Effects of Treatment Time during Subcritical Water Treatment and Its Re-treatment on the Properties of Rice Stem Extract

Boonnakhom TANGKHAVANICH, Takashi KOYASHI, and Shuji ADACHI†

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Rice stem was treated with subcritical water at 260°C for 0–20 min. The extracts treated for 5 min were subject to re-treatment for 0–60 min. In the further treatment, the total phenolic content, total carbohydrate content, radical scavenging ability, and metal chelating ability decreased with extended treatment time. Tyrosinase inhibition ability, however, did not depend on the treatment time. There were correlations between the total phenolic content and radical scavenging ability and between the total carbohydrate content and metal chelating ability under any treatment conditions.

Key words: antioxidant; further treatment; rice straw; subcritical water

1. Introduction

The 8 million tons of rice production in Japan was reported in year 2011 [1]. This brought about a million dry tons of agriculture residues, most of which is straw. Rice straw is treated as an agricultural waste and subject to burn or soil incorporation. Those two methods of disposal are considered to be undesirable to public health and environment [1,2]. Although other applications for rice straw have been proposed, the used quantity is still small compared to the produced amount. Rice straw consists of cellulose, hemicellulose, lignin, and other minerals. Phenolic compounds in rice straw, which are crosslinked with lignin in the cell wall structure [3], may have potential usages for their antioxidative abilities.

Water, which maintains its liquid state under a pressurized condition at temperature ranging from 100 to 374°C, is called subcritical water or compressed hot water. Its dielectric constant decreases to match that of organic solvents, and its ion product increases to over 1×10³ fold greater than that of water at room temperature [4,5]. With those properties and decreases in surface tension and viscosity, subcritical water has the ability to dissolve hydrophobic and hydrophilic substances, thus shows the ability to hydrolyse hemicellulose, lignin, and amorphous part of cellulose [6–8]. Though subcritical water has been extensively used as an extractant, decomposition of the extracted substances simultaneously occur during extraction [9–11]. The decomposition involves many reactions such as hydrolysis, thermolysis, and oxidation. Therefore, subcritical water is not only expected to extract the substances, but also to yield other beneficial substances via hydrolysis and decomposition [12].

This study is aimed towards the effects of treatment time during subcritical water treatment on the properties of the rice stem extract. The re-treatment of the extract was also employed to understand the effect of subcritical water treatment on the properties of the extract without extraction. The total carbohydrate and phenolic contents, radical scavenging ability, metal chelating ability, tyrosinase inhibition ability, and antioxidative ability of the extract against autoxidation of linoleic acid were measured.

2. Materials and Methods

2.1 Materials

A rice straw (Oryza sativa) sample was collected in Hyogo Prefecture, Japan. The sun-dried rice straw was kept at 4°C in a storage room. The stem was separated from the leaves and cut into 1–cm long pieces. L-Ascorbic acid (purity>99.5 % ) was purchased from Nacalai Tesque (Kyoto, Japan). Gallic acid and tyrosinase from mushroom were from Sigma–Aldrich Japan (Tokyo, Japan). Folin–Ciocalteu’s phenol reagent was from ICN Biochemicals (Aurora, OH, USA). 1,1-Diphenyl-2-picyrylhydratzyl (DPPH), iron (II) chloride tetrahydrate (FeCl₂.4H₂O), 3–(2-pyridyl)–5,6–diphenyl–1,2,4–triazine–4’,4”–disulfonic acid monosodium salt (ferro-
zine), L-tyrosine, and all other chemicals of reagent grade were from Wako Pure Chemical Industries (Osaka, Japan). Distilled water was used in all the experiments.

