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Scavenging Activity of Enzymatic Hydrolysates from Wheat Bran

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Summary

Wheat bran was destarched and deproteinated by α-amylase, protease and amyloglucosidase successively, and further hydrolyzed using *Bacillus subtilis* xylanases. The yield of enzymatic hydrolysates from wheat bran (EHWB) was 1.84 %. The total phenolics were 0.3712 g of ferulic acid equivalents per gram of EHWB. The antioxidant potency of EHWB was evaluated using different assays, such as iron ion chelation, reducing power, scavenging activity against 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and reactive oxygen species under *in vitro* conditions. EHWB exhibited an effective ferrous ion chelating activity and strong reducing power. It also showed a high DPPH radical scavenging activity (89.4 %) at 5.0 mg/mL, which was comparable to that of the synthetic antioxidant, butylated hydroxytoluene. EHWB also exerted a marked scavenging effect on $^{\cdot}OH$ with an EC_{50} value of 0.46 mg/mL, which was lower than that of mannitol (1.03 mg/mL), a classical hydroxyl radical scavenger, and obvious antioxidant activities toward $O_2^{\cdot -}$ and H_2O_2.

Key words: antioxidant activity, enzymatic hydrolysates, iron chelation, oxidative stress, wheat bran, xylanases

Introduction

Reactive oxygen species (ROS) are constantly formed in the human body during normal oxidative metabolism and removed by endogenous antioxidant defense consisting of enzymes with antioxidant activities (*e.g.* superoxide dismutase, catalase and glutathione peroxidase) and some relatively low molecular mass antioxidants (*e.g.* ascorbic acid, tocopherols, carotenoids, glutathione, ubiquinol (coenzyme Q), uric acid, melatonin, bilirubin, *etc.*) (*1,2*). ROS ($^{\cdot}OH$, $O_2^{\cdot -}$ and H_2O_2) can cause widespread damage to biological molecules leading to lipid peroxidation, protein oxidation, DNA base modification, *etc*. Furthermore, if ROS are formed additionally in the cells by exogenous sources or not adequately removed, oxidative stress may occur (*3*). Oxidative stress and ROS-mediated cell damage have been associated with aging and a variety of chronic health problems including various forms of neurodegenerative diseases, many types of cancers, or cardiovascular diseases (*4*). Therefore, much attention has been focused on the use of antioxidants, especially natural antioxidants to defend against oxidative stress by scavenging free radicals (*5*).

Epidemiological studies strongly suggest that diets rich in cereals play a crucial role in the prevention of chronic diseases such as cardiovascular disease and certain types of cancers (*6*). The beneficial health effects derived from the intake of diets rich in cereals have mainly been ascribed to dietary fibre, and/or to some of the components associated with the fibre, including phenolic acids (*7*). Phenolic antioxidants are reported to have

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strong *in vitro* and *in vivo* antioxidant activities associated with their ability to quench oxygen-derived free radicals, break radical chain reaction, and chelate metals (*8*). It is widely accepted that phenolic compounds, including ferulic, vanillic, *p*-coumaric, caffeic, and chlorogenic acids, are rich in the bran portion of cereal kernels and may contribute to the total antioxidant activities of wheat (*9*). These hydroxycinnamic acids exhibit *in vitro* chemoprotective and antioxidant properties, and are suggested to be mainly responsible for the beneficial effects of a diet rich in cereal bran (*10*).

In most experiments, aqueous alcohols and acetone with different levels of water are most commonly employed to extract the phenolic compounds from cereals (*11–13*). It is well known that significant levels of phenolics, mainly hydroxycinnamic acids, have been determined in wheat grains and are mostly found concentrated in the bran portion of wheat kernels, and the phenolic acids are primarily in an insoluble bound form, and esterified to the C-5 hydroxyl group of α-L-arabinfuranosyl substituents, which are linked to C-2 or C-3 on the xylopyranosyl backbone (*14*). The extraction solvents usually used, such as absolute ethanol, methanol, aqueous ethanol with different proportions, do not allow a complete release of antioxidant compounds, such as condensed tannins and polyphenols bound to dietary fibre, and, additionally, nonextractable polyphenols in cereal products with a high antioxidant activity are ignored, which were found in the residues of the aqueous-organic extraction (*15*). Liyana-Pathirana and Shahidi (*16*) reported that the contribution of bound phenolics to the total phenolic content was significantly higher than that of free and esterified fractions in wheat, and the bound phenolic fraction demonstrated a significantly higher antioxidant capacity than free and esterified phenolics. The covalently bound ferulic acids during the fermentation of wheat bran fibre in a human model colon were released (*17*). Although the solvent extraction is the major method to isolate bioactive compounds from plant origin, or to obtain plant extracts rich in bioactive compounds, there have been several controversial points such as extremely low recovery and strict regulations for the use of organic solvents in the food industry. As one of the extraction techniques, enzymatic hydrolysis gains more advantages over other conventional techniques because enzymes can convert water-insoluble materials into water-soluble materials and the method utilizes no organic solvent, nor any other toxic chemicals (*18*). For example, Heo *et al.* (*19*) reported that enzymatic hydrolysis of brown seaweeds gained high bioactive compound yield and showed enhanced biological activity compared with water and organic extract counterparts.

