innovation programme under grant agreement No. 668953−PROMINENT.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Abbreviations DF, dietary fibre; IDF, insoluble dietary fibre; PSE, protein separation efficiency; SC-CO_2, supercritical carbon dioxide; SDF, soluble dietary fibre

References

Abdul-Hamid, A., & Luan, Y. S. (2000). Functional properties of dietary fibre prepared from defatted rice bran. *Food Chemistry, 68*(1), 15–19.

Aiking, H. (2011). Future protein supply. *Trends in Food Science and Technology, 22*(2–3), 112–120.

Amagliani, L., O'Regan, J., Kelly, A. L., & O'Mahony, J. A. (2017). Composition and protein profile analysis of rice protein ingredients. *Journal of Food Composition and Analysis, 59*, 18–26.

Andersson, A. A. M., Landberg, R., Söderman, T., Hedkvist, S., Katina, K., Juvonen, R., Holopainen, U., Lehtinen, P., & Åman, P. (2011). Effects of alkylresorcinols on volume and structure of yeast-leavened bread. *Journal of the Science of Food and Agriculture, 91*(2), 226–232.

AOAC (1995). AOAC official method 991.43 total, soluble, and insoluble dietary fibre in foods. *Cereal Foods*, 7–9.

Avila Ruiz, G., Arts, A., Minor, M., & Schutyser, M. (2016). A hybrid dry and aqueous fractionation method to obtain protein-rich fractions from quinoa (Chenopodium quinoa Willd). *Food and Bioprocess Technology, 9*(9), 1502–1510.

Bechtel, D. B., & Pomeranz, Y. (1977). Ultrastructure of the mature ungerminated rice (*Oryza sativa*) caryopsis. The caryopisis coat and the aleurone cells. *American Journal of Botany, 64*(8), 966–973.

Day, L. (2013). Proteins from land plants - potential resources for human nutrition and food security. *Trends in Food Science and Technology, 32*(1), 25–42.

Douglas, S. G. (1981). A rapid method for the determination of pentosans in wheat flour. *Food Chemistry, 7*(2), 139–145.

Gnanasambandam, R., & Hettiarachchy, N. S. (1995). Protein concentrates from unstabilized and stabilized rice bran: preparation and properties. *Journal of Food Science, 60*(5), 1066–1069.

Hamada, J. S. (1997). Characterization of protein fractions of rice bran to devise effective methods of protein solubilization. *Cereal Chemistry, 74*(5), 662–668.

Hausalo, T. (1995). Analysis of wood and pulp carbohydrates by anion exchange chromatography with pulsed amperometric detection. In *Proceedings of the 8th ISWPC, June 6–9* (pp. 131–136). Helsinki.

Helm, R. M., & Burks, A. W. (1996). Hypoallergenity of rice protein. *Cereal Foods World, 41*(11), 839–843.

Holopainen-Mantila, U., Marjamaa, K., Merali, Z., Käsper, A., de Bot, P., Jääskeläinen, A. S., Waldron, K., Kruus, K., & Tamminen, T. (2013). Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers. *Bioresource Technology, 138*, 156–162.

Jayadeep, A., Singh, V., Sathyendra Rao, B. V., Srinivas, A., & Ali, S. Z. (2009). Effect of physical processing of commercial de-oiled rice bran on particle size distribution, and content of chemical and biofunctional components. *Food and Bioprocess Technology, 2*(1), 57–67.

Juliano, B. O. (1985). Polysaccharides, proteins and lipids of rice. In *Rice Chemistry and Technology* (Second Edition, pp. 59–174).

Juliano, B. O., & Bechtel, D. B. (1985). The rice grain and its gross composition. In *Rice Chemistry and Technology* (Second Edition, pp. 17–57).

Kies, A. K., de Jonge, L. H., Kemme, P. A., & Jongbloed, A. W. (2006). Interaction between protein, phytate, and microbial phytase. In vitro studies. *Journal of Agricultural and Food Chemistry, 54*(5), 1753–1758.

Kuk, M. S., & Dowd, M. K. (1998). Supercritical CO_2 extraction of rice bran. *Journal of the American Oil Chemists' Society, 75*(5), 623–628.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.

Latta, M., & Eskin, M. (1980). A simple and rapid colorimetric method for phytate determination. *Journal of Agricultural and Food Chemistry, 28*, 1315–1317.

Lehrfeld, J. (1994). HPLC separation and quantitation of phytic acid and some inositol phosphates in foods: Problems and solutions. *Journal of Agricultural and Food Chemistry, 42*(12), 2726–2731.

Marshall, W. E., & Wadsworth, J. I. (1993). *Rice science and technology.* CRC Press.

Mitsuda, H., Yasumoto, K., Murakami, K., Kusano, T., & Kishida, H. (1967). Studies on the proteinaceous subcellular particles in rice endosperm: Electron-microscopy and isolation. *Agricultural and Biological Chemistry, 31*(3), 293–300.

Mod, R. R., Conkerton, E. J., Ory, R. L., & Normand, F. L. (1978). Hemicellulose composition of dietary fiber of milled rice and rice bran. *Journal of Agricultural and Food Chemistry, 26*(5), 1031–1035.

Nivala, O., Mäkinen, O. E., Kruus, K., Nordlund, E., & Ercili-Cura, D. (2017). Structuring colloidal oat and faba bean particles via enzymatic modification. *Food Chemistry, 231*, 87–95.

Noguchi, A., Sato, O., Haque, Z., & Saio, K. (1981). Preparation of protein rich flour from rice by air classification and electrostatic separation. *Nippon Shokuhin Kogyo Gakkaishi, 28*(8), 405–411.