2.2 Subcritical water extraction

Extractions were done in a 117–mL SUS–316 stainless-steel vessel (30 mm i.d. × 165.5 mm) assembled by Taiatsu Techno (Osaka). Five grams of rice stem and 55 mL of water were placed in a vessel, and a lid of the vessel was tightly closed. Then it was heated to 260°C with a mantle heater (200 W, Sogo Laboratory Glass Works, Kyoto). The treatment temperature was adopted according to the previous study, where the highest total phenolic content was achieved [13]. A thermocouple was inserted into a tube installed in the vessel for measuring an internal temperature of the vessel. Excluded from a heat–up period (7.2°C/min), the designated temperature was maintained for 0–20 min. Although the pressure inside the vessel was not measured, it was estimated to be 0.33 to 3.6 MPa at 120 to 260°C from the vapor pressure and the expansion of air in the head space. After the treatment, the vessel was cooled down immediately to room temperature in an ice–water bath. The crude extracts were filtrated through an Advantec filter paper (No. 2, Toyo Roshi, Tokyo) to obtain the clarified extracts. Until used for analysis, the extracts were stored in a refrigerator at 4°C. The extracts treated for 5 min were subjected to re-treatment at 260°C. Similar to the previous treatment, the desired temperature was maintained for 0–60 min exclusive of the heat–up period. The obtained extracts were filtrated though No. 2 Advantec filter paper and kept at 4°C.

2.2 Total phenolic content

A 100–µL sample of the diluted straw extract was placed in a test tube with 400 µL of freshly prepared Folin–Ciocalteu reagent and 1 mL of 75 g/L sodium carbonate [14,15]. Volume of the mixture was adjusted to 5 mL by filling distilled water, and then the mixture was placed in the dark at room temperature for 2 h to complete the reaction. Absorbance at 765 nm was measured using an UV–1200 spectrophotometer (Shimadzu, Kyoto). The amount of phenolic compounds in the extracts was compared with that of gallic acid and was expressed as gallic acid equivalent (mg–gallic acid/g–straw).

2.3 Total carbohydrate content

Carbohydrate content of the extract was measured by phenol–sulfuric acid method with some modifications [16]. One milliliter of the diluted extract was mixed with a 25-µL aliquot of an 80 % (w/w) aqueous phenol solution and 2.5 mL of sulfuric acid in a test tube. After placed at room temperature for 10 min, the test tube was put in a 30°C water bath for another 10 min. Absorbance at 490 nm was then measured. The total carbohydrate content was calculated using glucose as standard.

2.4 Radical scavenging ability

A 4-mL sample of the diluted rice straw extract and 1 mL of 0.5 mmol/L DPPH in ethanol were mixed in a black tube, which prevents the light to avoid side reactions. After agitation, the mixture was left for 20 min at room temperature. The remaining radical quantity was calculated based on the absorbance measured at 516 nm. The percentage of radical scavenging ability was calculated as follows [17]:

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\text{Radical scavenging ability (\%) = (A – B + C)/A \times 100}
\]

where A is the absorbance of the DPPH, B is the absorbance of the mixture of sample and DPPH, and C is the absorbance of the diluted sample without DPPH. The radical scavenging ability was defined as the necessary amount of the extract to reduce the DPPH concentration by 50 %. The ability was compared with that of L-ascorbic acid (VC) and expressed in mmol-VC/g-straw.

2.5 Iron (II) chelating ability

The chelating ability was measured by the modified method of Carter [18]. A 500-µL sample of the rice stem extract was mixed with 25 µL of 1 mmol/L FeCl\(_2\)-4H\(_2\)O and 425 µL of distilled water. The mixture was left for 1 min at room temperature, and then was mixed with 50 µL of 5 mmol/L ferrozine. After being left at room temperature for another 10 min, the mixture was centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant was measured at 562 nm. The percentage of chelating ability was calculated using an equation similar to Eq. (1), where A is the absorbance of the FeCl\(_2\)-4H\(_2\)O, B is the absorbance of the mixture of sample and FeCl\(_2\)-4H\(_2\)O, and C is the absorbance of the sample without FeCl\(_2\)-4H\(_2\)O.

2.6 Tyrosinase inhibition ability

The tyrosinase inhibition ability was measured according to the method by Mason and Peterson with some modifications [19]. A 0.1–mL sample of the stem extract was mixed with 1 mL of 1 mmol/L L-tyrosine and 0.8 mL
of 1/15 mol/L potassium phosphate buffer (pH 6.8) in a test tube. The test tube was put into the water bath for 10 min at 37°C, and then 0.1 mL of a 400 U/mL mushroom tyrosinase solution was added. The mixture was left in the water bath for 30 min at 37°C, and then its absorbance at 475 nm was measured. The percentage inhibition of tyrosinase was calculated using the equation similar to Eq. (1), where A is the absorbance of the enzyme, B is the absorbance of the mixture of sample and enzyme, and C is the absorbance of the sample without enzyme. The ability of the extract for inhibiting the tyrosinase activity by 50% was compared with that of kojic acid, and expressed in mol–kojic acid/g–stem.

