Antioxidative Ability of Defatted Rice Bran Extract Obtained by Subcritical Water Extraction in Bulk Oil and Aqueous Dispersion Systems

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Defatted rice bran was treated with subcritical water between 120 and 240°C. The extraction temperature affected the total carbohydrate and protein contents of the extracts. In the tested temperature range, the total carbohydrate and protein contents were the highest at 180°C and 210°C. The DPPH radical scavenging activity of the extracts increased with increasing extraction temperature up to 240°C and showed a linear correlation with the total phenolic content. The antioxidative ability of the extracts was evaluated in both the bulk oil and aqueous dispersion systems of linoleic acid. The antioxidative ability differed in the two systems. The extract obtained at any temperature elongated the induction period for the oxidation of linoleic acid in the aqueous dispersion system. However, only the extract obtained at 240°C showed significant antioxidative ability in the bulk linoleic acid system.

Key words: defatted rice bran, subcritical water, antioxidative ability, bulk oil system, aqueous dispersion system

1. Introduction

In all commodities, paddy rice ranked fourth in the world for its production quantity of 678.7 million metric tons in 2009 [1]. The husk and rice bran, accounting for approximately 20 and 10% on weight basis of rice grains, respectively [1], are usually milled off the grains to obtain white rice. After being further treated with hexane to extract the bran oil, most defatted rice bran is discarded as agro-waste besides only a small part of it being utilized for feed or fertilizer. Defatted rice bran contains several functional substances such as dietary fiber [2], hypoallergenic protein [3], and phenolic compounds [4], thus making it a potential material for health foods.

Extraction by means of subcritical water, which maintains its liquid state under pressurized conditions from the boiling point under atmospheric pressure (100°C) to the critical point (374°C, 22.1 MPa), is relatively environmentally friendly compared to the processes that employ organic solvents as the extractants. The extraction ability of subcritical water lies in its characteristic properties of low relative dielectric constant and high ion product [5]. Subcritical water extraction involves dissolution and/or degradation of the treated materials. In our previous studies, the defatted rice bran extracts obtained by subcritical water extraction exhibited emulsifying and antioxidative abilities [6-8]. The antioxidative ability was investigated in bulk [8] and encapsulated [9] oil systems by using linoleic acid and methyl linoleate, respectively, as the substrate for oxidation. Despite the fact that defatted rice bran extracts are comprised of relatively hydrophilic substances and may hence exercise presumably higher antioxidative ability to retard the oxidation of oil in aqueous dispersion system, no study has to date been published looking into this matter.

In this study, we performed batch-wise subcritical water extraction on defatted rice bran in a pressure-resistant vessel at different temperatures. The effects of extraction temperature on the composition and antioxidative property of the extracts were studied in terms of the estimated yield and the total carbohydrate, protein, and total phenolic contents. The DPPH radical scavenging activity was estimated as well to study its correlation with the total phenolic content of the extracts. Estimation of the induction period for oxidation of bulk linoleic acid and investigation on the oxidation of linoleic acid dispersed in SDS buffer solution were performed to evaluate the antioxidative ability of the extracts in both the bulk oil and aqueous dispersion systems.
2. Materials and Methods

2.1 Materials

The defatted rice bran (*Oryza sativa*) was supplied by Tsuno Foods Industrial Co., Ltd. (Wakayama, Japan). The rice bran oil was earlier extracted using petroleum ether by the Soxhlet extraction according to the official method of AOCS Ba 3-38 [10]. The contents of oil, ash, water, and total carbohydrate were 0.921, 12, 12.8, and 58.5% by weight, respectively. D-(+)-Glucose (>98%), crystalline bovine serum albumin (abbreviated BSA, >98%), sodium dodecyl sulfate (SDS), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Ascorbic acid (>99.5%), sodium hydroxide, and sodium carbonate were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Gallic acid and Folin-Ciocalteu’s phenol reagent were from Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan) and ICN Biomedicals Inc. (Aurora, OH, USA), respectively. Unless otherwise noted, distilled water was used in the entire study.

