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Antioxidant activity of fractionated pigmented Thai Hawm Gra Dang Ngah 59 rice bran hydrolysates: Pretreatment-assisted enzymatic extraction

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ABSTRACT

Background: Hawm Gra Dang Ngah 59 rice (HGDN 59) is a pure line grown in the Takbai district, Narathiwat, located in the southern border province of Thailand. The previously reported HGDN 59 rice bran's potential as a component rich in nutrients-especially high-quality protein, has not been exploited. There is also insufficient research data on biological activity and tested methods of exposing and releasing bioactive hydrolysates from HGDN 59 rice bran hydrolysates (HGDN 59-RBH).

Objectives: This study aimed to determine the antioxidative activity of three different pretreatments following ultrafiltration fractionation of HGDN 59-RBH.

Methods: HGDN 59 rice bran was defatted and pretreated with acid, alpha amylase, and water prior to digestion with consecutive pepsin and trypsin. HGDN 59-RBH was further fractionated by ultrafiltration into three molecular weight (MW) fractions: < 3 kDa, 3-5 kDa, and > 5 kDa. The whole RBH and three MW fractions were assessed to determine protein yield, protein recovery, total phenolic content, and the following antioxidative assays: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferrous ion chelating activity, and ferric ion reducing antioxidation power (FRAP).

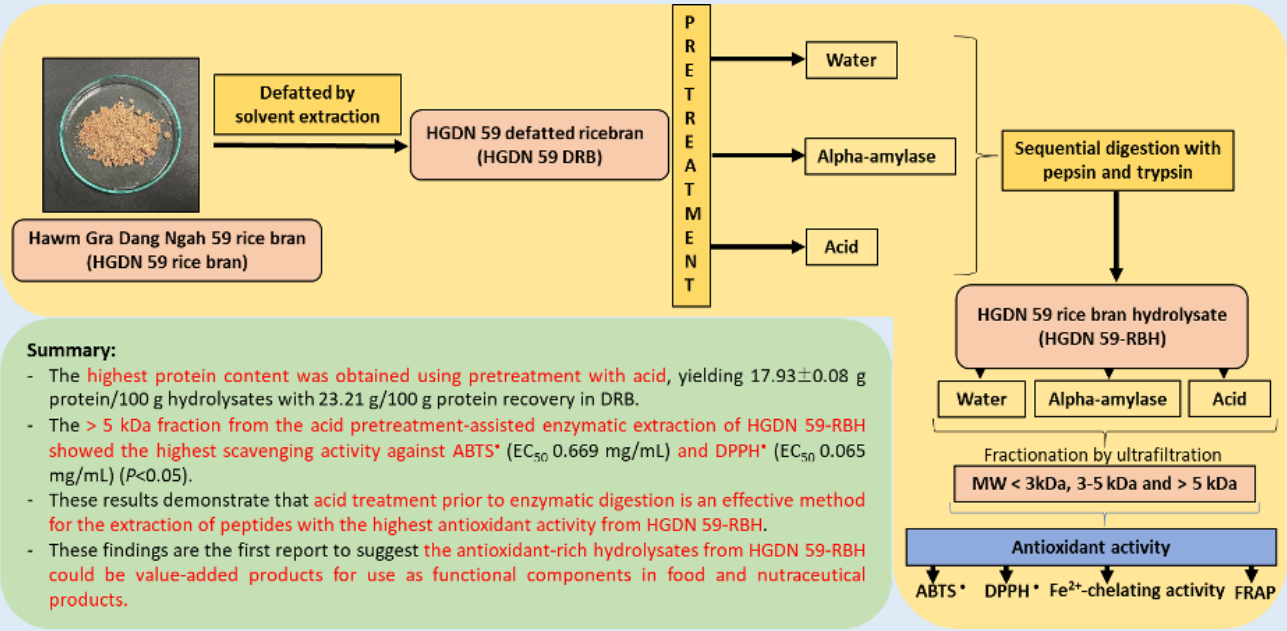
Results: The highest protein content was obtained using pretreatment with acid, yielding 17.93± 0.08 g protein/100 g hydrolysates with 23.21 g/100 g protein recovery in defatted rice bran (DRB). The > 5 kDa fraction from the acid pretreatment-assisted enzymatic extraction of HGDN 59-RBH showed the highest phenolic content and scavenging activity against ABTS radical (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) (EC_{50} 0.669 mg/mL) and DPPH

radical (2,2-diphenyl-1-picrylhydrazyl) (EC_{50} 0.065 mg/mL) (P<0.05).

Conclusions: The findings reveal that subjecting HGDN 59-RBH to acid treatment before enzymatic breakdown is a successful approach to extracting peptides with potent antioxidant properties. Consequently, the resulting hydrolysates, rich in antioxidants, hold promise as valuable additions to food and nutraceutical formulations, enhancing their functional attributes.

Keywords: Thai Hawm Gra Dang Ngah 59 rice bran, fractionated rice bran hydrolysates, pretreatment-assisted enzymatic extraction, antioxidant activity

Graphical Abstract:



INTRODUCTION

Hawm Gra Dang Ngah 59 (HGDN 59) is a certified local red rice variety developed by the Pattani Rice Research Center in Pattani, Thailand [1]. This pure line was developed from local HGDN, which was established hundreds of years ago, and continues to be grown in the Takbai district, Narathiwat, located in the southern border province of Thailand. Colored rice bran, a byproduct from rice milling, is considered a source of high-quality protein and several phytochemicals, such as phenolic and flavonoid compounds [2-8]. Colored rice bran is a natural source of biologically active compounds that may promote optimal health and reduce the risk of chronic diseases through biological activities, e.g., antioxidant activity [6,9-10]. As such, it may be considered a functional food. According to the Functional Food Center's definition, functional foods are natural or processed foods that consist of non-toxic active compounds, exhibit bioactivities, improve health, and provide a clinically proven and documented health benefit [11]. However, HGDN 59 rice bran's potential as a functional food requires investigation before such a claim can be made.

