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# Complex enzyme hydrolysis releases antioxidative phenolics from rice bran

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ABSTRACT

In this study, phenolic profiles and antioxidant activity of rice bran were analyzed following successive treatment by gelatinization, liquefaction and complex enzyme hydrolysis. Compared with gelatinization alone, liquefaction slightly increased the total amount of phenolics and antioxidant activity as measured by ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Complex enzyme hydrolysis significantly increased the total phenolics, flavonoids, FRAP and ORAC by 46.24%, 79.13%, 159.14% and 41.98%, respectively, compared to gelatinization alone. Furthermore, ten individual phenolics present in free or soluble conjugate forms were also analyzed following enzymatic processing. Ferulic acid experienced the largest release, followed by protocatechuic acid and then quercetin. Interestingly, a major proportion of phenolics existed as soluble conjugates, rather than free form. Overall, complex enzyme hydrolysis releases phenolics, thus increasing the antioxidant activity of rice bran extract. This study provides useful information for processing rice bran into functional beverage rich in phenolics.



## 1. Introduction

Rice bran is one of the major by-products of rice milling, making up about 8% of rice bran, and is composed of pericarp, aleurone, and subaleurone, as well as smaller amounts of germ and endosperm (Friedman, 2013). It has gained increasing attention worldwide due to its many beneficial nutritional and biological effects. However, it is presently underutilized due to its poor flavor and solubility, and its primary current use is in the production of fertilizer and animal feed. Therefore, how to best utilize rice bran has become an intense focus of research.

One benefit of rice bran is that it is a source of bioactive phenolics. These phenolic compounds have potent antioxidant and free radical scavenging properties, which prevent chronic diseases, such as cancer, diabetes, obesity and cardiovascular diseases (Lai, Chen, Chen, Chang, & Cheng, 2012; Okarter & Liu, 2010; Verschoyle et al., 2007). Our recently published study reported that the total amounts of phenolics and flavonoids were 13.1 and 10.4 times higher, respectively, in the rice bran than in the endosperm (Ti et al., 2014). Phenolics are found in multiple forms in cereal, including soluble free, soluble conjugates and insoluble bound forms (Adom & Liu, 2002; Wang et al., 2015). Of these forms, the soluble conjugate phenolics in cereals have not received as much attention as free and bound forms. Madhujith and Shahidi (2009) observed that there are higher amounts of soluble conjugate than free phenolics in barley. Furthermore, soluble conjugate phenolics, once released from ingested food by bacteria in the microbiota, may play an essential role in delivering antioxidants to the colon

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in a manner similar to bound phenolics (Saura-Calixto, 2011; Zhang et al., 2014). These reports suggest that the soluble conjugate form is an important source of phenolic compounds in cereals. However, little information is available on soluble conjugate phenolics and their antioxidant activity in rice bran.

A number of studies have demonstrated phenolic compounds typically occur in the insoluble bound form, rarely in the free form, in edible plants (Ahmad, Zuo, Lu, Anwar, & Hameed, 2016). For example, 74% of the total phenolics in rice are in the insoluble bound form. Furthermore, the antioxidative properties of bound phenolics are significantly higher than in free or soluble conjugated forms (Adom & Liu, 2002). A subsequent publication from Shao, Xu, Sun, Bao, and Beta (2014) reported that ferulic, *p*-coumaric, syringic, and isoferulic acids are mainly bound in rice bran. Therefore, there is a large amount of interest in finding an effective method to release the bound phenolic compounds. While there are some methods that have found to be successful at releasing phenolics from rice bran, such as subcritical water extraction (Wiboonsirikul et al., 2007), high hydrostatic pressure and far-infrafred radiation (Kim et al., 2015; Wanyo, Meeso, & Siriamornpun, 2014), the disadvantages of these methods, such as high energy consumption, expensive equipment, production on a small scale and low efficiency, limit their use in industry. An alternative approach of obtaining phenolics from rice bran is enzymatic release, which is a low cost method that requires only mild reaction conditions and is environmentally friendly. A recent study found that using a single cellulase treatment on rice bran increased the amount of free phenolic acids, such as protocatechuic and vanillic acids. However, this was a low efficiency process as it only slight increased the amount of free phenolic acids and failed to increase the total amount of phenolics (Wanyo et al., 2014).

The cell wall of rice bran is a complex three dimensional structure consisting of cellulose, polysaccharide and protein (Benoit et al., 2006). One possible method of degrading the cell wall to further release phenolics is complex enzyme hydrolysis using protease, cellulase and glucoamylase. The work presented here focuses on this method of enzymatic release of phenolics from rice bran.