2.2 Subcritical water extraction on defatted rice bran

Extraction was performed in a TVS-N2 stainless steel pressure-resistant vessel (≤20 MPa, Taiatsu Techno Corp., Osaka, Japan) with a working volume of 117 mL (30 mm i.d. × 165.5 mm height). Three grams of the defatted rice bran was added with 72 mL of distilled water into the vessel and heated to 120, 150, 180, 210, and 240°C using a mantle heater connected to a TXN-700B program temperature-controller (As One, Osaka, Japan). Although the pressure inside the vessel was not measured, it is estimated to be 0.33 to 3.6 MPa at 120 to 240°C from the vapor pressure of water and the expansion of air in the head space. The vessel was held at the extraction temperatures for 5 min and then immediately immersed into an ice bath to quench the extraction. The fluid extract was subjected to a two-step filtration: first filtration through a No. 5A, 40 μm filter paper (Kiriyama Glass Co., Ltd., Tokyo, Japan) and second filtration through a cellulose ester membrane filter with a pore size of 0.45, 3, or 5 μm (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) which was held in a KS-25 stainless syringe holder (Toyo Roshi Kaisha, Ltd.) mounted on a 50-mL syringe (TOP Corp., Tokyo, Japan). The filtrates were stored in amber glass bottles at 5°C with nitrogen-displaced head space for the following analyses.

2.3 Determinations of defatted rice bran extracts

2.3.1 Yield

The yield is defined as the weight of dry extract estimated for the initial volume of extractant (72 mL of distilled water) on the weight basis of the defatted rice bran (3 g of defatted rice bran) and thus takes the units of g/g-bran. Forty milliliter of the extract was lyophilized for over 24 h by using an FDU-1200 freeze dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan).

2.3.2 Total carbohydrate content

The total carbohydrate content of the defatted rice bran extracts was determined by the phenol-sulfuric acid method [11] using glucose as the standard. One milliliter of an appropriately diluted extract was added to 25 μL of 80% (w/w) aqueous phenol. The mixture was then added with 2.5 mL of concentrated sulfuric acid and kept in a 30°C water bath for 30 min. The total carbohydrate content of the sample was evaluated with a UV-1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan) at the wavelength of 490 nm.

2.3.3 Protein content

The Folin-Lowry method was employed for determining the protein content of the extract [12]. Because protein in the extract would be degraded into pieces during subcritical water treatment [13], the substances positive in the determination are regarded as protein even if they are low-molecular ones.

An alkaline copper solution was first prepared by mixing 100 mL of an aqueous alkaline solution containing 0.4% (w/v) NaOH and 2.0% (w/v) Na2CO3 with 2 mL of an aqueous copper solution containing 0.25% (w/v) CuSO4·5H2O and 0.5% (w/v) trisodium citrate. Two milliliter of the solution was then mixed with 0.4 mL of a properly diluted extract and the mixture was held at room temperature for 15 min before being added with 0.2 mL of the Folin-Ciocalteu’s reagent, which had been diluted twice with distilled water. The mixture was allowed another 30 min of standing at room temperature. The absorbance at 750 nm was measured with the spectrophotometer for quantification of protein using BSA as the standard.

2.3.4 Total phenolic content

Folin-Ciocalteu’s reagent was used for quantification of the total phenolic content in the defatted rice bran extracts with gallic acid as the standard. One tenth of a
milliliter of a properly diluted sample was added with 0.4 mL of the ten-time diluted Folin–Ciocalteu’s reagent and kept for 15 min. After that the sample was added with 1.0 mL of 7.5% (w/w) aqueous sodium carbonate and kept for another 15 min after which the sample was diluted with 3.5 mL of distilled water and further kept in the dark for 2 h. Spectrophotometric quantification was performed at 765 nm using the spectrophotometer.

2.3.5 DPPH radical scavenging activity

The radical scavenging activity of the defatted rice bran extracts was evaluated by a method modified from that previously reported by Fujinami et al. [14]. Dilution of the extracts was done with 50% (v/v) aqueous ethanol to obtain the appropriate concentrations for spectrophotometric analysis. A 0.2 mL aliquot of a 0.5 mmol/L DPPH in ethanol and 0.8 mL of the appropriately diluted extract were vigorously vortexed in a black 2-mL safe-lock tube (Eppendorf Co., Ltd., Tokyo, Japan). After 30 min of reaction at ambient temperature, the absorbance of the mixture was measured at 516 nm with the Shimadzu UV–1200 spectrophotometer. The 50% (v/v) aqueous ethanol and ascorbic acid were used as the blank and standard, respectively. All measurements were performed in triplicate. The DPPH radical scavenging activity of the extract was defined as the amount of the extract necessary to reduce the initial DPPH concentration by 50% and is expressed by the units of mmol-ascorbic acid equiv./g-bran. The index was determined by the equation reported by Wiboonsirikul et al. [6].