Wheat bran, an important by-product of the cereal industry, is produced worldwide in enormous quantities and recognized as a good source of dietary fibre. To the best of our knowledge, there appears to be little literature on the investigation of potential antioxidant activity of enzymatic hydrolysates from cereal brans. The main purpose of this study is to investigate the antioxidant capacity of enzymatic hydrolysates obtained from destarched and deproteinated wheat bran (EHWB) incubated with xylanases employing various *in vitro* assay systems, namely, iron ion chelation, reducing power, and scavenging activity of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical and reactive oxygen species (H_2O_2, $O_2^{\cdot-}$, $^{\cdot}OH$) in order to understand better the mechanisms of antioxidant activity.

Materials and Methods

Materials

Wheat bran was obtained from Beijing Gongdeli Flour Factory (Beijing, PR China). The bran was milled and passed through a 0.5-mm sieve. Xylanase from *Bacillus subtilis*, a commercial food-grade enzyme preparation, was from the Sunhy Biology Company (Wuhan, PR China). Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacillus licheniformis*, 120 Kilo Novo Units (KNU)/g), protease Alcalase 2.4 L (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 Anson Units (AU)/g), and amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 Novo Amyloglucosidase Units (AGU)/g) were from Novo Nordisk (Bagsvaerd, Denmark). Amberlite XAD-2 was obtained from Rohm and Haas Company (Philadelphia, USA). Ascorbate, 2-deoxy-D-ribose, ferulic acid, β-nicotinamide adenine dinucleotide (NADH), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), 2,2′-bipyridyl, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT) and DPPH radical were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide, ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA), potassium ferricyanide and ferric chloride were purchased from Shanghai Chemical Agents Company, PR China. All other chemicals and solvents used in this study were of analytical grade.

Preparation of EHWB

Wheat bran (100 g) was autoclaved for 45 min at 121 °C in order to destroy the activities of endogenous cell wall-degrading enzymes (*e.g.* endogenous arabinoxylanase) and subsequently swollen in a 2000-mL beaker with 1000 mL of water at 60 °C for 6 h. After that, 7.5 mL of α-amylase were added to the suspension, and then heated in boiling water bath for 40 min with continuous stirring. After the treatment with α-amylase, the pH of the suspension was adjusted to 7.5 with 275 mmol/L NaOH, and 3.0 mL of protease were then added to the sample. After incubation at 60 °C for 30 min, the pH of the suspension was acidified with 325 mmol/L of HCl to 4.5. Then, 3.5 mL of amyloglucosidase were added and the mixture was incubated at 60 °C for 30 min with continuous agitation. The suspension was centrifuged at 10 000×*g* for 10 min, and the residue was washed twice with hot and cold water alternately until no cloudiness was evident. The washed residue was dried at 40 °C overnight in a vacuum oven to obtain the destarched and deproteinated wheat bran. A mass of 10 g of the treated wheat bran was incubated in 200 mL of 0.5 % (by mass per volume) xylanase (in 50 mmol/L acetate buffer at pH=5.0) at 50 °C in the dark for 4 h with constant stirring. After heat inactivation of the enzyme at 100 °C for 10 min, the hydrolysate was centrifuged at 10 000×*g* for 20 min, and the supernatant was passed through a 0.45-μm filter. The filtrate was applied to an open column (80×2.5 cm i.d.) packed with Amberlite

XAD-2 (previously washed with 95 % (by volume) ethanol and then water). Elution was successively carried out with two column volumes of distilled water, three column volumes of 50 % (by volume) methanol and two column volumes of absolute methanol. The fractions eluted by methanol were combined to be concentrated and lyophilized with a freeze dry system to get EHWB (ALPHA1–4, Martin Christ GmbH, Germany).