The objectives of this study were as follows: (1) to determine the contents of free and soluble conjugate phenolics and flavonoiods and their antioxidant activities of rice bran extract at different stages of treatment; (2) to characterize changes in the compositions and contents of individual phenolics in the both free and soluble conjugate forms in rice bran extract during the enzymatic process.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Folin–Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), fluorescein disodium salt, and 2,20-azobis(2-amidinopropane)dihydrochloride (ABAP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile and acetic acid were obtained from Fisher (Suwanee, GA, USA). Individual phenolic standards were purchased from Aladdin Reagents (Shanghai, China). Alpha-amylase (20,000 uints/g), glucoamylase (100,000 uints/g), acid protease (50,000 uints/g) and acid cellulase (35,000 uints/g) were food grade and purchased from Youtell Biochemical Com., Ltd (Shanghai, China). All solvents used in chromatography were of HPLC grade and other chemicals were of analytical reagent grade.

### 2.2. Complex enzyme hydrolysis of rice bran

The fresh rice bran used in this study had been defatted using supercritical carbon dioxide (Rice Research Institute of Guangdong Academy of Agricultural Sciences, China) and was made up of 52.87% carbohydrate, 29.66% starch, 16.72% protein, 23.21% crude fiber and 12.35% ash based on dry weight (DW).

Complex enzyme hydrolysis was performed as described in a previous report with modifications (Wen et al., 2015). Briefly, 10 g rice bran were added into 50 mL of 0.01 M citric buffer (pH 6.0), and gelatinization was performed by heating the solution to 100 °C for 10 min. This mixture was cooled and kept at 70 °C in a thermostatic water bath. Liquefaction was then performed by adding alpha-amylase to the mixture and incubating for 10 min. The mixture was heated to 90 °C for 5 min to stop the liquefaction reaction, and the pH was adjusted to 4.1 using citric acid. Complex enzyme hydrolysis was then performed in triplicate, where this mixture was incubated with a complex of enzymes consisting of 0.5% glucoamylase, 1.5% protease and 1.5% cellulase, based on the weight of rice bran, for 190 min at 57.5 °C. Samples from each of the three stages were collected and phenolics were extracted and quantified.

### 2.3. Extraction of free phenolics

Free phenolic compounds were extracted according to a previously reported method, with modifications (Alrahmany, Avis, & Tsopmo, 2013). Briefly, 5 g rice bran extract was dissolved in 50 mL of acidified water (pH 3.0) and partitioned five times with 50 mL of ethyl acetate. The pooled ethyl acetate fractions were evaporated to dryness. The extract containing the free phenolics was reconstituted with MeOH to a final volume of 10 mL and then stored at −20 °C until analysis.

### 2.4. Extraction of soluble conjugate phenolics

The soluble conjugate phenolics were extracted from the water phase after extracting free phenolic compounds based on the previous methods (Adom & Liu, 2002; Madhujith & Shahidi, 2009). Briefly, the water phase was hydrolyzed with 40 mL 2 M NaOH at room temperature for 4 h with shaking under nitrogen gas. The solution was then acidified to pH 2.0 with 6 M HCl and extracted five times with ethyl acetate as previously described. The extract containing the soluble conjugate phenolics was reconstituted with MeOH to a final volume of 10 mL and then stored at −20 °C until analysis.

### 2.5. Determination of total phenolic content

The total phenolic content in both the free and soluble conjugate fractions was measured by the Folin–Ciocalteu colorimetric method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Briefly, a 125 μL aliquot of the above extract described above was diluted with 0.5 mL distilled water, and subsequently mixed with 125 μL Folin–Ciocalteu reagent. After 6 min, 1.25 mL 7% aqueous sodium carbonate solution was added, and the solution was diluted to a final volume of 3 mL. The reaction solution was incubated in dark for 90 min, and the absorbance was measured at 760 nm using a Shimadzu UV-1800 spectrometer (Shimadzu Inc., Kyoto, Japan). Gallic acid was used as the standard, and the results were expressed as mg gallic acid equivalents (GAE) per 100 g dry weight (DW) of rice bran.

### 2.6. Determination of total flavonoid content

The total flavonoid content was determined according to the colorimetric method with minor modifications (Min, Gu, McClung, Bergman, & Chen, 2012). A 300 μL aliquot of the above extract was mixed with 1.5 mL distilled water, and subsequently with 90 μL 5% $NaNO_2$ solution. After 6 min, 180 μL 10% $AlCl_3{\cdot}6H_2O$

solution was added to the solution, and the mixture was reacted for 5 min before adding 0.6 mL 1 M NaOH solution. Then, the mixture was diluted to a final volume of 3 mL with distilled water, and the absorbance at 510 nm was determined immediately using a Shimadzu UV-1800 spectrometer. (+)-catechin was used as the standard, and the results were expressed as mg (+)-catechin equivalents (CE) per 100 g DW of rice bran.