2.3.6 Antioxidative ability in bulk oil system

The antioxidative ability of the defatted rice bran extracts was evaluated against oxidation of linoleic acid under an accelerated condition according to the principle of the Rancimat method [9]. One gram of 10% (w/w) linoleic acid in methanol was added to an amber glass vial with an equivalent amount of 1.0 mg of dry extract. The solutions were thoroughly mixed and placed under reduced pressure for 3 h to completely eliminate water and methanol. The accelerated oxidation was performed in a 105°C heater (Dry thermo unit DTU-1B, Taitech Co., Ltd., Saitama, Japan). The vial was closed with a modified rubber stopper that allowed the flow of air through the head space. The mixture was first preheated for 5 min and then the head space was purged with fresh air at the flow rate of 10 L/h. The outlet air was bubbled through 50 mL of continuously agitated water controlled at 25°C, flowing the volatile oxidation products into the water. The conductance of the water was monitored over time with an LCR-meter (AD-5827, A&D Co., Ltd., Tokyo, Japan). The relative antioxidative ability of the extracts in bulk linoleic acid system was estimated by comparison of the induction period for oxidation characterized by an abrupt increase in conductance. Blank samples were used as the control.

2.3.7. Antioxidative ability in aqueous dispersion system

The extracts were tested for their antioxidative ability against induced oxidation of linoleic acid dispersed in a buffer solution by AAPH as the radical generator [15]. The aqueous dispersion contained 0.02 and 0.2 mmol/L of linoleic acid and SDS, respectively, within a continuous phase of 0.05 mmol/L NaH₂PO₄/Na₂HPO₄ buffer solution (pH = 7.4). Half a milliliter of a 0.2 mmol/L AAPH aqueous solution was added into a 100-mL laboratory bottle (Schott Duran, Schott AG, Mainz, Germany) containing 30 mL of the aqueous dispersion preheated for 10 min at 50°C. Subsequently, 1.0% (w/w) of the dried extract with respect to linoleic acid was added into the laboratory bottle. A pressure meter (BAT-221B-20 kPa, Daiichi Keiki Seisakusho Co., Ltd., Hyogo, Japan) was mounted to the bottle to monitor the change in the pressure ($P_0 - P$, $P_0$ = initial pressure, $P$ = pressure at a certain point of time) in the head space over time. To avoid the influence of thermal expansion of air on pressure, the valve of the pressure meter was closed after 10 min of equilibration. Throughout the experiment, the aqueous dispersion was held at the constant temperature of 50°C while intensely agitated with a magnetic stirrer to promote dissolution of oxygen into the liquid phase. Similar experiments were performed with a blank sample and 10 mg of ascorbic acid as the negative and positive controls, respectively.

3. Results and Discussion

3.1. Subcritical water extraction of defatted rice bran

Out of the initial volume of extractant of 72 mL, only approximately 66 mL was recoverable as crude fluid extract. Filtration of the crude fluid extracts was carried out to separate the defatted rice bran residue. The permeability of the extracts varied depending on the extraction temperature. After first filtration, second filtration through the 3 μm membrane was feasible for the filtrates of the extracts obtained at 210 and 240°C, though the former required a significantly higher pressure. The extract obtained at 240°C was even filterable through the 0.45...
μm membrane. However, filtration of the filtrates of the extracts obtained at 120, 150, and 180°C was not at all possible through the membrane of the pore size of 3 μm. The use of the 5 μm membrane filter allowed filtration of all the three filtrates. Nonetheless, the ones obtained at 150 and 180°C necessitated the exertion of remarkable pressure.

The filtrates of the extract obtained at 240°C were transparent after filtration through both the 0.45 and 3 μm membranes. After passing through the 3 μm membrane, the filtrate of the extract obtained at 210°C was also clear. However, after filtration through the 5 μm membrane, all the filtrates were cloudy. The particle size of extract became smaller as the treatment temperature rose which facilitated permeation through the membranes of smaller pore sizes. For the uniformity of the following analyses, second filtration was standardized by the use of the 5 μm membrane filter.