Determination of total phenolic content

The total phenolic content of EHWB was determined with the Folin-Ciocalteu method (*20*). Briefly, EHWB (3 mg) was dissolved in 10 mL of distilled water. An aliquot of 100 μL of appropriate dilution of the sample was shaken for 1 min with 500 μL of the Folin-Ciocalteu reagent freshly prepared in our laboratory, and 6 mL of distilled water. After the mixture was shaken, 2 mL of 15 % (by mass per volume) sodium carbonate were added and the mixture was shaken once again for 0.5 min. Finally, the solution was brought up to 10 mL by adding distilled water. After 2 h of reaction at ambient temperature, the absorbance at 750 nm was measured using glass cuvettes. Using ferulic acid as standard, the total phenolic content of EHWB was expressed as ferulic acid equivalents (mg FAE/g EHWB). Data are reported as mean±SD for at least three replications.

Measurement of metal ion chelating activity

The ferrous ion chelating potential of EHWB was measured according to the method of Yamaguchi *et al.* (*21*) with minor modifications. EDTA was used as the reference material. Briefly, 0.25 mL of $FeSO_4$ solution (1 mmol/L) and an equal volume of the tested sample solution of EHWB at different concentrations (1.0 to 5.0 mg/mL) were mixed; 1 mL of Tris-HCl buffer (pH=7.4) and 2,2′-bipyridyl solution (0.1 % in 0.2 mol/L of HCl) were added to the mixture together with 0.4 mL of 10 % (by mass per volume) hydroxylamine-HCl and 2.5 mL of ethanol, respectively. The control contained all the reaction reagents except EHWB or EDTA. The reaction mixture was adjusted to a final volume of 5 mL with water, shaken well and incubated for 10 min at room temperature. The absorbance was determined at 522 nm with a UV-1000 UV/VIS recording spectrophotometer (Rayleigh Analytical Instruments, Beijing, PR China). Lower absorbance of the reaction mixture indicated higher ferrous ion chelating ability. The capability to chelate ferrous iron was calculated by Eq. 1:

$$\text{Chelating activity}=(1-A_{\text{sample 522 nm}}/A_{\text{control 522 nm}})\times 100 \quad /1/$$

where A_{sample} was the absorbance in the presence of the tested sample or reference material, and A_{control} was the absorbance of the control without the tested sample or reference material. All determinations were performed in triplicate.

Measurement of Fe^{3+} to Fe^{2+} reducing activity

The reducing power of EHWB was determined according to the method of Dorman *et al.* (*22*). Briefly, 1.0 mL of aqueous sample at different concentrations (0.25–2.5 mg/mL) of EHWB was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH=6.6), and the reaction mixture was incubated with 2.5 mL of a 1 % (by mass per volume) aqueous potassium ferricyanide solution at 50 °C for 30 min. The reaction was terminated by adding 2.5 mL of 10 % (by mass per volume) TCA solution and the mixture was centrifuged at 3000×*g* for 10 min. A volume of 2.5 mL of aliquot of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1 % (by mass per volume) aqueous ferric chloride solution, and the absorbance was measured at 700 nm with a UV-1000 UV/VIS recording spectrophotometer (Rayleigh Analytical Instruments, Beijing, PR China). Higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as the reference material.

Measurement of DPPH scavenging activity

The scavenging activity of EHWB on DPPH radicals was measured according to the method of Brand-Williams *et al.* (*23*) with minor modifications. BHT was used as the reference material. Briefly, an aliquot of 0.2 mL of sample solution at different concentrations (0.2 to 5.0 mg/mL) of EHWB was mixed with 0.8 mL of Tris-HCl buffer (100 mmol/L, pH=7.4) and 1.0 mL of 0.5 mmol/L methanolic solution of $DPPH^{\cdot}$. The control contained all the reaction reagents except EHWB or BHT. The reaction mixture was shaken well and incubated for 30 min in the dark at room temperature, and the absorbance of the resulting solution was measured at 517 nm with a UV--1000 UV/VIS recording spectrophotometer (Rayleigh Analytical Instruments, Beijing, PR China). The radical scavenging activity of the tested samples was measured as a decrease in the absorbance of $DPPH^{\cdot}$ and was calculated by Eq. 2:

