Optimization of Polysaccharide Extraction and Its Composition in Pre-Germinated Brown Rice (pre-GBR)

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Abstract: The polysaccharides from pre-germinated brown rice (pre-GBR) was studied by water extraction and alcohol precipitation procedure. Besides the changes of proximate compositions in pre-GBR, the monosaccharide composition of pre-GBR were analyzed with gas chromatography(GC). A set of optimized water soaking extraction parameters were formed by analyzing and optimizing several major influential factors :extraction temperature of 95 ℃, extraction time of 4h, solid -liquid ration of 1:30 (w/v), and water extraction 4 times. Three groups were obtained from pre-GBR by hot water soaking extraction and alcohol precipitation: PGBRS-30, PGBRS-50 and PGBRS-80 group through different concentration of ethanol. PGBRS was composed of glucose, ribose, arabinose and mannose. The monosaccharide composition of PGBRS-30 and PGBRS-50 were similar with monosaccharide of xylose and the PGRS-80 group had less xylose.
Keywords: Pre-germinated brown rice (pre-GBR); Polysaccharides; Extraction; Monosaccharide composition

1.Introduction

Brown rice is a healthy option compared to the white rice mainly because there only hull of the kernel is removed pre-germinated brown rice (pre-GBR)is produced by soaking the whole kernel of brown rice in water until its embryo begins to bud[1].As the chemical compositions of the brown rice change drastically during the germination process [2], pre-GBR has been gaining a great deal of attention in recent years, especially in Asian countries [3]. Pre-GBR food products are easily and contain decomposed forms of high-molecular-weight polymers[4,5]. Moreover, the improvement of organoleptic qualities due to softening of texture and the increase in the amount of flavor components make it process-ready and cook[6].

Along with changing level of nutrients and increasing amount of essential amino acids, peptides and simple sugars, more importantly bioactive compounds were found in pre-GBR. The antioxidants substances of physiological and pharmacological functions significant improvement such as gamma aminobutyric acid (GABA), ferulic acid, oryzanol and prolylendopeptidase inhibitor[7,8]. Consumption of pre-GBR implies numerous health benefits that include antihyperlipidemia, antihypertension, psychosomatic improvements. Pre-GBR, is also known to reduce the risk of some chronic diseases, e.g., cancer, diabetes, and different cardio vascular diseases [6，8].

Recently, much attention has been paid to the study of plant polysaccharides for their immunologic and biological effects[9,10]. Although a wide number of studies have documented the advantage and bioactivity of pre-GBR, characterization of the exact composition of Pre-GBR polysaccharides is overdue. Some studies reported that the grain and bran layer of pre-GBR are composed of starch or no-starch polysaccharides, however, the functional polysaccharides in pre-GBR is relatively less mentioned[11]. The objectives of this study are to evaluate the optimum extraction procedure of water-soluble polysaccharide and to determine their composition.This study also looks at the changes of the polysaccharide's proximate compositions, to provide reference information for further study on pre- GBR polysaccharides.

2. Materials and methods

2.1 Materials and reagents

Paddy rice of oryza sativa L., cultivar Huai-6 was purchased from a local rice-milling factory in Jiangsu province, China.

Analytical grade monosaccharide standards were purchased from Beijing Biological Technology Co. Ltd, the standard glucose solution was prepared in our laboratory, Methanol was used for chromatographically grade and all the other chemicals were obtained from Tanggu chemical Reagent Company (Tianjin, China).

2.2 Pre-GBR and its extract powder preparation

The preparation of pre-GBR samples were conducted following the methods of Suzuki and Maekawa [12].The brown rice was prepared by removing the husk of the paddy rice using a laboratory de-husker, the germination was initiated using distilled water soaking for12h, and the process took place in a germinating chamber for 24h period at 28–30℃. The relative humidity of the chamber was 90–95% controlled by an automatic sprinkler. The germination rate was above 99±1%, and the pre-GBR were dried at 50℃ for 3h [13] and then ground into powder using a mill. All samples passing through a 100-mesh sieve were packed in hermetically sealed plastic bags and stored at 4 ℃.