3.2. Yield, total carbohydrate and protein contents

The yield increased with the increase of extraction temperature up to 210°C with the maximum value of 0.547 g/g-bran, but decreased when the temperature reached up to 240°C (Fig. 1).

The temperature dependencies of the total carbohydrate and protein contents were similar to those for subcritical water treatment of black rice [6] and wheat [16] brans. The total carbohydrate content increased with increasing extraction temperature up to 180°C, giving the maximum of 0.306 g/g-bran, and decreased thereafter. It indicated that carbohydrate was increasingly extracted from the defatted rice bran at higher temperatures up to 180°C and it then either decomposed or transformed into other substances at further increased temperatures [17, 18].

The total protein content also increased with the increase in extraction temperature, and it was 0.222 g/g-bran at 210°C. The proteinous compounds showed no obvious degradation with further temperature increase to 240°C, remaining at a close value of 0.216 g/g-bran.

Monosaccharides were significantly degraded at 180 to 260°C, while amino acids such as branched ones were stable at these temperatures [19]. This implies that monosaccharides start to be degraded at lower temperatures in comparison to amino acids. Although the degradation of protein might proceed at higher temperatures, the extraction time would probably be too short for significant degradation of protein [8].

The defatted rice bran extracts also demonstrated compositional changes in response to extraction temperature. The carbohydrate and protein fractions in the extract are shown by the closed symbols in Fig. 1. The carbohydrate fraction escalated with increasing extraction temperature, maximizing at 150°C, and displayed thereafter a shrinking trend. It recorded a maximum carbohydrate fraction of 0.658 g/g-extract. Although the total carbohydrate content peaked at 180°C, the other constituent substances also increased simultaneously but
to a greater extent and thus, declining the carbohydrate fraction. As for the protein fraction, despite the fact that the protein content stabilized above 210°C, the highest value of 0.607 g/g-extract was obtained at 240°C within the tested range. The result would in part be attributable to the sudden substantial decrement in the total carbohydrate content at 240°C.

Although the estimation of the total carbohydrate and protein contents was carried out by glucose and BSA as the respective standards, these results still served as conservative indicators to the fact that carbohydrate and protein are the major constituent substances in the extracts, implying that compositional alteration might be achievable by manipulating the extraction temperature.

3.3. Total phenolic content and DPPH radical scavenging activity

The total phenolic content increased with the increase in extraction temperature within the tested range, reaching 38.9 mg-gallic acid/g-bran at 240°C (Fig. 2).

Only relatively smaller amounts of phenolic substances was extractable at the extraction temperatures below 180°C. The phenomenon indicated that high temperatures were necessary to loosen or destroy the lignin structure consisted predominantly of cross-linked phenolic substances [20]. The DPPH radical scavenging activity of the extract has the same trend as the total phenolic content, with the highest value of 0.271 mmol-ascorbic acid equiv./g-bran at 240°C within the tested range. As shown in the inset of Fig. 2, the linear correlation between the total phenolic content and the radical scavenging activity with a coefficient of determination of 0.99 indicated that the phenolic compounds might be one of the major substances contributing to the radical scavenging property of the extracts. The DPPH radical scavenging activity of rice bran extracts obtained by subcritical water extraction has been reported to be related to the product compounds from the Maillard reaction [21], but the phenomenon was not observable in the present study. The phenolic fraction increased to a greater degree than the total phenolic content at higher temperatures presumably because of the significant decrement of the estimated yield of extraction due to the comparatively noticeable shrinkage of the total carbohydrate content.

3.4. Antioxidative ability in bulk oil system

The antioxidative ability of the extracts was studied in bulk linoleic acid system by estimating the induction period for oxidation of linoleic acid. The extracts obtained at the extraction temperatures from 120 to 210°C exhibited no obvious differences in antioxidative ability comparing with the control sample that recorded an induction period of 23.7 min (Fig. 3). Only the extract obtained at 240°C demonstrated a remarkable antioxidative ability with an induction period of 111 min. The induction period prolonged obviously as the amount of the added extract doubled (inset of Fig. 3).