2.7 HPLC analysis

The extract was analyzed by HPLC using an LC–10AD HPLC pump (Shimadzu) equipped with a Hydrosphere C18 separation column (3 mm i.d.×150 mm, YMC, Kyoto), a guard column (10 mm i.d.×30 mm, YMC), and an SPD-10A UV detector (Shimadzu). A 20–μL sample of the 100-fold diluted extract was applied to the column and eluted at 0.3 mL/min under gradient mode. From 0 to 10 min, the methanol concentration in the mobile phase linearly increased from 0 to 61%. The methanol concentration was kept at 61% from 10 to 25 min, and was raised to 70% from 25 to 35 min. The rice stem extracts showed the absorption maximum around 280 nm, which probably indicates various phenolic substances originating from lignin [13]. Hence, the absorbance at 280 nm of the effluent was recorded.

2.8 Statistical analysis

All the experiments were done in triplicate. The results obtained were analyzed by using Microsoft® Excel 2010 with a two–way analysis of variance (ANOVA). The standard derivations for total phenolic content, radical scavenging ability, tyrosinase inhibition ability, total carbohydrate content, and metal chelating ability were about 11.6%, 16.9%, 11.5%, 15.3%, and 13.6%, respectively.

3. Results and Discussion

3.1 Effects of treatment time on the properties of the extracts

Figure 1 shows the properties of the extracts obtained at the different treatment times. The total phenolic content of the extracts scarcely depended on the treatment time. The phenolic compounds in the extracts would be produced by hydrolyzing lignin in rice stem cell wall. It was reported that the phenolic compounds extracted from lignin are degradable under the sub– and supercritical conditions [20,21]. However, no significant degradation was recognized in this study due to the relatively low temperature compared with the temperatures in the previous studies. The radical scavenging ability of the extract did not change with increasing treatment time. It also showed a linear correlation with the total phenolic content (R²=0.76), suggesting that the phenolic compounds in the rice stem extract were the main sources of its radical scavenging ability. Tyrosinase inhibition ability of the extract gradually increased with increasing treatment time up to 10 min, and then it became steady. The steadiness of tyrosinase inhibition ability of the extracts obtained after 10 min might be because the compounds responsible for the tyrosinase inhibition ability have high stabilities in subcritical water. Rice straw was also reported to contain p-coumaric acid and salicylic acid, which possess the mushroom tyrosinase inhibition ability [22–26]. However, those phenolic compounds are reported to decompose in subcritical water with the temperature above 150°C [27,28]. Therefore, one of other reasons for the steady tyrosinase inhibition could be that the decomposition products of the phenolic compounds also possess the tyrosinase inhibition ability.

Total carbohydrate content in the extract decreased almost linearly with increasing treatment time. The carbohydrate content originated from hydrolyzed products of cellulose and hemicellulose in subcritical water. Cellulose and hemicellulose were hydrolyzed to shorter poly-β–, di–, or monosaccharides, and the resulting saccharides would be further decomposed [5,7]. Decomposition of these saccharides is, therefore, the reason for decrease in the total carbohydrate content in the extract after elongated treatment time. Metal chelating ability also decreased gradually with increasing treatment time. This was in similar tendency to that of the total carbohydrate content, and there was a linear correlation between them (R²=0.94). Carbohydrates are potential ligands to metals, and their chelating ability would come from the interactions with iron to form complexes through deprotonated hydroxyl groups [29,30].

3.2 Properties of the extracts after re-treatment

The extracts obtained at a treatment time of 5 min were subject to further treatment in subcritical water. During the treatment, the time required to reach the desired treatment temperature is defined as heat–up time. Generally, the heat–up time of ca. 36 min was
needed to reach 260°C. The extract without re-treatment obtained just after reaching the desired temperature had the highest total carbohydrate content, and its total phenol content was not statistically different from those obtained with increasing treatment time (Fig. 1). This would suggest that the extraction would already occur during the heat-up period. The total carbohydrate and phenolic contents of the extracts obtained without re-treatment at 5 min (Fig. 1) were compared with those of the extracts with re-treatment obtained just after reaching the desired temperature (Fig. 2), and they were not statistically different (p<0.05). Hence, it suggests that the heat-up time for the re-treatment would have negligible effect on the properties of the rice stem extract.