### 2.7. Determination of phenolic composition

The individual phenolic compounds in the above extract was analyzed by HPLC using an Agilent 1200 HPLC system (Waldbronn, Germany) equipped with an Agilent 1200 series VWD detector, autosampler, and a 250 × 4.6 mm i.d., 5 μm Agilent Zorbox SB-$C_{18}$ column (Palo Alto, CA, USA). The mobile phase was a 0.4% aqueous solution of acetic acid (solution A) and acetonitrile (solution B), using the following gradient program: 0–40 min, solution B 5–25%; 40–45 min, solution B 25–35%; 45–50 min, solution B 35–50%. Other chromatographic conditions included an injection volume of 20 μL, a flow rate of 1.0 mL/min, a run time of 50 min, column temperature of 30 °C and detection wavelength of 280 nm. Before analysis, the samples were filtered through a 0.25-μm membrane filter (Millipore, Billerica, MA, USA). Individual compounds were identified based on the retention time and the chromatography of authentic standards. Accurate amounts of 10 phenolics were added to rice bran extract after complex enzyme hydrolysis in Section 2.2 and then they were extracted as described in Section 2.3. The extract was analyzed by HPLC, and the recovery percentage of these phenolics was calculated based on the amount found and the amount spiked, which ranged from 94.2 to 99.1%. The concentrations of each compound were calculated based on a standard curve, and the results were expressed as μg per gram DW of rice bran.

### 2.8. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted based on the method described by Benzie and Strain (1996). Fresh FRAP working solution was prepared daily by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL TPTZ solution (10 mM TPTZ in 40 mM HCl), and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ solution, which was warmed to 37 °C prior to use. A 30 μL aliquot of the above extract was diluted with 90 μL of distilled water, and then allowed to react with 900 μL of the FRAP solution for 30 min in dark at room temperature. The absorbance was determined at 593 nm using a Shimadzu UV-1800 spectrometer. Trolox was used to establish a standard curve, and the FRAP antioxidant activity was expressed as mg Trolox equivalents (TE) per 100 g DW of rice bran.

### 2.9. Oxygen radical scavenging capacity (ORAC) assay

According to the previous methods (Qiu, Liu, & Beta, 2010; Zhang, Zhang, Zhang, & Liu, 2010) with slight modifications, the ORAC assay was performed in black-walled 96-well plates (Corning Scientific, Corning, NY). Briefly, the above extract dilutions were prepared with 75 mM phosphate buffer (pH 7.4). The outside wells of the plate were not used because there was much more variation from them than from the inner wells. Each well contained 20 μL of extract solution or 20 μL of Trolox standard (range: 6.25–50 μM) and 200 μL of fluorescein (final concentration 0.96 μM). The reaction mixture was incubated at 37 °C for 20 min. Subsequently, 20 μL of 119 mM ABAP solution was added to each well using a multichannel pipette. The fluorescence intensity was detected using a Fluoroskan Ascent FL plate-reader (Thermo Labsystems, Franklin, MA, USA) at excitation of 485 nm and emission of 538 nm for 35 cycles every 4.5 min. The ORAC value was expressed as micromole Trolox equivalents per gram DW of rice bran.

### 2.10. Statistical analyses

All experiments were repeated 3 times and data are expressed as mean ± standard deviation (SD). Data were analyzed by one-way ANOVA followed by the SNK-q test using SPSS13.0 software (SPSS Inc. Chicago, IL, USA). The level of significance was set at $p < 0.05$.

## 3. Results

### 3.1. Total phenolic content

Table 1 presents the contents of free, soluble conjugate, and total phenolics in the rice bran extract, and the contributions of the free and soluble conjugate phenolics to the total phenolics following treatment. After gelatinization, the amounts of free, soluble conjugate and total phenolics were 13.83, 75.36 and 89.19 mg GAE/100 g DW, respectively. Liquefaction had no significant influence on the phenolic content. Meanwhile, complex enzyme treatment significantly increased the contents of free, soluble conjugate, and total phenolics by 2.17-, 1.33-, and 1.46-fold more than gelatinization ($p < 0.05$). The contribution of free phenolic fraction to the total soluble phenolics increased from 15.51% in gelatinization group to 23.01% in complex enzyme hydrolysis group, while the corresponding soluble conjugate phenolic fraction decreased from 84.49% in gelatinization group to 76.99% in complex enzyme hydrolysis group.

### 3.2. Total flavonoid content

Table 2 presents the free, soluble conjugate, and total flavonoid content in rice bran extract, as well as the contributions of free and soluble conjugate fractions to the total flavonoids following enzymatic processing. After gelatinization, the amounts of free, soluble conjugate and total flavonoids in rice bran extract were 26.85, 15.60 and 42.45 mg CE/100 g DW, respectively. Compared to gelatinization, liquefaction slightly, but significantly, increased the amounts of free, soluble conjugate, and total flavonoids by 8.94%, 5.64% and 7.72%, respectively ($p < 0.05$), while complex enzyme treatment increased the amounts by 69.3%, 96.1% and 79.1%, respectively. Meanwhile, the contribution of free flavonoid fraction to the total soluble flavonoids decreased from 63.26% in gelatinization group to 59.77% in complex enzyme hydrolysis group, and the corresponding soluble conjugate flavonoid fraction increased from 36.74% in gelatinization group to 40.23% in complex enzyme hydrolysis group.