The complex nature of rice bran suggests that the assimilation of nutrients may be limited without further processing. Thus, various experimental studies have tested methods of exposing and releasing bioactive hydrolysates and peptides from rice bran protein, including chemical, enzymatic, and physical methods [4,8,12-14]. *In vitro,* gastrointestinal enzymatic hydrolysis (pepsin-trypsin system) is used to extract protein from rice bran for the production of rice bran hydrolysates because it does not affect the nutritional value or antioxidant activity [13,15-17]. In order to improve protein extraction yield and antioxidant capacity, pretreatments with water, alpha-amylase, or acid are applied to loosen up the rice bran structural matrix, providing the proteolytic enzymes access to the protein substrate. Moreover, the antioxidant potential varies based on various factors, including protein origin, molecular weight, and amino acid composition [18-19]. Consequently, it is common practice to fractionate protein hydrolysates through ultrafiltration before assessing the functional attributes of each fraction.

We aim to promote HGDN 59 rice bran as an alternative source of functional ingredients. This study aims to investigate the efficiency of three different pretreatments of HGDN 59 rice bran hydrolysates (HGDN 59-RBH) with regard to antioxidant activity. Considering the practicality of the process that can be applied using standard commercially available equipment, we compared water, alpha-amylase, and acid-assisted enzymatic hydrolysis using pepsin and trypsin, followed by membrane ultrafiltration. This research evaluates the effectiveness of fractions derived from HGDN 59-RBH in scavenging free radicals, chelating metal ions, and reducing ferric ions, thus providing valuable insights into their potential applications. These findings advance our understanding of the efficiency of pretreatment-assisted enzymatic hydrolysis in producing effective antioxidants. Notably, the antioxidant-rich hydrolysates from HGDN 59-RBH could be value-added products for functional components in food and nutraceutical products.

MATERIALS AND METHODS

Materials: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), The following chemicals and enzymes were sourced from Sigma Aldrich: Pepsin from porcine gastric mucosa, Trypsin from bovine pancreas, potassium persulfate, trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), gallic acid, Trolox, and Folin-Ciocalteu phenol reagent. All solvents and chemical reagents utilized were of reagent grade. Deionized water (DI water) was exclusively used to prepare chemical reagents. The Pierce™ bicinchoninic acid (BCA) protein assay kit from Thermo Fisher was employed for protein determination, with bovine serum albumin serving as the protein standard.

METHODS

Preparation of DRB: HGDN 59 rice bran was obtained from the Pattani Rice Research Center, Pattani, Thailand. The removal of lipids from the rice bran was carried out by solvent extraction. In brief, ten grams of rice bran were homogenized with 50 mL hexane for 1 min at 10,000 rpm (IKA Labortechnik homogenizer, Selangor, Malaysia). The homogenates underwent agitation at room temperature for 12 hours on an orbital shaker (IKA Labortechnik KS501 digital) set at 300 rpm. Subsequently, they were filtered through Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The defatted rice bran was then gathered, and the hexane was eliminated by drying it in a hot air oven at 55°C for 4 hours. The resulting defatted rice bran was ground and preserved at -20°C.

The proximate and mineral compositions of DRB: The proximate content of DRB was assessed following the guidelines outlined by the Association of Official

Analytical Chemists (AOAC). The mineral profile was also evaluated using Energy Dispersive X-ray Fluorescence (EDX-RF) spectroscopy.

Pretreatment of DRB

Water pretreatment: Ten grams of DRB were combined with 100 mL of DI water in a 250-mL Erlenmeyer flask. The pretreatment was conducted at room temperature for 1 hour with continuous agitation at 100 rpm.

Acid pretreatment: The Initial pretreatment involved combining ten grams of DRB with 100 mL of DI water in a 250-mL Erlenmeyer flask. A suitable amount of 6 N HCl was used to adjust the mixture's pH to 2. Subsequently, the pretreatment was conducted at room temperature for 1 hour with continuous agitation at 100 rpm.

Alpha-amylase pretreatment: Initially, the same procedure was followed: ten grams of DRB mixed with 100 mL of DI water in a 250-mL Erlenmeyer flask. The pH was adjusted to 6.2 using 6 N HCl. After pre-incubation in a water bath set at 55°C for 15 minutes, alpha amylase (5,000 U) was added. The pretreatment continued at 55°C for 1 hour with constant agitation at 100 rpm.

In vitro gastrointestinal enzymatic hydrolysis's pretreatment of DRB

Hydrolysis with pepsin: The pre-treated samples were adjusted to pH 2 using an appropriate amount of 6 N HCl. The samples were then pre-incubated in a water bath set at 37°C for 15 min before the addition of pepsin (1 part per 100 parts of crude protein in DRB), and the hydrolysis proceeded for 2 h with constant agitation at 100 rpm.

Hydrolysis with trypsin: The pepsinolysis was deactivated by adjusting the hydrolysates to a pH of 8 using an appropriate amount of 6 N NaOH. The samples were then pre-incubated in a water bath set at 37°C for 15 min before the addition of trypsin (1 part per 100 parts of crude protein in DRB). This hydrolysis proceeded for 2 h with constant agitation at 100 rpm. The samples underwent heating at 95°C for 10 minutes to deactivate enzyme activity. Following centrifugation at 3,000×g for 15 minutes, RBH was obtained. Subsequently, the RBH was neutralized to pH 7 with an appropriate quantity of 6 N HCl, freeze-dried, and stored at -80°C until further analysis.

