

Inhibition of wheat bran and its active components on α -glucosidase *in vitro*

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ABSTRACT

Background: Wheat bran is a traditional Chinese medicine; however, it is mostly used as feedstuff in China. Wheat bran is widely accepted as an important ingredient in many low-glycemic index foods in modern western societies; however, its glycemic control mechanism is unknown. **Objective:** To determine potent α -glucosidase inhibitory compounds from wheat bran and to identify the inhibition on α -glucosidase. **Materials and Methods:** Ethanolic extract of wheat bran was prepared to evaluate the inhibitory activity on α -glucosidase, then fractionation of the extract was guided by *in vitro* enzyme-inhibition assay, and the potent α -glucosidase inhibitory compounds were identified by high performance liquid chromatography and atmospheric pressure chemical ionization-mass spectrometry; finally the enzyme inhibition process was studied using the Michaelis-Menton and the Lineweaver-Burk equations. **Results:** Both baker's yeast and rat intestinal enzymes were mostly inhibited (87.9% and 66.8% inhibition, respectively) at concentration 0.6 mg/mL of the ethanolic extract of wheat bran. The petroleum ether fraction in the ethanolic extract of wheat bran showed significant activity against rat intestinal α -glucosidase, and revealed a dose-dependent effect. The inhibition was 76.57% at 0.3 mg/mL and 100% at 0.6 mg/mL. The active fraction 13 of petroleum ether fraction was identified as alkylresorcinols (ARs). ARs showed strong inhibition towards α -glucosidase and its IC_{50} value was found to be 37.58 μ g/mL. The enzyme kinetic studies showed that, in the presence of ARs, the Michaelis-Menton constant (K_m) remains constant whereas the maximal velocity (V_{max}) decreases, revealing a non-competitive type of inhibition. **Conclusion:** The therapeutic potentiality of ARs in the management of the postprandial hyperglycemia will proliferate the utilization of wheat bran in controlling type 2 diabetes.

Key words: Alkylresorcinols, inhibition, wheat bran, α -glucosidase

INTRODUCTION

Diabetes mellitus (DM), (types 1 and 2) is recognized as one of the most serious global health problems with high morbidity and mortality, and DM is also one of the most costly and burdensome chronic diseases.^[1] There is an increasing trend in its epidemic proportions throughout the world, especially in China. This increasing trend in DM has been of serious medical concern, and there are an estimated 90 million sufferers in China currently. There needs to be sincere effort on the treatment of diabetic patients.^[2-4] However, the management of diabetes

without any side effects poses a challenge to the medical fraternity.^[5-7]

Type 2 diabetes (non-insulin-dependent DM) accounts for more than 90% of all diabetes cases.^[4,8] Type 2 diabetes is characterized by hyperglycemia, as a result of the diminishing insulin secretion and insulin resistance of the peripheral tissues, such as the liver, skeletal muscle, and adipose tissue.^[9] α -Glucosidase inhibitor is usually used to prevent or medically treat type 2 diabetes, which can effectively block the uptake of postprandial blood glucose. Some hypoglycemic drugs have some side effects, many studies have been conducted to find more effective α -glucosidase inhibitors from natural sources, such as herbal medicine and compounds derived from food.^[10,11]

Wheat (*Triticum aestivum* L) is one of the major crops cultivated in China. Wheat bran is a by-product of

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the commercial flour milling process, and the annual output reaches 20 million tons. In traditional Chinese medicine, wheat bran has had a long medicinal history. The “Compendium of Materia Medica” has the earliest recorded use of wheat bran in the treatment of beriberi, cold sweat, and night sweat.^[12] In modern western societies, wheat bran of whole wheat is widely accepted as an important ingredient in several low-glycemic index food. A recent epidemiological study showed that the consumption of wheat bran can decrease the risk of type 2 diabetes.^[13] Liu’s experiments first showed that wheat bran and germ extracts had α -glucosidase inhibitory activities *in vitro* and phosphatidic acids were investigated to be the potent inhibitor in the germ.^[7] However, α -glucosidase inhibitors from wheat bran have not, so far, been reported. Hence, a detailed study of the bioactive phytochemicals of anti-diabetic in wheat bran may help elucidate the mechanism of reducing blood sugar of wheat bran, and promote effectiveness, less side effects, and relatively low-cost functional foods in preventing and treating DM, following an in-depth processing of wheat bran.