**Table 1**
The contents of free, soluble conjugate and total phenolics in rice bran extract following enzymatic processing, and the percentage contributions of free and soluble conjugate fractions to the total soluble phenolic fraction.

| Stage | Phenolics (mg GAE/100 g DW) | | |
|---|---|---|---|
| | Free | Soluble conjugate | Total |
| G | 13.83 ± 1.22a[a] (15.51)[b] | 75.36 ± 4.79a (84.49) | 89.19 ± 6.01a |
| L | 14.95 ± 0.31a (16.33) | 76.59 ± 0.25a (83.67) | 91.53 ± 0.06a |
| E | 30.04 ± 1.60b (23.01) | 100.49 ± 0.85b (76.99) | 130.52 ± 0.75b |

G: gelatinization; L: liquefaction; E: complex enzyme hydrolysis; GAE: gallic acid equivalents.

[a] Values with different letters in each column are significantly different following enzymatic processing ($p < 0.05$).

[b] Values in parentheses indicate percentage contribution to the total content.

**Table 2**
The contents of free, soluble conjugate and total flavonoids in rice bran extract following enzymatic processing, and the percentage contributions of free and soluble conjugate fractions to the total soluble flavonoid fraction.

| Stage | Flavonoids (mg CE/100 g DW) | | |
|---|---|---|---|
| | Free | Soluble conjugate | Total |
| G | 26.85 ± 0.68a[a] (63.26)[b] | 15.60 ± 0.06a (36.74) | 42.45 ± 0.61a |
| L | 29.25 ± 0.60b (63.96) | 16.48 ± 0.27b (36.04) | 45.73 ± 0.87b |
| E | 45.45 ± 1.00c (59.77) | 30.59 ± 3.04c (40.23) | 76.04 ± 2.04c |

G: gelatinization; L: liquefaction; E: complex enzyme hydrolysis; CE: (+)-catechin equivalents.
[a] Values with different letters in each column are significantly different following enzymatic processing ($p < 0.05$).
[b] Values in parentheses indicate percentage contribution to the total content.

### 3.3. Phenolic composition

The quantities of 10 phenolics, including ferulic acid, protocatechuic acid, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, coumaric acid, gallic acid, syringic acid, quercetin and (−)-epicatechin, were measured in the free and soluble conjugate fractions of the rice bran extract following enzymatic processing. The contents of the 10 phenolics and the percentage contribution of the free and soluble conjugate fractions to the total contents, are shown in Table 3. The compositions of the free and soluble conjugate phenolics in rice bran extract were similar following enzymatic processing as measured by HPLC, but the total amounts of the individual phenolics differed significantly ($p < 0.05$). Each of the measured phenolics was present in both free and soluble conjugate forms in the rice bran extract with the exception of chlorogenic acid, which was present only as a soluble conjugate. Coumaric acid was primarily found in the free form, while the other phenolics were mainly in the soluble conjugate form following enzymatic processing. Interestingly, syringic acid and (−)-epicatechin were primarily found as conjugates following gelatinization, but were primarily in the free form following complex enzymatic hydrolysis.

Generally, the total amount of each phenolic compound significantly increased following enzymatic processing ($p < 0.05$) with the exception of syringic acid, which did not change significantly ($p > 0.05$). The specific increases of each phenolic compound were 177.4% for ferulic acid, 496.5% for protocatechuic acid, 121.6% for p-hydroxybenzoic acid, 285.7% for chlorogenic acid, 154.0% for caffeic acid, 84.9% for coumaric acid, 65.6% for gallic acid, 526.8% for quercetin, and 138.4% for (−)-epicatechin. Compared to gelatinization alone, liquefaction significantly increased the amounts of free gallic acid and (−)-epicatechin, decreased the amount of free protocatechuic acid, and had no significant influence on the amounts of free ferulic acid, *p*-hydroxybenzoic acid, coumaric acid, syringic acid and quercetin. The amounts of each phenolic present as soluble conjugate in rice bran extract were also measured (Fig. 1). Liquefaction significantly increased the soluble conjugate forms of ferulic acid, protocatechuic acid, chlorogenic acid, coumaric acid, quercetin and (−)-epicatechin, decreased syringic acid, and did not significantly influence *p*-hydroxybenzoic, caffeic and gallic acids.