Yield, protein yield, and protein recovery: The yield of the RBH was calculated based on the following equation:

$$\text{Yield}(\%) = 100\left(\frac{\text{amount(g) of RBH after freeze drying}}{\text{amount(g) of DFRB}}\right)$$

The protein content in the DRB and in the RBH was determined by a Kjeldahl method using a conversion factor of 6.25. The protein recovery was calculated as follows:

$$\text{Protein recovery}(\%) = 100\left(\frac{\text{amount(g) of protein in RBH after freeze drying}}{\text{amount(g) protein in DFB}}\right)$$

Degree of hydrolysis (DH): DH was determined as the proportion of peptide bonds cleaved by the hydrolytic reaction, expressed as a percentage. Every peptide bond cleavage generates a new alpha-amino group, which can be measured according to Adler-Nissen [20] and Benjakul and Morris [21]. In brief, 0.1 mL of sample was combined with 2 mL of 0.2125 M sodium phosphate buffer, pH 8.2, and 1 mL of 0.02% (w/v) 2,4,6-trinitrobenzenesulfonic acid (TNBS). The mixture was incubated in darkness at 50°C for 30 minutes and then combined with 2 mL of 0.1 M sodium sulfite. After a 15-minute incubation period, the absorbance at 420 nm was measured. The alpha-amino acid content was quantified in terms of equivalents of L-leucine. The DH was calculated as follows:

$$DH(\%) = 100 \times \left(\frac{L_t - L_0}{L_{max}}\right)$$

where L_0 and L_t are the amount of alpha-amino acid before and after hydrolysis at time t, respectively. L_{max} is the total amount of alpha-amino acid after acid hydrolysis in 6 N HCl at 100°C for 24 h [22].

Fractionation of RBH by ultrafiltration: RBH was

dissolved in DI water at 1.5 mg/mL and centrifuged at 8,000×g for 15 min. The fractionation was performed sequentially using centrifugal ultrafiltration (Vivaspin-6, Sartorius) with molecular weight cut-off (MWCO) of 5 kDa and 3 kDa. The RBH was fractionated into three molecular weight (MW) fractions: the fraction larger than 5 kDa (> 5 kDa), the fraction between 3 and 5 kDa (3-5 kDa), and the fraction smaller than 3 kDa (< 3 kDa). The gathered fractions were lyophilized and stored at -80°C for subsequent analysis.

Total phenolic content: The method described by Fernandes and colleagues [23] was adapted for use in a 96-well plate format. A sample was combined with 80 µL of 7.5% (w/v) sodium carbonate and 100 µL of a Folin-Ciocalteu reagent diluted with deionized water (1:10, v/v). After a 30-minute incubation at 40°C, the absorbance was measured at 765 nm. A standard curve using gallic acid (50-1000 µM) was generated to determine the total phenolic content, expressed as gallic acid equivalents (µmol/mg sample).

Antioxidant activities of whole RBH and RBH fractions: The freeze-dried RBH samples were dissolved in DI water at the following concentrations (mg/mL): 1, 3, 5, 10, 20, and 40. The freeze-dried whole RBH and the three freeze-dried MW fractions were prepared at 1 mg/mL.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity: An ABTS stock solution was made to prepare for the assay by combining 7.4 mM ABTS with 2.6 mM potassium persulfate in equal volumes. This mixture was left in the dark at room temperature for 16 hours. A working ABTS solution was then prepared by diluting the stock solution with 5 mM phosphate-buffered saline (PBS), pH 7.4, until it reached an absorbance of 0.70 ± 0.02 at 734 nm. Samples (10 µL) in deionized water were mixed with 190 µL of the working ABTS solution. After a 15-minute incubation at room temperature, the absorbance at 734 nm was measured. A standard curve using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was generated, and the ABTS radical-scavenging activity was expressed as Trolox equivalents (µM/mg sample).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity: We followed a modified version of the procedure outlined by Sowmya and Schindra [24], tailored for use in a 96-well plate configuration. Initially, 100 µL of samples was combined with 0.4 mM DPPH reagent dissolved in methanol to attain an initial absorbance reading of 0.90 ± 0.02 at 515 nm. The reaction was then conducted at 37°C for 30 minutes in the absence of light. A Trolox standard curve (ranging from 10 to 120 µM) was established in a 1:1 (v/v) mixture of methanol and water, and subsequently, the DPPH radical-scavenging activity was determined and expressed as Trolox equivalents (µM/mg sample).

Ferrous ion chelating activity: An assay procedure followed Chai et al. [25], with modifications for a 96-well plate format. The solution added to the wells of a 96-well plate was 50 µL of 0.3 mM ferrous sulfate, 50 µL of sample, and 100 µL of 0.5 mM ferrozine reagent. The experiment was conducted under dark conditions at room temperature for a duration of 10 minutes. Following this, the absorbance was measured at 562 nm. A standard curve using EDTA (ranging from 8 to 62 µM) was established, and the iron-chelating activity was quantified and expressed as EDTA equivalents (µmol/mg sample).

Ferric ion reducing antioxidation power (FRAP): The capacity of RBH to convert ferric iron to ferrous iron through the donation of single electrons was assessed following the method described by Adjimani and Asare [26], adapted for use in a 96-well plate setup. A sample (175 µL) was combined and allowed to incubate with 295 µL of 0.2 M sodium phosphate buffer, adjusted to pH 6.6, and 295 µL of 1% (w/v) potassium ferricyanide at 50°C for 20 minutes. Subsequently, 295 µL of 10% (w/v) TCA

solution was introduced, and the mixture underwent centrifugation at 200×g for 10 minutes. The resulting supernatant (115 µL) was transferred into a well of a 96-well plate containing 115 µL of deionized water and 20 µL of 0.1% (w/v) ferric chloride hexahydrate. Following a 10-minute incubation at room temperature, the plate was subjected to analysis at 700 nm. A standard curve using gallic acid (ranging from 8 to 1000 µM) was established, and the FRAP was determined and expressed as gallic acid equivalents (µmol/mg sample).