As mentioned above, only the extract at 240°C could be easily filtered through the 0.45 μm membrane, and its total carbohydrate and phenolic contents were low and high, respectively. These facts suggested that the compounds in the extract at 240°C were low-molecular-mass substances and had high solubility in the linoleic acid.

3.5. Antioxidative ability in aqueous dispersion system

No research into the antioxidative ability of defatted rice bran extracts in different systems has been published to our knowledge. Therefore, the oxidation of the aqueous dispersion of linoleic acid was carried out to examine the oxidation reaction in the samples treated with the extracts by monitoring the pressure change of the closed system (Fig. 4). The antioxidative ability of the extracts in the aqueous dispersion system of linoleic acid also demonstrated extraction temperature depen-
The negative control sample showed a rapid increase in the pressure change, \( P_0 - P \), upon the commencement of the experiment. After 5 h of oxidation, the pressure change in the closed system containing the sample treated with the extract obtained at 240°C was 4.5 kPa, while the negative and positive controls were 8.1 and 3.6 kPa, respectively. The samples treated by the addition of the extracts obtained at 120, 150, and 180°C underwent almost similar pressure changes, arriving at a final pressure change ranging from 6.5 to 6.6 kPa after 5 h of oxidation. At the same length of oxidation time, the pressure dropped down to 5.7 kPa in the system treated with the extract obtained at 210°C, falling in between those recorded by the extracts obtained at 240°C and 120–180°C.

In addition, the oxidation rate constant of linoleic acid could be obtained by Eq. (1) through the plot of pressure changes against oxidation time (Fig. 5).

\[
\frac{(P - P_\infty)}{(P_0 - P_\infty)} = \exp[-k(t-t_{\text{ind}})]
\]

(1)

where \( P \) is the pressure at any time, \( P_0 \) is the initial pressure, \( P_\infty \) is the pressure of the system at sufficiently long time, \( t_{\text{ind}} \) is the induction period, and \( k \) is the rate constant for oxygen consumption.

The induction period and the rate constant for oxygen consumption were evaluated from the \( x \)-intercept and the slope of each line, respectively, and are shown in Fig. 6 as functions of extraction temperature. The induction period increased until the extraction temperature of up to 240°C, although it was much shorter than that of L-ascorbic acid. On the other hand, the rate constant for the oxygen consumption did not depend on the extraction temperature up to 180°C but became smaller at higher temperatures. The rate constants for linoleic acid
with the extracts were in the almost same level as those for the negative and positive controls.

4. Conclusion

The extracts exhibited different antioxidative profiles in the bulk oil and aqueous dispersion systems of linoleic acid. The extracts obtained by subcritical water extraction at different temperatures ranging from 120 to 240°C contained multifarious components. The total phenolic content showed a good correlation with the DPPH radical scavenging activity through the tested extraction temperature range. The extraction temperature indicated no significant effect on the rate constant for oxygen consumption in the bulk linoleic acid except for the extract obtained at 240°C. The extracts obtained at any extraction temperature within the tested range elongated the induction period of linoleic acid in the aqueous dispersion system. Especially, the extract at 240°C exhibited the longest induction period although the rate constants obtained at 240°C became smaller than those at lower temperatures.

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和文要約

脱脂米糠の亜臨界水処理による抽出物のパルク系および水分散系における脂質に対する抗酸化性

脱脂米糠を120℃～240℃の亜臨界水で処理して抽出物を得た。処理温度は抽出物の全糖およびタンパク質の含有率に影響を及ぼし、処理温度が210℃まではタンパク質含有率は全糖のそれより低かったが、240℃ではその関係が逆転した。抽出物のDPPHラジカル消去能は抽出温度の上昇とともに240℃まで増加し、全フェノール性物質の含有率と相関した。パルク系およびミセルへの可溶化系でのリノール酸の酸化に対する抽出物の抗酸化性を評価したところ、抽出物の抗酸化性は2つの系で異なっていた。ミセル可溶化系では、抽出物を加えない対照に比べて、いずれの温度における抽出物もリノール酸の酸化を遅延し、とくに240℃での抽出物の効果が大きかった。一方、パルク系でのリノール酸の酸化に対しては、240℃での抽出物のみが抗酸化性を示した。