2.3 Determination of proximate compositions in non-GBR and pre-GBR

Moisture contents of the samples were determined by oven-drying at 105 ℃ to a constant weight. The crude protein content was calculated from nitrogen content, using the Kjeldahl method (Gerhardt, Germany)and multiplied by a factor of 5.95. Total free amino acids (TFAA),crude fat and free fatty acid(FFA)content were determined in accordance with the standard methods described by Association of Official Analytical Chemists(AOAC)[14].Crude fat was measured by extracting the ground rice samples with petroleum ether using the Soxhlet apparatus. FFA was extracted in a benzene solution, and the extracted solution was titrated with potassium hydroxide to detect by a gas chromatograph[15].The total sugar and reducing sugar were determined using phenol–sulphuric acid method with d-glucose as standard followed Dubois's [16].

All measurements were triplicated for accuracy and expressed as a percent of dried matter(DM) basis.

2.4 Extracted of pre-GBR polysaccharides

The pre-GBR powder (10g) was degreased with petroleum ether reflux for 6 h at 60±2℃ using the Soxhlet extractor, following by 95% ethanol immersing reflux for 1h at 70±2 ℃ to remove fats. The defatted pre-BGR powder was diluted and extracted at different temperature, time, solvent/material ratio and repeated extraction times as single-factor-test. The mixtures were centrifuged at 4000 revolutions per minute (rpm) for 10 min, the supernatant was added together and concentrated to one third of the original volume with a rotary evaporator at 80±1 ℃ under vacuum. After cooling, the proteins in the extract were removed using the Sevag reagent (n-butanol/chloroform, 1:4), and anhydrate ethanol were added to a final concentration of 80% (v/v). The mixture was kept at 4℃ for 24 h, and then centrifuged at 4000 rpm for 10 min. After collecting the precipitate, that was washed with 95% ethanol. Further washing was done using anhydrate ethanol to obtain crude polysaccharides. All the samples were lyophilized to analyze.

The flow chart can be shown as following:

Pre-powder→ defatted → water extraction→ supernatant concentration →protein removing →precipitated by ethanol→ washed by anhydrate ethanol →washed by acetone →washed by petroleum ether→collection lyophilized.

2.5 Determination of pre-BGR polysaccharide

The crude pre-BGR polysaccharide was dissolved in distilled water. The composition of the polysaccharide was determined with the anthrone-sulfuric acid method[17]. First, the polysaccharide solution was diluted to appropriate concentration:2mL of diluted polysaccharide solution was removed to mix with 1 mL of phenol test solution. After10 min,5-mLof concentrated sulfuric acid was added into the solution. The mixture was then vortexed and left to stand for 20 min. The concentration of pre-BGR polysaccharides was determined by UV-spectrophotometry at 490 nm [18]. The measurementswere conducted in triplicates.

The concentration of polysaccharidesolution was computed employing regression equation with glucose concentration of X and absorbance of A as:

$A = 71.53X - 0.0174$ (correlation coefficient $r = 0.9934$).

Extraction rate of polysaccharide (%) = theconcentration ×the volume of polysaccharide solution/ crude polysaccharide dry weight×100%

2.6 Optimization of extraction conditions

Single-factor-test was employed to determine the preliminary range of the extraction variables including extraction temperature, extraction duration, solid–liquid ratio and number of times extraction needed.

Extraction temperatures considered are60℃, 70℃, 80℃, 90℃ and 95℃; at each of these temperatures,water soaking for 1h and solid-liquid ratio maintained was 1:30.

Extraction time was conducted by soaking defatted pre-GBR powder for 1h, 2h, 3h, 4h and 5h; during each of these extraction, solid-liquid ratio 1:30 at 80℃.

Solid-liquid ratio were ranged from 1:20 to 1:50; for each of these solid-liquid ratios,water soaking duration was 1h at 80℃.