In Fig. 2, the total phenolic content in the extract decreased with increasing re-treatment time. In subcritical water, it has been reported that lignin in rice stem is hydrolyzed into phenolic compounds, and the resulting phenolic compounds are further decomposed to degraded products [20,21]. It has also been reported that the major phenolic compounds, which are p-coumaric acid, p-hydroxybenzoic acid, ferulic acid, and vanillic acid [23,24], are rapidly decomposed in subcritical water [27,28], and they would become smaller phenols, which are more stable under subcritical condition. The radical scavenging ability also decreased progressively with extended treatment time. The total phenolic content and the DPPH radical scavenging activity showed strong linear correlation ($R^2=0.95$). This would suggest that the decomposed products from phenolic compounds would not possess the DPPH radical scavenging ability.

The extracts submitted to further treatment for 0, 20, 40 and 60 min were chosen for HPLC analysis at 280 nm. The HPLC chromatograms for the extracts exhibited two major peaks at retention time of ca. 16 min (Fig. 3). These two major peaks decreased with increasing treatment time. However, the minor peaks materialized between ca. 18-22 min were stable. The decrease of the major peaks can be explained by the total phenolic content, which decreased rapidly at the beginning and became steady at longer treatment time. Most of the phenolic compounds in the rice stem extracts showed their

![Fig. 1](image1.png)

**Fig. 1** Total phenolic content (△), total carbohydrate content (○), DPPH radical scavenging ability (◇), metal chelating ability (□), and tyrosinase inhibition ability (●) of the extracts obtained at different treatment time.

The treatment time indicates the time elapsed after reaching 260°C.

![Fig. 2](image2.png)

**Fig. 2** Total phenolic content, total carbohydrate content, DPPH radical scavenging ability, metal chelating ability, and tyrosinase inhibition ability of the extracts obtained after its re-treatment. The symbols are the same as in Fig. 1.

![Fig. 3](image3.png)

**Fig. 3** HPLC analysis of the extracts obtained after its re-treatment for (a) 0 min, (b) 20 min, (c) 40 min, and (d) 60 min.
peaks, when the concentration of methanol in the mobile phase was at 51%. This suggests that the phenolic compounds in rice stem have moderate polarity.

The treatment time negligibly affected the tyrosinase inhibition ability of the extracts. It was reported that \(p\)-coumaric and ferulic acid derived amides, which were obtained by coupling reactions, also possess the ability to inhibit tyrosinase activity [31,32]. The black melanin was caused by transformation of L-tyrosine to ortho-dopaquinone. This transformation occurs in two steps: hydroxylation of L-tyrosine to L-dopa, and then oxidation of L-dopa to ortho-dopaquinone [33]. In the re-treatment, the free radical scavenging ability of rice stem decreased, while its tyrosinase inhibition ability was being steady. This may suggest that the tyrosinase inhibition ability of rice stem extract did not involve the inhibition of catalytic reactions of L-dopa to ortho-dopaquinone caused by tyrosinase.

The total carbohydrate content and the metal chelating ability almost linearly decreased with increasing re-treatment time and showed a strong correlation between them \((R^2=0.86)\). However, the metal chelating ability disappeared after 20 min of re-treatment time. In subcritical water, carbohydrates are decomposed [5,7], and the longer reaction time would result in the formation of more degraded products. The disappearance of metal chelating ability after 20 min of re-treatment would mean that the degraded products from carbohydrate do not possess the metal chelating ability.

4. Conclusions

The long-term treatment and re-treatment resulted in decrease in the total phenolic content, total carbohydrate content, radical scavenging ability, and metal chelating ability of the rice stem extracts. However, the tyrosinase inhibition ability of the extracts did not decrease with increasing treatment time. This would indicate that the degradation products possess the ability to inhibit tyrosinase activity. Therefore, the short-time treatment would be more suitable for obtaining carbohydrate and phenolic compounds with higher contents from rice stem using subcritical water at 260°C.

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References

15) V. L. Singleton, J. A. Rossi; Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagent. Am. J.