$$\text{Scavenging activity}=(1-A_{\text{sample}}/A_{\text{control}})\times 100 \quad /2/$$

where A_{sample} was the absorbance in the presence of the sample of EHWB or reference material, and A_{control} was the absorbance of the control containing all the reaction reagents except the sample or reference material. All determinations were performed in triplicate.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging ability of EHWB was determined using 2-deoxy-D-ribose oxidative degradation mediated by hydroxyl radicals as described by Halliwell *et al.* (*24*). The reaction mixture contained 500 μL of the sample of EHWB solution dissolved in KH_2PO_4-KOH buffer (50 mmol/L, pH=7.4) at various concentrations (0.1–2.0 mg/mL), 100 μL of 2.8 mmol/L 2-deoxy-D-ribose in KH_2PO_4-KOH buffer, 200 μL of a premixed 100 μmol/L $FeCl_3$ and 104 μmol/L EDTA solution (1:1, by volume), 100 μL of 1 mmol/L hydrogen peroxide and 100 μL of 100 μmol/L ascorbic acid. The reaction mixture was incubated at 37 °C for 60 min. Thereafter, 1.0 mL of 0.5 % thiobarbituric acid in 10 % trichloroacetic acid was added and the mixtures were vortexed and heated in a water bath at 100 °C for 15 min. The reaction was stopped by a 5-minute ice water bath. The mixtures were centrifuged at 12 000×*g* for 5 min at room temperature, and the absorbance of the supernatants was measured at 532 nm with a UV-1000 UV/VIS recording spectrophotometer (Rayleigh Analytical Instruments, Beijing, PR China). The potassium phosphate buffer was

used as a blank. Mannitol was used as the reference material. The scavenging activity against hydroxyl radicals was calculated using Eq. 2.

The EC_{50} values of EHWB and mannitol were estimated by a nonlinear regression algorithm (Data processing system, v. 3.01). The EC_{50} value stands for the concentration of an antioxidant required to scavenge 50 % of the hydroxyl radicals in the reaction mixture under the experimental conditions. All the tested compounds were dissolved in KH_2PO_4-KOH buffer (50 mmol/L, pH= 7.4). All determinations were performed in triplicate.

Superoxide anion radical scavenging assay

The superoxide anion scavenging activity of EHWB was assessed using the method described by Nishikimi *et al.* (*25*) with slight modifications. Superoxide anion radical was generated in a nonenzymic system. A volume of 1 mL of NBT solution (156 μmol/L) and 1 mL of NADH solution (468 μmol/L) in phosphate buffer (100 mmol/L, pH=7.4), and 1 mL of the sample solution of EHWB with different concentrations (1.0–5.0 mg/mL) were mixed. The reaction was started by adding 1 mL of PMS solution (60 μmol/L) in phosphate buffer (100 mmol/L, pH=7.4) to the mixture. The reaction mixture was incubated at room temperature for 5 min, and the absorbance at 560 nm was read against blank samples. Ascorbate was used as the reference material. All tests and analyses were run in triplicate. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using Eq. 2.

Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging ability of the samples (EHWB solutions) was determined according to the method described by Ruch *et al.* (*26*). A solution of hydrogen peroxide (2 mmol/L) was prepared in phosphate buffer (0.1 mmol/L, pH=7.4) at 20 °C. Hydrogen peroxide concentration was determined spectrophotometrically at 230 nm using a molar absorptivity for hydrogen peroxide of 81 L/(mol·cm) (*27*). An aliquot of 3.4 mL of the sample dissolved in phosphate buffer (0.1 mmol/L, pH=7.4) at various concentrations (1.0–5.0 mg/mL) was mixed with 600 μL of a hydrogen peroxide solution. Ascorbic acid was used as the reference compound. The concentration of hydrogen peroxide was measured by reading the absorbance values of the reaction mixtures at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The scavenging capacities against hydrogen peroxide of both sample and standard compounds were calculated using Eq. 2. All tests and analyses were run in triplicate.