Different extraction numbers were performed to observe the effect of this parameters on the outcome of the experiment. Each of these extractions times is conducted by solid-liquid ratio of 1:30, soaked in 80℃ water for 1h, then centrifuged at 4000 rpm for 10 min. After first cycle, the supernatant was collected, and the precipitate was repeated extraction as same steps, and the supernatant was added combined from first and second step. The same procedure was repeated 3, 4, and 5 times to get five sets of data in total.

An orthogonal test was set up according to the L9 (3^4) orthogonal table. The extraction rate of polysaccharides was used as an indicator. Using four a fore mentioned variables, i.e., temperature, extraction time, solid-liquid ratio, and extent of extraction the experimental matrix was set up.

2.7 Purification

The purification of pre-BGR polysaccharides was followed the method of Yang's [19]. Anhydrous ethanol was added to the concentrated polysaccharide solution to further fractionate by incremental increases of ethanol concentration including 30, 50 and 80% (v/v) [20]. To prepare PGBRS-X polysaccharide, the final concentration of X% (v/v)was kept at 4 ℃ for 12h and centrifuged at 8000 rpm for 10 min, then the precipitate was collected and washed twice with anhydrous ethanol. After all these processes, the clean precipitate was dried to obtain PGBRS-X.

2.8 Analysis of monosaccharide's by gas chromatography

The group of afore mentioned hydrolyzed polysaccharide samples and mixing standard monosaccharide 5 mg were added into 10 mg hydroxylamine hydrochloride, 2 mg internal standard- insitolhexa acetate and 0.5 mL pyridine and 0.5 mL acetic anhydride to be acetylated[21].The sample and mixed monosaccharide standards were separated by a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan) fitted with a fused silicacapillary column (DB-17, Agilent Technologies, Inc., Santa Clara, CA,USA; 30 m×0.25 mm×0.25μm) and hydrogen flame ionization detector(HFID).The injector and detector both were set at 280 ℃while oven temperature was 190◦C. Nitrogen was used as the carriergas, at a flow rate of 20.0 mL/min.

2.9 Statistical Analysis

Data were expressed as mean±standard deviation of three replicated determinations. A one way of variance analysis (ANOVA) was used to determine the significance of differences between treatments (SPSS 16.0). Statistical significance was set at $p<0.05$.

3. Results and discussions

3.1 Proximate compositions in non-GBR and pre-PBR

There was a significant increase of crude protein, total free amino acids, sugars and free fat acidcontents after brown rice germination(Table1). During the germination process, some enzymes are activated and some non-protein nitrogen substances transformed into organic protein nitrogen substances, and with degradation of protein and crude fat, the free amino acid and free fat acid increased, there were many similar reports on increasing amino acid and fat acid[22,23], However, available literature do not have much discussion regarding the changes of total sugar during the germination of brown rice, it showed that there was significant increase of total sugar(above11.91%) whereas no difference in reducing sugar, these increased total sugar maybe come from the germ and bran composition sugar which were degraded.

TABLE 1. Proximate components of un-germinated (UGBR) and pre-germinated brown rice (pre-GBR)

Parameter	Crude protein	TFAA	Total sugar	Reducing sugar	Crude fat	Free fat acid
UGBR(%)	8.51±0.05	1.54±0.35	74.62±0.05	10.41±0.03	1.07±0.03	1.46±0.15
Pre-GBR(%)	9.37±0.05 [a]	2.01±0.33 [a]	83.51±0.35 [a]	11.97±0.05	1.11±0.03	1.97±0.05 [a]

Note: TFAA = total free amino acids, the letter means the significant difference in the same line at $p < 0.05$.

3.2 Single factor experiment

Single factor analysis showed that the yield of polysaccharides increased with the increasing extraction temperature, number of extractions, water soaking duration, and showed a single peak curve with the increasing of solid/liquid ratios (Fig.1). The extraction temperature, time and water soaking extraction times exhibited positive correlation with the yield of polysaccharide of pre-GBR. With increasing temperature, a continuous increase of the polysaccharide yield was observed. The maximum polysaccharide yield was achieved at 95℃

among the three temperature tested.