**Table 3**
Change in individual phenolics in rice bran extract following enzymatic processing and the percentage contribution of the free and soluble conjugate fractions to the total soluble phenolic fraction.

| Phenolic acid | Stage | Free (μg/g) | Soluble conjugate (μg/g) | Total (μg/g) |
|---|---|---|---|---|
| Ferulic acid | G | 14.4 ± 0.1a[a] (20.1)[b] | 57.3 ± 0.8a (79.9) | 71.7 ± 0.9a |
| | L | 14.8 ± 0.1a (19.1) | 62.6 ± 1.3b (80.9) | 77.4 ± 1.2b |
| | E | 79.0 ± 2.3b (39.7) | 119.9 ± 7.8c (60.3) | 198.9 ± 10.1c |
| Protocatechuic acid | G | 2.7 ± 0.1b (48.0) | 3.0 ± 0.5a (52.0) | 5.7 ± 0.4a |
| | L | 1.2 ± 0.2a (8.3) | 13.4 ± 0.2b (91.7) | 14.6 ± 0.4b |
| | E | 6.3 ± 0.3c (18.5) | 27.7 ± 3.1c (85.5) | 34.0 ± 3.4c |
| *p*-Hydroxybenzoic acid | G | 1.9 ± 0.07a (12.5) | 13.4 ± 2.0a (87.5) | 15.3 ± 2.0a |
| | L | 1.8 ± 0.05a (10.5) | 15.6 ± 0.2b (89.5) | 17.4 ± 0.3b |
| | E | 6.6 ± 1.1b (19.5) | 27.3 ± 4.6c (80.5) | 33.9 ± 4.6c |
| Chlorogenic acid | G | nd | 5.6 ± 0.1a (100) | 5.6 ± 0.1a |
| | L | nd | 6.4 ± 0.3b (100) | 6.4 ± 0.3b |
| | E | nd | 21.6 ± 0.6c (100) | 21.6 ± 0.6c |
| Caffeic acid | G | nd | 6.3 ± 0.2a (100) | 6.3 ± 0.2a |
| | L | nd | 5.9 ± 0.2a (100) | 5.9 ± 0.2a |
| | E | 3.5 ± 0.4c (21.8) | 12.5 ± 1.4b (78.2) | 16.0 ± 1.8b |
| Coumaric acid | G | 4.7 ± 0.7a (64.0) | 2.6 ± 0.1a (36.0) | 7.3 ± 0.7a |
| | L | 6.0 ± 0.9b (66.4) | 3.0 ± 0.2b (33.6) | 9.0 ± 1.1b |
| | E | 10.5 ± 1.2c (78.0) | 3.0 ± 0.1b (22) | 13.5 ± 1.3c |
| Gallic acid | G | 0.4 ± 0.06a (13.7) | 2.8 ± 0.3a (86.3) | 3.2 ± 0.3a |
| | L | 0.8 ± 0.1b (22.2) | 2.8 ± 0.3a (77.8) | 3.6 ± 0.4a |
| | E | 1.0 ± 0.2b (18.9) | 4.3 ± 0.5b (81.1) | 5.3 ± 0.7b |
| Syringic acid | G | 1.5 ± 0.1a (39.1) | 2.3 ± 0.1c (56.8) | 3.8 ± 0.1a |
| | L | 1.4 ± 0.3a (41.3) | 2.0 ± 0.1b (57.9) | 3.4 ± 0.4a |
| | E | 2.0 ± 0.1b (55.7) | 1.6 ± 0.1a (44.3) | 3.6 ± 0.2a |
| Quercetin | G | 1.7 ± 0.3a (41.5) | 2.4 ± 0.1a (58.5) | 4.1 ± 0.4a |
| | L | 1.8 ± 0.1a (35.3) | 3.3 ± 0.7b (64.7) | 5.1 ± 0.6b |
| | E | 2.8 ± 0.1b (11.1) | 22.9 ± 1.8c (88.9) | 25.7 ± 1.8c |
| (−)-Epicatechin | G | 1.5 ± 0.1a (17.7) | 7.1 ± 0.8a (82.3) | 8.6 ± 0.7a |
| | L | 2.9 ± 0.4b (23.7) | 9.2 ± 0.8b (76.3) | 12.1 ± 1.2b |
| | E | 12.4 ± 1.3c (60.5) | 8.1 ± 0.4ab (39.5) | 20.5 ± 1.7c |

G: gelatinization; L: liquefaction; E: complex enzyme hydrolysis.
[a] Values with different letters in each column are significantly following enzymatic processing ($p < 0.05$).
[b] Values in parentheses indicate percentage contribution to the total.