Statistical analysis: The analysis of RBH hydrolysis was a randomized design with 3 independent biological replicates (n = 3). A one-way analysis of variance (ANOVA) was performed on SPSS software version 11.5. The differences were separated by a P-value of 0.05 ($P<0.05$). The multiple comparisons of means were performed using Tukey's test.

RESULTS AND DISCUSSION

The proximate and mineral compositions of DRB: Table 1 summarizes the proximate and mineral compositions of DRB from HGDN 59. The results revealed that the composition of DRB was in line with reports by Jiamyangyuen et al [27]; Sirikul et al [28]; Wang et al [29]; Kumari et al [30]. The fat content in defatted HGDN 59 rice bran decreased, whereas the protein content in defatted HGDN 59 rice bran increased compared to full-fat HGDN 59 rice bran [31]. The selective reduction of the fat content in DRB was achieved by hexane extraction. This is a simple and effective method to remove fat, thus avoiding rancidity and stabilizing the rice bran material. The protein content in DRB is generally 10-15%, depending on the cultivar [32]. This protein is the predominant source of health benefits in DRB [32]. Therefore, the results showed that the defatting step successfully removed fat and increased the protein content in the rice bran sample before pre-hydrolysis treatment, showing that hexane treatment did not deplete protein content.

Table 1. Proximate and mineral compositions of defatted rice bran

Compositions	Content
Moisture (g/100g)	10.72
Protein (g/100g dry weight)	13.4
Ash (g/100g dry weight)	9.97
Fat (g/100g dry weight)	3.4
Carbohydrate (g/100g dry weight)	62.5
Dietary fiber (g/100g dry weight)	37.5
Calcium (g/kg dry weight)	0.821
Potassium (mg/kg dry weight)	11.0
Magnesium (mg/kg dry weight)	6.4
Phosphorus (mg/kg dry weight)	13.4
Iron (mg/kg dry weight)	0.2024
Manganese (mg/kg dry weight)	0.1718
Zinc (mg/kg dry weight)	0.0534

Regarding the mineral composition of the DRB, phosphorus was the predominant mineral component, followed by potassium and magnesium. Meanwhile, the minor mineral components were calcium, iron, manganese, and zinc. Compared to full-fat rice bran, phosphorus, potassium, and magnesium increased in total mineral content [9,30]. Rice bran contains substantial amounts of both macro- and micro-minerals, which are crucial for regulating various biological processes within the body [9,33]. The results obtained from the EDX-RF technique confirm the mineral profile, which shows that phosphorus and potassium are the main minerals in DRB (Figure 1).

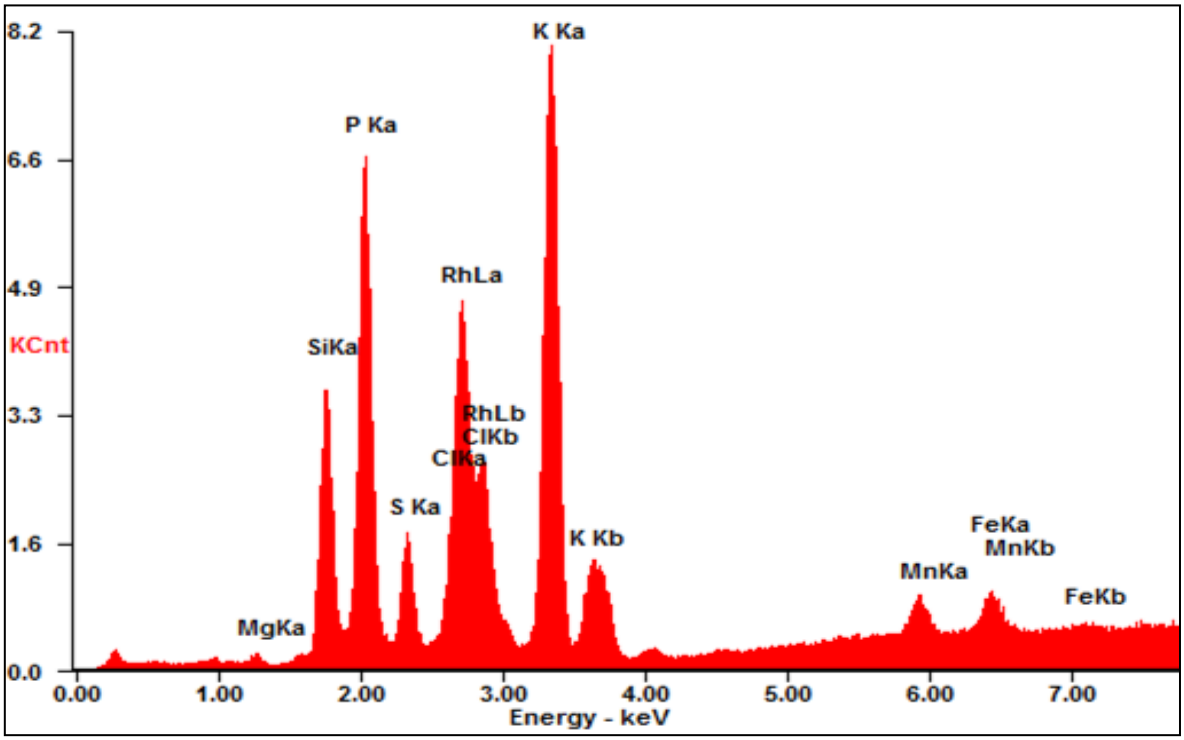


Figure 1. An EDX-RF spectrum of DRB

Effect of the pretreatments on the RBH yield, protein content, protein recovery and degree of hydrolysis of RBH: Rice bran consists of several structurally and chemically distinct cell-based layers, including the pericarp, aleurone and subaleurone (the outermost layer of starchy endosperm), seed coat and germ layer [34]. The dietary fiber content of bran is 20-30% [35]. Rice bran protein is associated or physically entrapped within the matrices of carbohydrates such as polysaccharides and fibers, and extraction requires the disruption of those matrices [12,15,36]. This work performed three different pretreatments using water, alpha-amylase, or acid to aid protein extraction prior to digestion with consecutive *in vitro* gastrointestinal enzymes (pepsin and trypsin). The final yield of RBH from the alpha-amylase pretreatment was markedly more significant than that of the water and acid pretreatments ($P<0.05$; Table 2).