Just like temperature, increase in extraction duration and extraction cycle resulted higher yield. With an increase of extraction duration from 1h to5h, an apparent increase of PBGR yield was observed. However, this trend doesn't holdfor the increase from 4h to 5h (Fig.1b). Along the similar vein, the yield increasing of PBGR was not rapid as the extraction times changed from 4 to 5 times (Fig1d), therefore, other number of cycles 3, 4, and 5 were chosen as three levels of orthogonal experiment. In case of solid-liquid ratio, the pre-GBR yield significantly rises until the solid - liquid ratio reaches 1:30(Fig.1c),beyond that a plateau happens. Therefore, at solid-liquid ratio of 1:30, the highest yield occurs; also, the yield at1:50 ratio's close to that of the 1:40. This can be attributed to the fact that liquid to solid ratio exceeds a certain value, other substances become more soluble than polysaccharide and hinder the dissolution of the polysaccharide. Therefore, the solid to liquid ratio of 1:20, 1:30 and 1:40 were selected as three levels of orthogonal experiment.

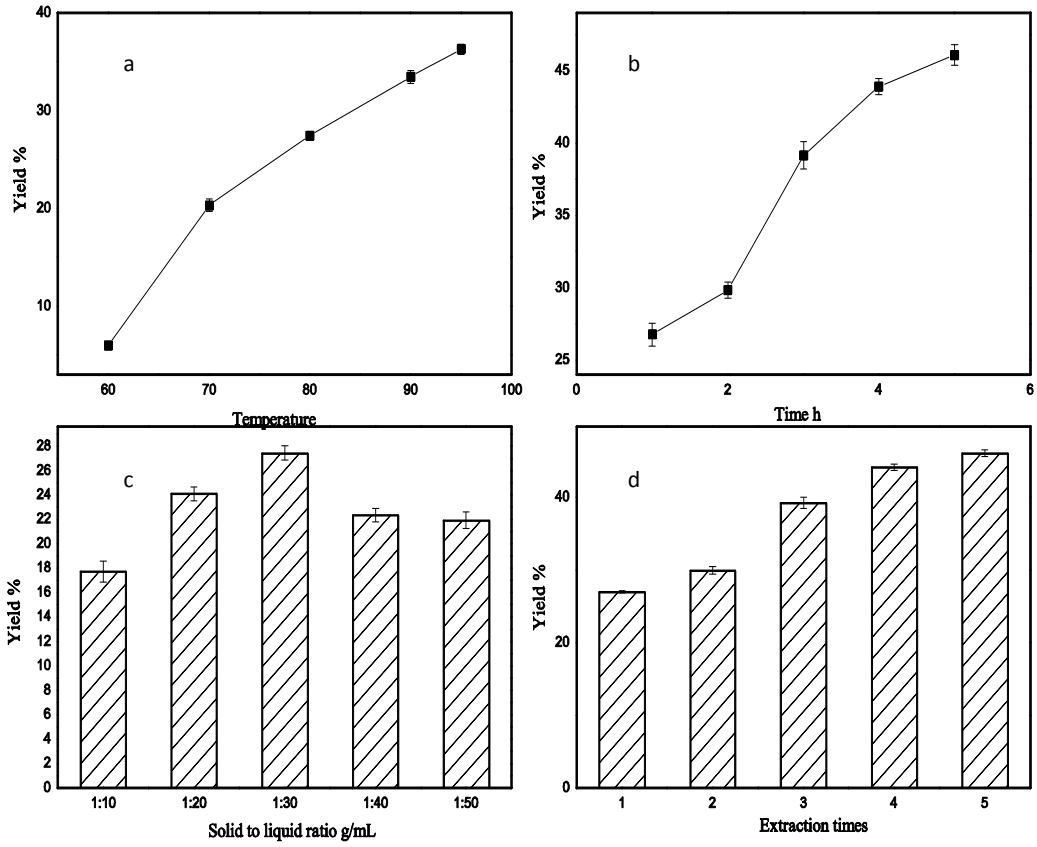

FIG.1. Effect of single factor on polysaccharides yield (a :temperature, b:time, c:solid to liquid ratio, d: extraction times)