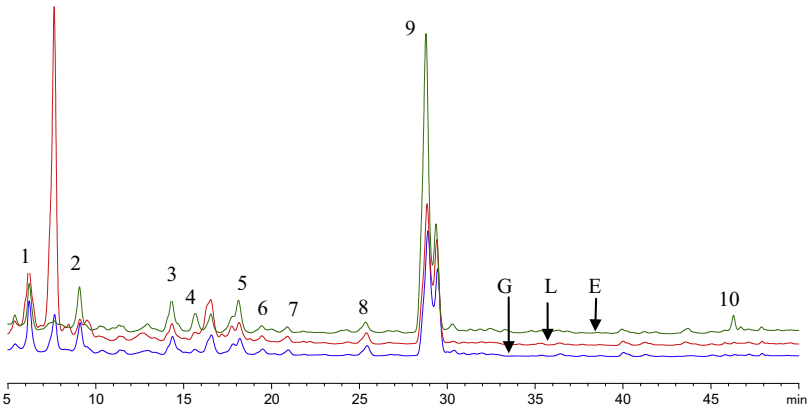


**Fig. 1.** HPLC chromatogram of the soluble conjugate phenolics in rice bran extract at different stages of gelatinization (G), liquefaction (L) and complex enzyme hydrolysis (E). Peaks: 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, chlorogenic acid; 5, caffeic acid; 6, syringic acid; 7, (−)-epicatechin; 8, coumaric acid 9, ferulic acid; 10, quercetin.

Similarly, compared to liquefaction, complex enzyme hydrolysis significantly increased each of the free phenolic compounds measured with the exception of gallic acid, which did not significantly change. Moreover, complex enzyme hydrolysis significantly increased the soluble conjugate of ferulic acid, protocatechuic acid, p-hydroxybenzoic acid, chlorogenic acid, caffeic acid and quercetin, decreased the amount of syringic acid, and had no significant effect on coumaric acid, gallic acid and (−)-epicatechin. Interestingly, free caffeic acid levels, which were undetectable after gelatinization and liquefaction, were found 3.5 μg/g in complex enzyme hydrolysis group. As shown in Fig. 1, an unknown peak followed by gallic acid present as soluble conjugate in liquefaction group was higher than that in gelatinization group, and then almost disappeared in complex enzyme hydrolysis group. Thus, the further studies are required to identify the unknown compound.

### 3.4. FRAP and ORAC analysis of antioxidant activity

Table 4 shows the antioxidant activity of the free, soluble conjugate, and total phenolic fractions in the rice bran extract, and the contributions of free and soluble conjugate fractions to the total antioxidant activity following enzymatic processing expressed as FRAP and ORAC values.

**Table 4**
Antioxidant activity of the free, soluble conjugate and total fractions of rice bran extract following enzymatic processing, and the percentage contributions of free and soluble conjugate fractions to the total antioxidant activity.

| Stage | Antioxidant Activity | | |
|---|---|---|---|
| | Free | Soluble conjugate | Total |
| *FRAP value (mg TE/100 g DW)* | | | |
| G | 60.11 ± 5.85a[a] (42.79)[b] | 80.36 ± 7.30a (57.21) | 140.47 ± 1.45a |
| L | 75.65 ± 5.98b (45.78) | 89.61 ± 1.12b (54.22) | 165.26 ± 4.85b |
| E | 152.24 ± 4.48c (41.86) | 211.77 ± 9.23c (58.14) | 364.02 ± 4.75c |
| *ORAC value (μmol TE/g DW)* | | | |
| G | 20.96 ± 1.42a (21.97) | 74.43 ± 3.16a (78.03) | 95.39 ± 1.74a |
| L | 24.27 ± 0.30b (25.15) | 72.23 ± 3.48a (74.85) | 96.50 ± 3.79a |
| E | 38.42 ± 2.08c (28.37) | 97.02 ± 2.87b (71.63) | 135.44 ± 4.96b |

G: gelatinization; L: liquefaction; E: complex enzyme hydrolysis; TE: Trolox equivalents.

[a] Values with different letters in each column are significantly different following enzymatic processing ($p < 0.05$).

[b] Values in parentheses indicate percentage contribution to the total antioxidant activity.

After gelatinization, the free, soluble conjugate and total FRAP values were 60.11, 80.36 and 140.47 mg TE/100 g DW, respectively. Liquefaction significantly increased the free, soluble conjugate and total FRAP values by 25.85%, 11.51% and 17.65%, respectively ($p < 0.05$). Complex enzyme treatment further increased the free, soluble conjugate and total FRAP values by 2.53-, 2.64- and 2.59-fold, respectively, compared to following gelatinization ($p < 0.05$). The contribution of free FRAP fraction to the total soluble FRAP decreased from 42.79% in gelatinization group to 41.86% in complex enzyme hydrolysis group in rice bran extract, while the corresponding soluble conjugate FRAP fraction increased from 57.21% in gelatinization group to 58.14% in complex hydrolysis group.