Although the RBH yield was higher, the protein content and recovery resulting from the alpha-amylase pretreatment were notably lower than those from the water and acid pretreatments, respectively ($P<0.05$). In other work on defatted rice bran using various methods, the protein content obtained ranges from 10-38% [37]. All treatments resulted in a similar degree of hydrolysis, ranging between 14-15%. Phongthai et al. [13] reported protein recovery using alkaline extraction prior to pepsin-trypsin hydrolysis of 16.80 ± 0.29%. Concurrently, this study found 23.95 ± 0.54%, 23.21 ± 0.93%, and 18.80 ± 0.57% using water, acid, and alpha-amylase

pretreatments prior to pepsin-trypsin hydrolysis, respectively. Results showed that a pH lower than 8 during acid, water or alpha-amylase pretreatments enhanced solubilization and improved recovery. However, the protein content obtained by Phongthai et al. [13] using alkaline extraction was higher than those obtained by pretreatment in this study. The results indicated that the different pre-hydrolysis treatments influenced yield, protein content, and protein recovery. There are three categories for protein extraction: physical, chemical, and enzymatic methods [12]. Pretreatments with water, acid, and alpha-amylase were selected in this study. However, detailed information on protein hydrolysates obtained by autoclave and alkaline pretreatments was not provided due to low protein content and recovery (data not shown).

Table 2. Effects of different pretreatments of DFRB on RBH yield, protein content, protein recovery, and degrees of hydrolysis (DH) of RBH

Pretreatment	RBH yield (%)	Protein content (%)	Protein recovery (%)	DH (%)
Water	18.91 ± 0.04[b]	17.29 ± 0.40[a]	23.95 ± 0.54[a]	14.08 ± 0.59
Alpha-amylase	21.21 ± 0.91[a]	12.10 ± 0.37[b]	18.80 ± 0.57[b]	14.10 ± 0.39
Acid	17.67 ± 0.75[b]	17.93 ± 0.08[a]	23.21 ± 0.93[a]	14.77 ± 0.56

The mean and standard deviation were calculated from three independent batches of rice bran hydrolysates (n = 3). Different letters (a-b) in the same column indicate significant differences among the pretreatments (P<0.05).

Prior removal of carbohydrate components from bran enables protease treatment to enhance protein extraction due to an increase in the solubility of protein hydrolysates [36]. The protein-solvent interaction is the result of electrostatic repulsion between the protein molecules, which facilitates their separation [38]. A possible reason for low protein content and protein recovery with alpha-amylase pretreatment could be the high pH used in comparison to other pretreatment methods. Alkaline pH causes ionization of the carboxylic groups and deprotonation of the amine groups. This increases the negatively charged species, which encourages their interaction with the solvent. Increased pH also affects the proportion of protein that falls into the insoluble fraction: “in the range of pH from 2-9, less than 10% of the protein in rice bran is extractable” [39].

However, Bedin and colleagues [40] found that an increase in temperature and pH resulted in an increase in extracted protein. Moreover, increasing temperature resulted in an elevated extraction yield, with a reduced impact on the duration used for extraction [40]. In contrast, Sgarbieri [41] reported that very high temperatures in the range of 75-95°C can reduce protein activity and cause thermal denaturation of proteins. However, the alpha-amylase pretreatment in this study was conducted at 55°C but still resulted in significantly lower protein content and protein recovery than the water and acid pretreatments.

Fractionation of RBH by ultrafiltration and total phenolic content of whole RBH and RBH fractions: Following pretreatment and digestion, the resulting RBH was fractionated into three molecular weight (MW) fractions using ultrafiltration. The relative mass proportions of the three MW fractions, which are RBH > 5 kDa, 3-5 kDa, and < 3kDa, are presented in Table 3. The average weight proportions of the three MW fractions were 26.5%, 3.5%, and 70% (by weight) for RBH < 3 kDa, 3-5 kDa, and > 5 kDa, respectively, regardless of the pretreatment.

Table 3. Proportion of RBH after fractionation by ultrafiltration

Pretreatment	Mass proportion (g/100 g)		
	< 3 kDa	3-5 kDa	> 5 kDa
Water	26.1 ± 0.5	2.8 ± 0.2	71.2 ± 0.3
Alpha-amylase	26.2 ± 4.9	3.3 ± 1.0	70.4 ± 4.7
Acid	26.6 ± 2.9	4.0 ± 0.5	69.5 ± 3.2

The mean and standard deviation were calculated from three independent batches of rice bran hydrolysates (n = 3).

The data presented in Figure 2 delineates the total phenolic content associated with each pretreatment applied to both the entirety of RBH and its three MW fractions. Across the whole RBH, the total phenolic content ranged from 0.31 to 0.40 µmol GAE/mg RBH. Remarkably, the RBH > 5 kDa treated with acid exhibited the highest total phenolic content, followed by water pretreatment and alpha-amylase pretreatment, as graphically represented in Figure 2.