Statistical analysis

Data were reported as the mean±standard deviation of triplicate determinations. Data were analyzed by an analysis of variance (ANOVA; $p<0.05$) and the means separated by Duncan's multiple range test. Results were processed by Statistica software (v. 6.0).

Results and Discussion

The yield of EHWB and total phenolic content

Hydrolytic products of the destarched and deproteined wheat bran incubated with xylanase were applied to an open column (80×2.5 cm i.d.) packed with Amberlite XAD-2, which is a polymeric adsorbent capable of binding aromatic compounds. Initially, some water-soluble non-phenolic compounds were eluted by application of distilled water to the column. After that, 50 % aqueous methanol and absolute methanol were successively applied to the column, and the eluted fractions were combined to be concentrated and lyophilized to get EHWB. The yield of EHWB was 1.84 %, and total phenolic compounds were (0.3712±0.0025) g of FAE per gram of EHWB.

Metal chelating activity

Transition metal, particularly iron, plays a crucial role in oxygen radical reactions and subsequent oxidative damage to biological materials. These reactions often involve iron-catalyzed activation of oxygen to form superoxide anion radical, hydrogen peroxide, and highly reactive hydroxyl radical *via* the Fenton reaction and Haber-Weiss cycle (*28*). As metal chelation is one of the important properties of antioxidants, the chelating of ferrous ion by EHWB was estimated by 2,2′-bipyridyl competition assay. The 2,2′-bipyridyl can quantitatively form red complexes with Fe^{2+}. In the assay, EHWB interfered with the formation of ferrous and 2,2′-bipyridyl complex, suggesting that they have chelating activity and capture the ferrous ion before 2,2′-bipyridyl. It can be seen in Table 1 that the ferrous ion chelating effect of EHWB linearly increased with increasing concentration of EHWB ranging from 1.0 to 5.0 mg/mL. Although EHWB had an overall lower chelating effect than EDTA, a maximum chelating effect (64.05 %) was apparently shown at 5.0 mg/mL of EHWB. Ferrous ion chelating capacity was significant since it reduced the concentration of the catalysing transition metal in the Fenton reaction and lipid peroxidation (*29*). Reduction of Fe^{2+} concentrations in the Fenton reaction can protect against oxidative damage.

Table 1. Fe^{2+} chelating activity of various EHWB concentrations

Sample	Chelating activity/%				
	γ(EHWB)/(mg/mL)				
	1.0	2.0	3.0	4.0	5.0
EHWB	15.49±2.38	30.33±1.13	42.27±3.48	62.70±5.22	64.05±4.60
EDTA	90.06±4.46	—	—	—	—

Data are expressed as means±SD of triplicate measurements

Fe^{3+} to Fe^{2+} reducing activity

The antioxidant activity has been attributed to a variety of mechanisms, including free radical chain breaking, transition metal chelation, decomposition of peroxides, singlet oxygen quenching, prevention of continued hydrogen abstraction, reductive capacity, radical scav-

enging and inhibition of oxidative enzymes, in real food and biological systems (*30*). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (*31*). In the present study, the reducing power of EHWB was investigated. Ascorbate, a known reducing agent as well as a reductone, was used as a reference material for the purpose of comparison. It can be seen in Table 2 that the reducing power of EHWB increased with increasing concentration of the sample of EHWB solution. However, as anticipated, the reducing power of ascorbate was relatively more pronounced than that of EHWB. EHWB at a concentration of 2.5 mg/mL was comparable with that of ascorbate at a concentration of 0.25 mg/mL. The reducing properties are thought to be associated with the development of reductones, which have been shown to exert antioxidant action by terminating the free radical chain reactions by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (*29*). EHWB were capable of reducing Fe^{3+}, which was related to the capability of donating electrons of the present phenolics, suggesting that EHWB may act as the terminators of free radical chains, transforming reactive free radical species into more stable nonradical products.