Antioxidant activity was also measured by ORAC assay. After gelatinization, the free, soluble conjugate and total ORAC values were 20.96, 74.43 and 95.39 μmol TE/g DW, respectively. Liquefaction significantly increased the free ORAC by 15.80% ($p < 0.05$) and had no significant effect on the soluble conjugate and total ORAC values. Complex enzyme treatment significantly increased the free, soluble conjugate and total ORAC values, which were 1.83, 1.30, and 1.42 times higher, respectively, compared to values after gelatinization ($p < 0.05$). The contribution of free ORAC fraction to the total soluble ORAC increased from 21.97% in gelatinization group to 28.37% in complex enzyme hydrolysis group in rice bran extract, while the corresponding soluble conjugate ORAC fraction decreased from 78.03% in gelatinization group to 71.63% in complex enzyme hydrolysis group.

## 4. Discussion

### 4.1. Effect of complex enzyme hydrolysis on the total soluble phenolic content

Rice bran is a good source of proteins and phytochemicals, such as vitamin E, oryzanol and phenolics. High fiber cereal is underutilized as a source of phenolics because of the low solubility due to the binding of the phenolics to the matrix of the cell wall. Therefore, there is a need to explore the best method to release phenolics from rice bran. Results from the study demonstrate that liquefaction only slightly increases the total soluble phenolic and flavonoid content. However, treatment by gelatinization and liquefaction destroy the crystalline structure of starch, the main component of rice bran, allowing enzymes to attach to their substrates (Blazek & Gilbert, 2010; Li et al., 2015). Thus, gelatinization and liquefaction are important treatments by which to prepare rice

bran for optimal release of phenolics using a complex of enzymes. Phenolic acids can be easily esterified with arabinose, xylose or galactose units of hemicelluloses (Chen et al., 2014). Phenolic compounds, including flavonoids, can also bind starch or other polysaccharides through hydrogen bonds or chelarion (Yu, Vasnathan, & Temelli, 2001). Treatment with the enzymes glucoamylase, cellulase and protease caused hydrolysis of starch, polysaccharides, protein and fiber in the rice bran and disrupted the interactions between the phenolics and cell wall components, thus the corresponding bound phenolics were released into free or soluble conjugates (Tables 1 and 2). Pretreatment of rice bran by gelatinization and liquefaction is necessary to increase the efficiency of phenolic release because it allows access of the enzymes to their corresponding substrates (Li et al., 2015). This was demonstrated in a report by Wanyo et al. (2014) that showed a single cellulase treatment had no significant effect on the total amounts of phenolic acids and flavonoids in rice bran. Furthermore, hydrolysis by a complex of enzymes is more effective than use of a single enzyme, which is consistent with previously published studies. Kammerer, Claus, Schieber, and Carle (2005) reported amplification in phenolic compounds extraction from grape pomace when using a combination of pectinolytic and cellulolytic enzymes. However, this is contradicted by a report by Xu et al. (2014), where enzyme hydrolysis only accelerated the release of phenolics, rather than amplified it, in Noble grape skin. However, this phenotype may be a result of anthocyanin being the major phenolics in Noble skin and existing in a free form in cell vacuoles.

In addition, Schmidt, Gonçalves, Prietto, Hackbart, and Furlong (2014) also found that fermentation with *Rizhopus* oryzae increased the amount of free phenolics in rice bran. However, it is difficult to compare the value from this study with that from our study due to different extraction and quantification methods. In our study, the free phenolics were firstly extracted by water, and the total phenolics were determined based on gallic acid equivalents, while the free phenolics were extracted by methanol, and the total phenolics were determined based on ferulic acid equivalents in the report of Schmidt et al. (2014). Moreover, this study failed to quantify the soluble conjugate phenolics. Results from our study demonstrate that the total soluble phenolics were predominantly present as soluble conjugates in rice bran extract, while the total soluble flavonoids were mainly free forms (Tables 1 and 2). This is likely because phenolic acids with a carboxyl group are a main component of the total soluble phenolic fraction relative to flavonoids, and are easily esterified with reducing sugars or soluble oligosaccharides generated from the hydrolysis of starch and fiber. Chen et al. (2015) worked on non-darkening cranberry beans, and also found that the soluble conjugate phenolics were the largest contributors to the total phenolic content. Therefore, it is important to perform a comprehensive evaluation of the phenolic profile in cereals, including an analysis of the conjugate phenolics.