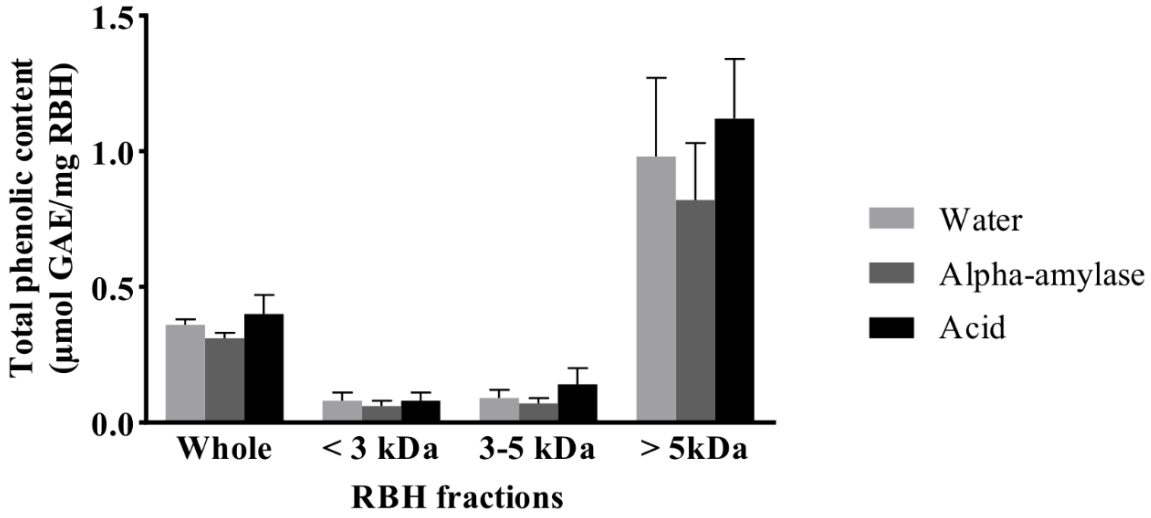


Figure 2. The total phenolic content of the whole RBH and the three MW fractions for each pretreatment. The mean and standard deviation were calculated from three independent batches of RBH (n = 3).

Antioxidant activities of whole RBH and RBH fractions

ABTS radical scavenging activity: The ABTS radical scavenging activity of the whole RBH and the three MW fractions with different pretreatments is shown in Figure 3A. The whole RBH and the RBH fractions pretreated with acid demonstrated higher ABTS radical scavenging activity compared to water and alpha-amylase pretreatments. Of all the conditions tested, the RBH > 5 kDa treated with acid displayed the most pronounced ABTS radical scavenging activity ($P<0.05$). Depending on pretreatments, the RBH > 5 kDa had the lowest EC_{50} value amongst all the samples ($P<0.05$), but showed less inhibitory effect than Trolox as a positive control (0.49 mg/mL) (Figure 3B). Notably, the RBH > 5 kDa treated with acid showcased the highest ABTS radical scavenging activity and the lowest EC_{50} value. This coincides with this fraction containing the highest content of phenolic compounds. This finding aligns with the research conducted by Chen et al. [42], which highlighted that rice protein hydrolysates with a molecular weight exceeding 10 kDa exhibited the most potent ABTS radical scavenging activity. This indicates that the higher MW fraction had a higher ability to trap ABTS radicals than the lower MW fraction. Therefore,

HGDN 59-RBH > 5 kDa may consist of more potent water-soluble antioxidants, such as bioactive peptides, compared to low-MW HGDN 59-RBH fractions (< 3 kDa and 3-5 kDa) and whole RBH. The ABTS radical scavenging activity observed in the three MW fractions of RBH obtained in this study surpasses the levels reported for three fractions of rice bran protein hydrolysates in prior studies [13].

DPPH radical scavenging activity: The DPPH radical scavenging assay evaluates the ability of substances to neutralize free radicals by transferring electrons from a donor molecule to the radical within a methanol solution. Consistent with the findings of ABTS radical scavenging activity, the RBH > 5 kDa treated with acid displayed the most substantial DPPH activity, followed by water and alpha-amylase pretreatments; this variation was statistically significant ($P<0.05$) (Figure 3C). The EC_{50} value of the whole RBH was higher than the positive antioxidant Trolox, whose EC_{50} value was 0.02 mg/mL (Figure 3D). The RBH > 5 kDa significantly exhibited the lowest EC_{50} value ($P<0.05$), aligning with the outcomes observed in ABTS radical scavenging activity. This indicates that the compounds of RBH > 5 kDa exhibit potent antioxidant activity compared to those of whole RBH and other RBH fractions. This corroborates earlier research that the high-MW fraction of rice and rice bran protein hydrolysates (> 10 kDa) separated by ultrafiltration had higher DPPH scavenging activities than the low-MW fraction [16,43]. Consistent findings were also reported, indicating that protein hydrolysates from Zanthoxylum piperitum [44] and Douchi [45] with higher molecular weights exhibited enhanced DPPH radical scavenging activity. Moreover, the DPPH activity observed in this study's three MW fractions of RBH obtained in this study surpassed the levels reported for three fractions of rice bran protein hydrolysates in previous research [13].