DDPH scavenging assay

In this study, EHWB was assessed against DPPH radicals to determine the free radical scavenging ability, and the results are shown in Table 3. EHWB exhibited a strong scavenging activity on the DPPH radical in *in vitro* assay. A significant increase of the scavenging activity was observed at the concentration range of 0.2–1.0 mg/mL of EHWB, and the antioxidant inhibition reached a maximum plateau at around 90 % from 2.0 to 5.0 mg/mL. Even if EHWB had an overall lower free radical scavenging activity against DPPH radical than BHT, they showed a high DPPH radical scavenging activity (89.4 %) at 5.0 mg/mL, which was comparable to that of the synthetic antioxidant, BHT. It is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability (*32,33*). Several investigations were conducted to study free radical scavenging capacity of the extracts of wheat and wheat bran against stable DPPH radical (*34,35*). Their results demonstrated that the present phenolic compounds contributed to the significant free radical scavenging capacity. The DPPH radical scavenging activity of phenolic antioxidants depends mainly on different structural features such as O-H bond dissociation energy, resonance delocalization of the phenol radical and steric hindrance derived from bulky groups substituting hydrogen in the aromatic ring. In this study, EHWB showed a dose-dependent scavenging of DPPH free radical within the tested concentration range, which may be attributable to the hydrogen-donating ability of the present phenolics.

Hydroxyl radical scavenging activity

Hydroxyl radicals are generated by a mixture of Fe^{3+}, ascorbate and H_2O_2 in the presence of a slight molar excess of EDTA over the salt, as can be seen in the following reactions (*24*).

$$Fe^{3+}\text{-EDTA}+\text{ascorbate} \rightarrow Fe^{2+}\text{-EDTA}+\text{+oxidized ascorbate}$$

$$Fe^{2+}\text{-EDTA}+H_2O_2 \rightarrow OH^- + {}^{\cdot}OH + Fe^{3+}\text{-EDTA}$$

$${}^{\cdot}OH+\text{deoxyribose} \rightarrow \text{fragments} \xrightarrow[\Delta]{\text{TBA}+\text{acid}} \text{MDA}$$

$$2\text{TBA}+\text{MDA} \rightarrow \text{chromogen}$$

In the presence of hydroxyl radical scavengers, the antioxidants compete, to some extent, with deoxyribose for the hydroxyl radicals and decrease the rate of deoxyribose degradation. A series of reaction fragments derived from the deoxyribose degradation by hydroxyl radicals eventually results in the formation of malonaldehyde (MDA) under heat and acid conditions. The MDA reacts with thiobarbituric acid to form a pink chromogen. The relative extents of inhibition of deoxyribose de-

Table 2. The effect of EHWB on Fe^{3+} reduction (A_{700nm})

Sample	A_{700nm}					
	γ(EHWB)/(mg/mL)					
	0.25	0.5	1.0	1.5	2.0	2.5
EHWB	—	0.247±0.014	0.406±0.007	0.539±0.012	0.792±0.023	0.824±0.018
Ascorbate	0.879±0.016	—	—	—	—	—

Data are expressed as means±SD of triplicate measurements

Table 3. Scavenging activity of different concentrations of EHWB against DPPH radical

Sample	Scavenging activity/%						
	γ(EHWB)/(mg/mL)						
	0.2	0.5	1.0	2.0	3.0	4.0	5.0
EHWB	23.5±0.46	58.3±0.29	83.5±0.98	85.8±0.21	87.9±0.11	88.7±0.54	89.4±0.42
BHT	90.7±0.38	—	—	—	—	—	—

Data are expressed as means±SD of triplicate measurements

gradation will give an indication of hydroxyl radical scavenging activity. Consequently, the ability to diminish the amount of colour formation has been adapted as one measurement of antioxidative properties.

In the presence of EDTA, mannitol, a classical scavenger of hydroxyl radicals, significantly inhibited deoxyribose degradation in a dose-dependent manner with an EC_{50} value of 1.03 mg/mL, as shown in Fig. 1. It was observed that EHWB exhibited a stronger concentration--dependent inhibition of deoxyribose and nearly 82.1 % inhibition was observed at EHWB concentration of 2.0 mg/mL (Fig. 1). The EC_{50} (0.46 mg/mL) of EHWB was evidently lower than that of mannitol. The results indicate that EHWB might be a potent hydroxyl radical scavenger. Smith *et al.* (*36*) reported that molecules that can suppress deoxyribose degradation are those that can chelate iron ions and render them inactive or poorly active in a Fenton reaction. In another assay system of the present study, EHWB showed the ferrous ion chelating ability. Based on the results, it is reasonable to conclude that metal ion chelating capacity is not the only mechanism for explaining the hydroxyl radical quenching activity of phenolic compounds. Other mechanisms, such as hydrogen-abstraction reaction and electron-transfer process (*37*), also regulate the hydroxyl radical scavenging activity.