### 4.2. Effect of complex enzyme treatment on the phenolic composition

Changes in individual phenolics in rice bran extract, including ferulic, protocatechuic, p-hydroxybenzoic, chlorogenic, caffeic, coumaric, gallic and syringic acids, were examined following enzymatic processing as well as the flavonoids quercetin and (−)-epicatechin, the main phenolic compounds in rice bran (Liu et al., 2015; Ti et al., 2015). Generally, liquefaction and complex enzyme treatment together increased the total soluble amount of each phenolic compound studied with the exception of syringic acid (Table 3). Syringic acid levels may have failed to increase due to degradation upon exposure to high temperatures. Of the individual phenolics, ferulic acid was present at the highest levels, followed by protocatechuic acid, while syringic acid was the lowest following complex enzyme treatment. Based on these results, it can be concluded that the increases in the phenolic compounds are mainly due to the release of insoluble bound phenolics, such as ferulic acid, protocatechuic acid and quercetin. Interestingly, the release of syringic acid differs from that of ferulic acid. This rise in free syringic acid levels likely resulted from the hydrolysis of the soluble conjugate form, rather than the insoluble bound form, because complex enzyme treatment significantly decreased the amount of soluble conjugate syringic acid, but had no effect on the total soluble amount of syringic acid. The release of (−)-epicatechin differed from syringic acid because the increase in free (−)-epicatechin mainly resulted from the hydrolysis of the insoluble bound form, rather than the liberation of the soluble conjugate form, based on the lack of effect complex enzyme treatment on the amount of the soluble conjugate (−)-epicatechin. In summary, the manner in which each specific phenolic was released differed based on their attachments to cell wall components, as well as the specificity of the enzymes.

This study found that complex enzyme treatment resulted in a larger release of individual phenolics than liquefaction. For example, liquefaction released 5.7 μg/g of ferulic acid from rice bran, while complex enzyme treatment released 121.5 μg/g, which was 21.3 times that of gelatinization. Ferulic acid and its dimers are covalently linked to arabinosyl residues in cell wall polysaccharides and lignin (Grabber, 2005). Yu, Maenz, Mckinnon, Racz, and Christensen (2002) reported that *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase act synergistically to release ferulic acid from oat hulls, where the highest efficiency measured was 69%. This present study showed that complex enzyme treatment released more soluble conjugate phenolics than the corresponding free form, which is in accordance with previous research. Alrahmany et al. (2013) found that both cellulase and alpha-amylase treatment released more soluble conjugate than free phenolic acids from oat bran, including ferulic, coumaric, caffeic and vanillic acids, and the amount released significantly differed between the tested enzymes.

### 4.3. Effect of complex enzyme hydrolysis on antioxidant activity

The potential health benefits of phenolic compounds are mainly attributed to their antioxidant activity. Due to the complexity of food systems, a single assay is unable to accurately measure all the individual antioxidants in one food system (Qiu et al., 2010). Thus, multiple assays are necessary to provide complete information on the scavenging of different radicals (Shahidi & Zhong, 2015). The antioxidant activity of different forms of phenolics in rice bran extract was measured using FRAP and ORAC assays. The FRAP assay is based on single electron transfer, while the ORAC assay is based on transfer of a hydrogen atom (Prior, Wu, & Schaich, 2005). This present study demonstrated that complex enzyme treatment significantly increased the free FRAP and ORAC antioxidant activity in rice bran extract. Generally, the two methods of measuring antioxidant activity gave similar results and were consistent with the change in phenolics during enzymatic processing. Overall, there is a positive correlation between antioxidant activity and the total amounts of phenolics and flavonoids in rice bran (Min et al., 2012; Ti et al., 2014). This finding is in accordance with a previous study by Alrahmany and Tsopmo (2012), which found that three different enzymatic treatments increased the free ORAC in oat bran. Our previously published study also showed that digestion with pepsin and pancreatin significantly increased the free ORAC in brown rice after cooking (Ti, Zhang, Li, Wei, & Zhang, 2015).

Until now, little information has been available on the effect of processing methods on the antioxidant activity of soluble conjugates in cereal. Results of this study show that complex enzyme treatment also significantly increases antioxidant activity of

soluble conjugates as measured by two methods, and the soluble conjugate fraction was the primary contributor to the total soluble antioxidant activity of rice bran extract. Taken together, our results indicate that complex enzyme treatment increases the total soluble antioxidant activity of rice bran extract by releasing bound antioxidants. A large amount of bound antioxidative phenolics are located in the aleurone layer and embryo, and were released upon enzymatic treatment (Naxzk & Shahidi, 1989; Ti et al., 2015) because starch, fiber and protein (the main component of the aleurone layer and embryo) were hydrolyzed by the enzymes. Xu et al. (2014) reported that cellulase treatment increased the antioxidant activity of grape seed extract, and suggested that enzyme hydrolysis modifies the galloylated form of phenolics, and amplifies antioxidant activity in the form of phenolic acids, particularly gallic acid. Along those lines, another mechanism by which complex enzyme treatment may increase the antioxidant activity of rice bran extract may be by significantly increasing gallic acid levels, a phenotype noted in our study.

## 5. Conclusion

Overall, large amounts of phenolics were released as free and soluble conjugate forms from rice bran by treatment, especially after complex enzyme hydrolysis. Furthermore, a major proportion of phenolics in rice bran extract existed in the soluble conjugate form, a form often not evaluated in previous studies. Therefore, this study clarified the contributions of the free and soluble conjugate forms of phenolics and flavonoids to the total soluble content in rice bran extract following enzymatic processing. The results of this study provide useful information for processing rice bran into functional beverage rich in phenolics and flavonoids and amplified antioxidant activity.

## Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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