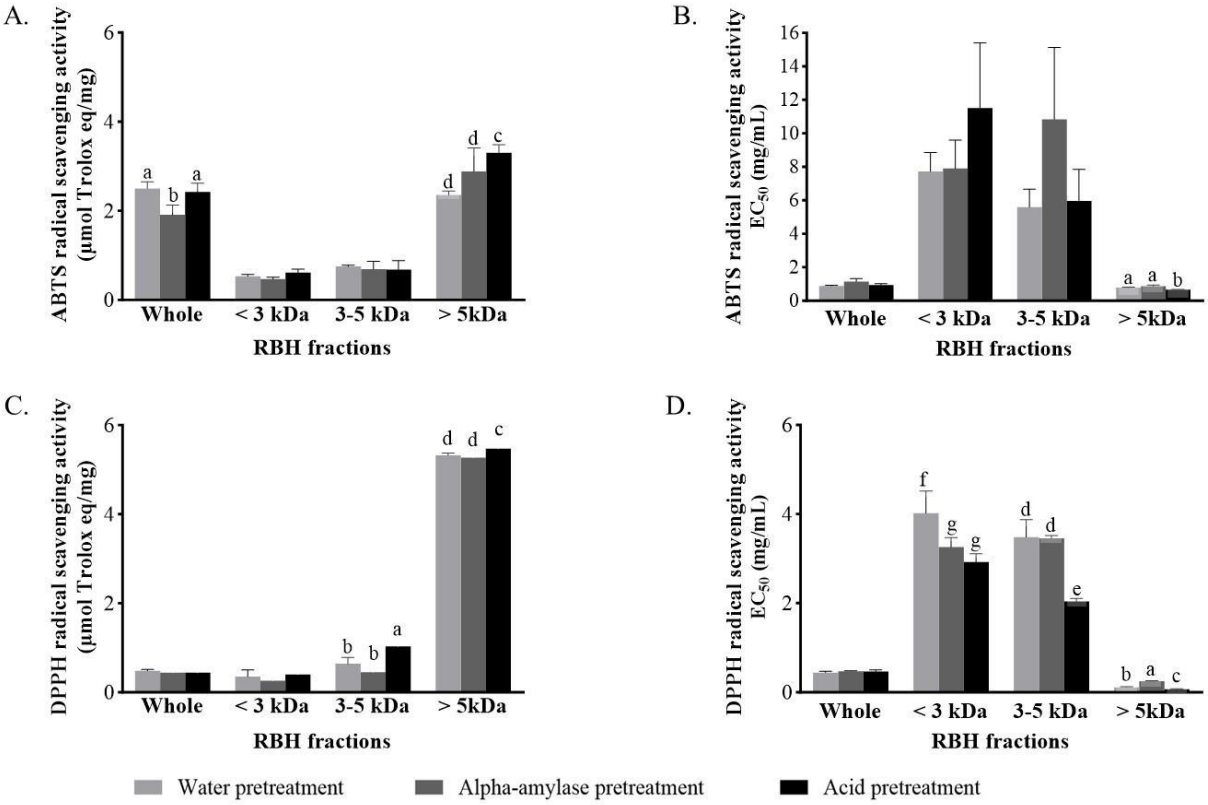


Figure 3. Antioxidative activities and EC_{50} values of whole RBH and its three MW fractions treated with various pretreatments: (A and B) ABTS radical scavenging activity and (C and D) DPPH radical scavenging activity. The mean and standard deviation were computed from three separate batches of rice bran hydrolysates (n = 3). Bars labeled with different letters denote significant differences in results ($P<0.05$) among the pretreatments of RBH.

Ferrous ion chelating activity: Ferrous ion is the redox-active form of the iron ion involved in the Fenton reaction, which produces hydroxyl radicals from the breakdown of peroxides. Metal ion-chelation is the mechanism for analyzing the ability of antioxidants to exclude ferrous ions from participating in the reaction with peroxides. The water and acid pretreatments of the entire RBH yielded superior ferrous iron chelating activity compared to the alpha-amylase pretreatment ($P<0.05$) (Figure 4A). As shown in Figure 4B, the EC_{50} values obtained from whole RBH with water, alpha-amylase, and acid pretreatments were inferior to the effective iron sequester EDTA (EC_{50} 0.05 mg/mL). The EC_{50} of RBH fractions was not available. Among RBH fractions, the RBH > 5 kDa and 3-5 kDa showed the most and second-most powerful ferrous ion chelating activity, which agrees with similar studies for black bean protein fraction 3-10 kDa [10]. The ferrous ion chelating activity demonstrated a robust positive correlation with the presence of amino acids histidine and arginine in hydrolysates [10].

Ferric ion reducing antioxidation power (FRAP): The FRAP assay measures the ability of antioxidants to donate an electron that reduces ferric ions to ferrous ions. The whole RBH and three fractions with acid pretreatment demonstrated higher FRAP activity compared to the water and alpha-amylase pretreatments ($P<0.05$) (Figure 4C). The highest FRAP in the RBH > 5 kDa was similar to that reported for rice bran protein hydrolysates MW 5-10 kDa [13]. This increase may occur through the mechanism of hydrophobicity by positively charged rice bran peptides, mainly related to aromatic amino acid content [46].