3.3 Performance of orthogonal design

Based on the single-factor test described above, the orthogonal table of $L_9(3^4)$ was selected to determine the optimum extraction conditionin the orthogonal test, which was based on three levels as shown as Table 2:

TABLE 2. Levels and factors table of orthogonal experiment

Level	Temperature(℃) A	Time(h) B	solid-liquid ratio(mL/g) C	Times D
1	80	3	1:20	3
2	90	4	1:30	4
3	95	5	1:40	5

The results of orthogonal design and variance analysis showed that primary and secondary factor on pre-germinated brown rice polysaccharides yield (Table 3), extraction temperature (A)> solid to liquid ratio (C)> extraction time (B)> extraction times (D). The optimum conditions are shown by the K value of the four factors: $A_3C_2B_1D_2$, the actual factors are temperature of 95℃solid-liquid ratio of 1:30, extraction time of 3h, and extraction cycle of 4. Further experiments showed that the yield of water-soluble polysaccharides of pre-GBR was 44.01% using the best conditions of orthogonal design.

TABLE 3. Results of orthogonal test of polysaccharides of pre-BGR

Number	A	B	C	D	Yield/%[a]
1	1	1	1	1	26.65
2	1	2	2	2	31.43
3	1	3	3	3	21.23
4	2	1	2	3	32.17
5	2	2	3	1	26.10
6	2	3	1	2	21.36
7	3	1	3	2	42.01
8	3	2	1	3	34.49
9	3	3	2	1	40.75
K_1	26.437	33.61	27.5	31.167	
K_2	26.543	30.673	34.783	31.6	
K_3	39.083	27.78	29.78	29.297	
R	12.646	5.83	7.283	2.303	

[a] The yields were calculated based on produced pre-germinated brown rice crude polysaccharides(g)/ germinated brown rice powder(g)

For the reaction dynamics of solid/liquid extraction, the extractioneffect of plant tissue was mainly determined by the penetration speed of the active substance from the inside of the cells to the surface. Rice (Oryza sativa L.) had been consumed for starchy food and most of the storage polysaccharides in seeds was soluble in water.

Energy consumption should be considered in the practical process, the traditional hot-water soaking extraction may save natural sources and was much effective in yield of extraction, so water extraction methods had been apply in many plant polysaccharide [24,25],especially in soaking pre-germination seeds, hot water extraction would be an fit method in research the composition of pre-GBR, the optimum extraction process in our study had some advantages and much value in production.

3.4 GC analysis of monosaccharides

The monosaccharides found in PGBRS-30, PGBRS-50, and PGBRS-80 were determined to be ribose or rhamnose, arabinose, mannose and glucose, the most dominant monosaccharide is glucose in PGBRS, groups of PGBRS-30 and PGBRS-50 have xylose, whereas the PGBRS-80 has not. Three groups have some other unknown peaks which may be impurities or some other pentoses and hexoses in addition to the above-described monosaccharides.

Polysaccharides, including structural and storage polysaccharides, are very important component of plant cell wall and energy substance of starchy seeds; it also possesses beneficial bioactivity for human health[17]. It is to be mentioned here that the structural characteristics are highly related to the bioactivity. For pre-GBR polysaccharides, the glucose is the major monosaccharide's that provide energy in metabolism. Similar result is reported in water-soluble polysaccharides, when isolated from the emergency barley and wheatseeds[26].

4. Conclusions

There was a significant increase of crude protein, total free amino acids, sugars and free fat acid contents in pre-GBR. The optimal technology of germinated brown rice polysaccharide extraction is water bath temperature 95℃, solid to liquid ratio of 1:30, extraction time of 4 h, repeat 4 times. Under these conditions, the polysaccharide obtained at the best extraction effect rate is 44.01%. The influential parameters of extraction procedure including water bath temperature, extracting time, solid to liquid ratio, each extraction time. the most of water-extraction were the main four factors in pre-GBR extraction.