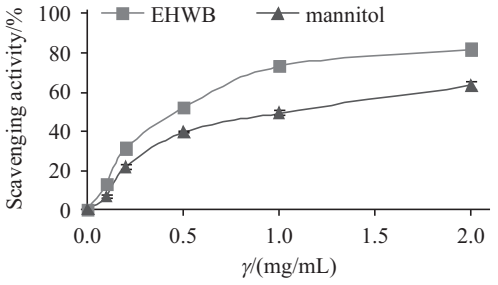

Fig. 1. Hydroxyl radical scavenging activity of EHWB and the standard (mannitol). The data are expressed as mean±SD (*N*=3)

Superoxide anion radical scavenging activity

Superoxide anions are the most common free radicals *in vivo* and are generated in a variety of biological systems, either by autoxidation processes or by enzymes. Superoxide anions can damage biomacromolecules directly or indirectly by forming hydrogen peroxide, hydroxyl radical, peroxylnitrile, or singlet oxygen during pathophysiologic events (*38*). Therefore, the PMS/NADH--NBT system was used to test whether EHWB scavenge superoxide anion radicals. The superoxide anion radicals are generated from dissolved oxygen by the coupling reaction of PMS-NADH mixture at pH=7.4, which can reduce NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion radicals in the reaction mixture. Fig. 2 shows the inhibitory effect of EHWB on superoxide anion radicals. EHWB exerted a concentration-dependent scavenging activity with 39.2 % of superoxide anion radical scavenging activity at 5.0 mg/mL, which was comparable with that of ascorbate at 3.0 mg/mL.

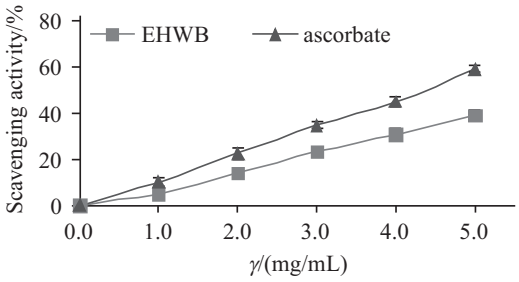

Fig. 2. Superoxide anion radical scavenging activity of EHWB and the standard (ascorbate). The data are expressed as mean±SD (*N*=3)

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent; however, its potential to produce highly reactive oxygen species, such as hydroxyl radical through Fenton reaction, is very high (*39*). Hydrogen peroxide can inactivate a few enzymes directly, usually by oxidation of essential thiol groups. Hydrogen peroxide can cross biological membranes rapidly, once inside the cell, it can react with $Fe^{2+}$and Cu^{2+} ions to form cytotoxic hydroxyl radical (*40*). Thus, the removing of hydrogen peroxide is very important for antioxidant defense in the biological cell of food systems. The decomposition of hydrogen peroxide into water may occur according to the following reaction:

$$H_2O_2+2H^++2e^- \rightarrow 2H_2O$$

As shown in Fig. 3, EHWB exhibited a concentration-dependent scavenging activity against hydrogen peroxide, which was relatively lower than that of ascorbate. EHWB showed 32.9 % of hydrogen peroxide scavenging activity at 5.0 mg/mL, which was comparable with that of ascorbate at 4.0 mg/mL.

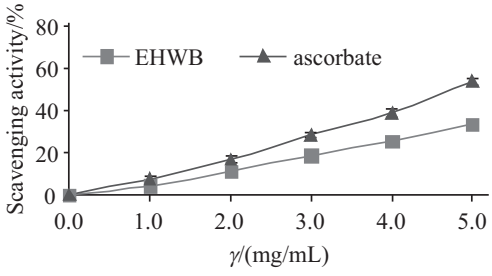

Fig. 3. Hydrogen peroxide scavenging activity of EHWB and the standard (ascorbate). The data are expressed as mean±SD (*N*=3)

Conclusions

In the present study, the results have shown for the first time that the enzymatic hydrolysates from destarched and deproteined wheat bran exhibited effective antioxidant activities in the employed *in vitro* experiments, including metal chelation analysis, reducing power test, DDPH radical and reactive oxygen species (H_2O_2, $O_2^{\cdot-}$, $^{\cdot}OH$) scavenging activity assays, which indi-

cates that EHWB may be a potential source of natural antioxidants for the food industry.

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