Park, D.-J., Ku, H.-K., & Mok, C. (1993). Microparticulation / air classification of rice bran: Characteristics and application. *Korean Journal of Food Science and Technology, 25*(6), 769–774.

Pelgrom, P. J. M., Vissers, A. M., Boom, R. M., & Schutyser, M. A. I. (2013). Dry fractionation for production of functional pea protein concentrates. *Food Research International, 53*(1), 232–239.

Pelgrom, P. J. M., Boom, R. M., & Schutyser, M. A. I. (2015). Method development to increase protein enrichment during dry fractionation of starch-rich legumes. *Food and Bioprocess Technology, 8*(7), 1495–1502.

Rosa-Sibakov, N., Sibakov, J., Lahtinen, P., & Poutanen, K. (2015). Wet grinding and microfluidization of wheat bran preparations: Improvement of dispersion stability by structural disintegration. *Journal of Cereal Science, 64*, 1–10.

Saio, K., & Noguchi, A. (1983). The microstructure of polished, milled and air-classified rice and rice bran. *Nippon Shokuhin Kogyo Gakkaishi, 30*(6), 331–338.

Saunders, R. M. (1990). The properties of rice bran as a foodstuff. *Cereal Foods World, 35*(7), 632–636.

Schutyser, M. A. I., Pelgrom, P. J. M., van der Goot, A. J., & Boom, R. M. (2015). Dry fractionation for sustainable production of functional legume protein concentrates. *Trends in Food Science & Technology, 45*(2), 327–335.

Selle, P. H., Ravindran, V., Caldwell, R. A., & Bryden, W. L. (2000). Phytate and phytase: Consequences for protein utilisation. *Nutrition Research Reviews, 13*(2), 255–278.

Shibuya, N., Nakane, R., Yasui, A., Tanaka, K., & Iwasaki, T. (1985). Comparative studies on cell wall preparations from rice bran, germ, and endosperm. *Cereal Chemistry, 62*(4), 252–258.

Sibakov, J., Myllymäki, O., Holopainen, U., Kaukovirta-Norja, A., Hietaniemi, V., Pihlava, J. M., Poutanen, K., & Lehtinen, P. (2011). Lipid removal enhances separation of oat grain cell wall material from starch and protein. *Journal of Cereal Science, 54*(1), 104–109.

Silventoinen, P., Sipponen, M. H., Holopainen-Mantila, U., Poutanen, K., & Sozer, N. (2018). Use of air classification technology to produce protein-enriched barley ingredients. *Journal of Food Engineering, 222*, 169–177.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). *NREL standard, determination of structural carbohydrates and lignins in biomass, laboratory analytical procedure (LAP)*. National Renewable Energy Laboratory: Golden.

Tanaka, K., Yoshida, T., Asada, K., & Kasai, Z. (1973). Subcellular particles isolated from aleurone layer of rice seeds. *Archives of Biochemistry and Biophysics, 155*(1), 136–143.

Tang, S., Hettiarachchy, N. S., & Shellhammer, T. H. (2002). Protein extraction from heat-stabilized defatted rice bran. 1. Physical processing and enzyme treatments. *Journal of Agricultural and Food Chemistry, 50*(25), 7444–7448.

Thiex, N. J., Manson, H., Andersson, S., & Persson, J.-A. (2002). Determination of crude protein in animal feed, forage, grain, and oilseeds by using block digestion with a copper catalyst and steam distillation into boric acid: Collaborative study. *Journal of AOAC International, 85*(2), 309–317.

Vaintraub, I. A., & Lapteva, N. A. (1988). Colorimetric determination of phytate in unpurified extracts of seeds and the products of their processing. *Analytical Biochemistry, 175*(1), 227–230.

Van Der Goot, A. J., Pelgrom, P. J. M., Berghout, J. A. M., Geerts, M. E. J., Jankowiak, L., Hardt, N. A., Keijer, J., Schutyser, M. A. I., Nikiforidis, C. V., & Boom, R. M. (2016). Concepts for further sustainable production of foods. *Journal of Food Engineering, 168*, 42–51.

Villareal, R. M., & Juliano, B. O. (1981). Properties of albumins of milled rice. *Phytochemistry, 20*(8), 1785–1789.

Wada, T., & Lott, J. N. A. (1997). Light and electron microscopic and energy dispersive X-ray microanalysis studies of globoids in protein bodies of embryo tissues and the aleurone layer of rice (*Oryza sativa* L.) grains. *Canadian Journal of Botany, 75*(7), 1137–1147.

Wang, M., Hettiarachchy, N. S., Qi, M., Burks, W., & Siebenmorgen, T. (1999). Preparation and functional properties of rice bran protein isolate. *Journal of Agricultural and Food Chemistry, 47*(2), 411–416.

Wang, J., Suo, G., de Wit, M., Boom, R. M., & Schutyser, M. A. I. (2016). Dietary fibre enrichment from defatted rice bran by dry fractionation. *Journal of Food Engineering, 186*, 50–57.

Wouters, A. G. B., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive Reviews in Food Science and Food Safety, 15*(4), 786–800.

Zhao, Q., Selomulya, C., Xiong, H., Chen, X. D., Ruan, X., Wang, S., Xie, J., Hailong, P., Sun, W., & Zhou, Q. (2012). Comparison of functional and structural properties of native and industrial process-modified proteins from long-grain indica rice. *Journal of Cereal Science, 56*(3), 568–575.

Zhu, S. M., Lin, S. L., Ramaswamy, H. S., Yu, Y., & Zhang, Q. T. (2017). Enhancement of functional properties of rice bran proteins by high pressure treatment and their correlation with surface hydrophobicity. *Food and Bioprocess Technology, 10*(2), 317–327.