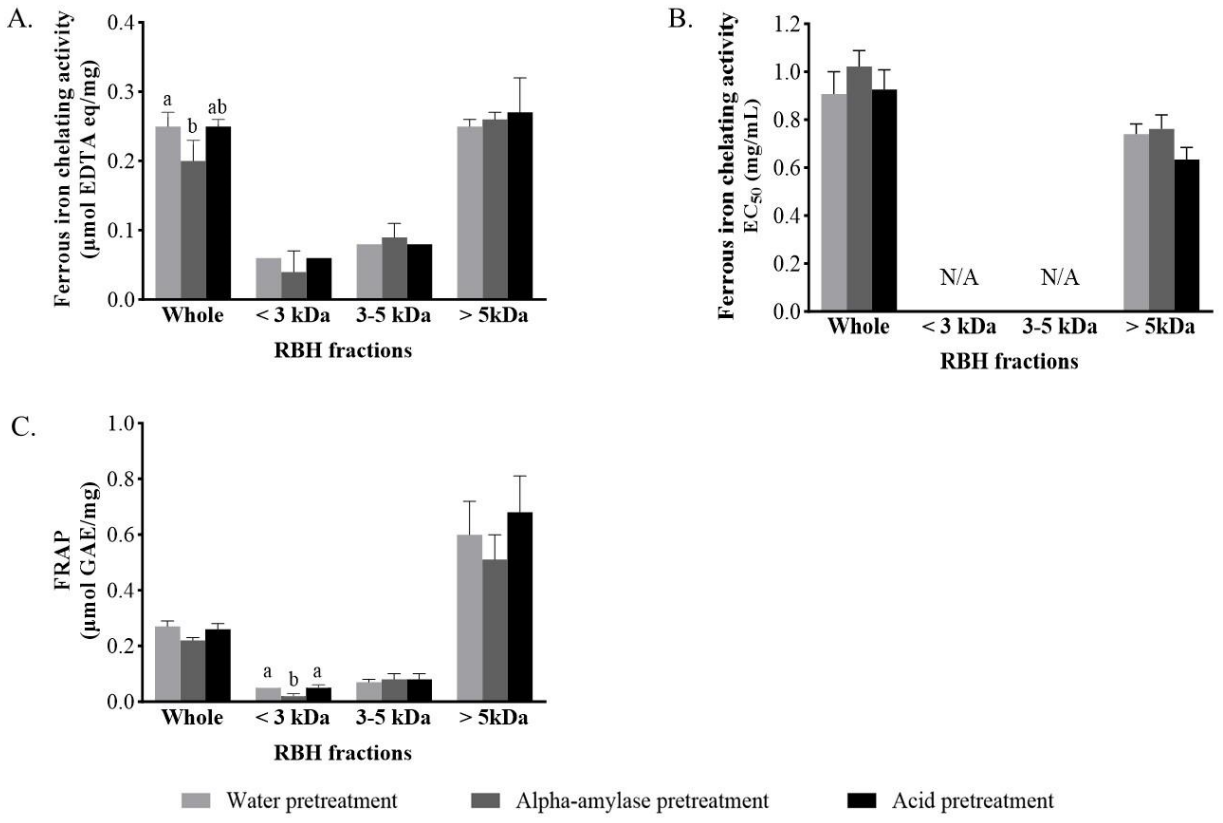


Figure 4. Antioxidative activities and EC_{50} values of whole RBH and its three MW fractions treated with various pretreatments: (A and B) Ferrous iron chelating activity. Antioxidative activities of whole RBH and its three MW fractions treated with various pretreatments; (C) FRAP. The mean and standard deviation were computed from three separate batches of rice bran hydrolysates (n = 3). Bars labeled with different letters denote significant differences in results ($P<0.05$) among the pretreatments of RBH. N/A means not available.

From the results, regardless of the pretreatment, the RBH > 5 kDa fraction exhibited more significant antioxidant activities and higher phenolic content than the other fractions. This leads to speculation that phenolic compounds may be responsible for the observed antioxidant activities. Rice bran is a rich source of polyphenols and flavonoids known to interact covalently and non-covalently with protein [47]. In particular, the alkaline conditions during trypsin digestion can induce the oxidation of polyphenols to form quinones, electrophiles that reactively form conjugation with nucleophilic side chains of proteins [47-48]. In addition, polysaccharides could contribute to the RBH's antioxidant activities. Hydrolysis of Sang-yod rice bran was found to release beta-glucan, a high molecular weight polysaccharide [48]. Antioxidant activities of beta-glucan from various sources (e.g., barley, oat, yeast cell wall, mushroom) have been reported [49-50].

CONCLUSIONS

Pre-proteolysis treatments with water, alpha-amylase, or acid impact the extraction and the antioxidant activities of protein hydrolysates from HGDN 59-RBH. The highest protein yield was obtained through pretreatment by acid, yielding 17.93±0.08 g protein/100 g hydrolysates with 23.21 g/100 g protein in DRB recovery. The whole and three fractions of HGDN 59-RBH have different total phenolic contents and antioxidant capacities. Among the 3 pretreatments, the > 5 kDa fraction of HGDN 59-RBH from the acid pretreatment-assisted enzymatic extraction possessed the highest antioxidant potential according to ABTS radical scavenging activity and DPPH radical scavenging activity. These results represent the first indication that HGDN 59-RBH could serve as a promising natural antioxidant source. Nonetheless, further research is needed to explore the bioactive compounds and the sequences of bioactive peptides in HGDN 59-RBH and assess their potential applications in the development of functional food and nutraceutical products.

Abbreviations: HGDN 59: Hawm Gra Dang Ngah 59, HGDN 59-RBH: HGDN 59 rice bran hydrolysates, MW: molecular weight, ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical, DPPH: 2,2-diphenyl-1-picrylhydrazyl radical, FRAP: ferric ion reducing antioxidation power, DRB: defatted rice bran, EC_{50}: half maximal effective concentration, TCA: trichloroacetic acid, EDTA: ethylenediaminetetraacetic acid, GAE: gallic acid equivalent, DI water: deionized water, BCA: bicinchoninic acid, AOAC: association of official analytical chemistry, EDX-RF: energy dispersive x-ray fluorescence, HCl: hydrochloric acid, NaOH: sodium hydroxide, DH: degree of hydrolysis, TNBS: 2,4,6-trinitrobenzenesulfonic acid, MWCO: molecular weight cut off, PBS: phosphate buffer saline, ANOVA: one-way analysis of variance

Conflict of Interest: The authors declare that they have no competing interests.

Author Contributions: Pornpen Panomwan: Concept and design, Data analysis/interpretation, Statistical analysis, Critical revision of manuscript, Final approval Nantawat Tatiyaborworntham: Data acquisition, Data analysis/interpretation, Statistical analysis, Draft manuscript, Critical revision of manuscript, Final approval. Both authors contributed equally to this work.

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