Three sections of alcoholpurification were obtain from polysaccharides of pre-GBR, themonosaccharide composition of PGBRS-30, PGBRS-50 and PGBRS-80 were rhamnose, arabinose, mannose and glucose, monosaccharide may contain non-standard items, and PGBRS-30, PGBRS-50 also containing xylose. Three samples also had some other unknown peaks, which may happen due to presence of some impurities or some other pentoses and hexoses.

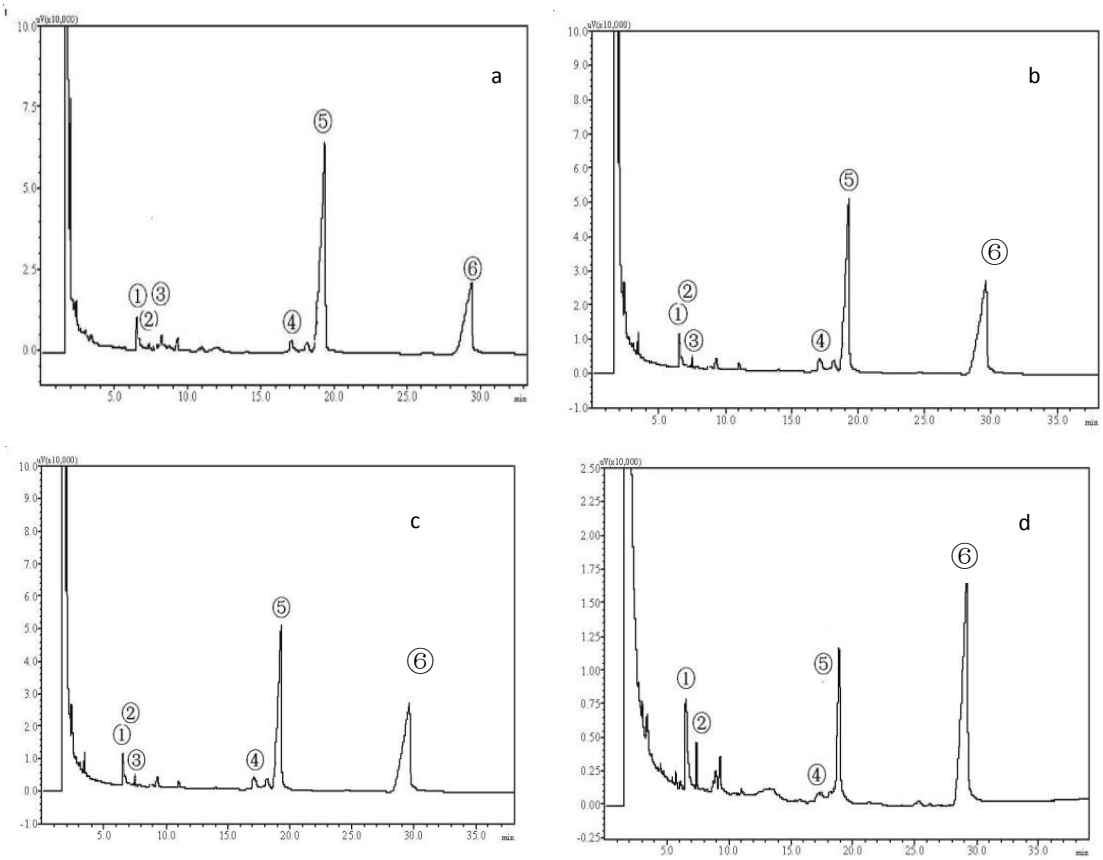

FIG. 2 **GC profiles of monosaccharides of (a)standard sample, (b)PGBRS-30,(c) PGBRS-50and (d) PGBRS-80.**

Note: ①ribose and rhamnose ②arabinose ③xylose ④mannose ⑤glucose ⑥galactose

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