MATERIALS AND METHODS

Material and chemicals

Wheat bran (*T. aestivum* L.) used in the study was supplied by Jiangnan Flour Milling, Zhenjiang, Jiangsu Province, China. The bran contained the pericarp, seed coat, aleurone, and a small quantity of embryo of the wheat seed. The bran was cleaned and stored in a cool and dry place prior to use. A voucher specimen was deposited in the school of Food and Biology Engineering, Jiangsu University. α -Glucosidase isolated from baker’s yeast and the substrate *p*-nitrophenyl-alpha-D-glucopyranoside (*p*NPG) were purchased from Wako Pure Chem Co. (Osaka, Japan). Mammalian α -glucosidase (rat intestinal acetone powder) was purchased from Sigma-Aldrich Co. (St. Louis, USA). All the reagents used were of analytical grade. Glass (double)-distilled water was used to prepare the reagents. The methanol was of high performance liquid chromatography (HPLC) grade and was distilled before use.

Preparation of wheat bran extracts

The wheat bran was crushed into powder with a grinder. The dried samples (1,000 g) were extracted three times with 95% ethanol for 48 h, with continuous stirring at room temperature (25°C). The crude extract was obtained by filtration and the extract was evaporated with a rotary evaporator, under reduced pressure at 45°C to give wheat bran ethanolic extract (WBE, 69.7 g). The WBE was dissolved by water, and then extracted by different solvents in the following sequence: Petroleum ether, diethyl ether, ethyl acetate, and *n*-butanol saturated by water. Finally, the

dried petroleum ether fraction (petroleum ether fraction of wheat bran ethanolic extract WBEP, 41.25 g), diethyl ether fraction (diethyl ether fraction of wheat bran ethanolic extract WBED, 0.4 g), ethyl acetate fraction (ethyl acetate fraction of wheat bran ethanolic extract WBEE, 0.7 g), *n*-butanol fraction (*n*-butanol fraction of wheat bran ethanolic extract WBEB, 5.35 g), and water fraction (water fraction of wheat bran ethanolic extract WBEW, 16.15 g) were obtained. The extract and fractions were stored in a freezer at -20°C until use.

α -Glucosidase inhibitory assay

The dried residue was redissolved in 50% dimethyl sulfoxide (DMSO), and subjected to α -glucosidase inhibitory *in vitro* assay. With the baker’s yeast and rat intestinal α -glucosidase using *p*NPG as substrate, the assay method was a slight modification of the procedure used by Shinde.^[14] Rat intestinal acetone powder (200 mg) was manually homogenized with 10 mL ice-cold 50 mM phosphate buffer. The mixture was centrifuged at 8,000 rpm for 25 min. The supernatant was then applied to a Sephadex G-100 column, and eluted with ice-cold 50 mM phosphate buffer to isolate α -glucosidase for the assay. The obtained α -glucosidase (2 μ L, 1-2.5 U/mL) was premixed with 20 μ L of the extracts or fractions at varying concentrations and incubated for 5 min at 37°C. Then, 1 mM *p*NPG (20 μ L) was added to 50 mM of phosphate buffer to initiate the reaction, and the mixture was further incubated at 37°C for 15 min. The reaction was terminated by the addition of 50 μ L of 1 M Na₂CO₃, and the final volume was made up to 150 μ L in 50 mM phosphate buffer at pH 6.8. α -Glucosidase activity was determined spectrophotometrically at 405 nm on a Biorad 550 microplate reader (Hercules, CA) by measuring the quantity of *p*-nitrophenol released from *p*NPG. Background absorbance was determined using a non-enzyme control containing the phosphate buffer and was subtracted from the absorbance of samples and controls. Individual blanks for test samples were prepared to correct background absorbance. The control sample contained 20 μ L 50% DMSO in place of test extracts or fractions. Percentage of enzyme inhibition was calculated as $(1-B/A) \times 100\%$, where A represents absorbance of control without test samples and B represents absorbance in the presence of test samples. The assay was performed in triplicate. The concentration of the extracts or fraction required to inhibit 50% of α -glucosidase activity under the assay conditions was defined as the IC₅₀ value. The results are reported as %I \pm SD.

Fractionation of WBEP

Petroleum ether fraction of wheat bran ethanolic extract (WBEP, 10 g) was selected for further research, which was adsorbed on silica gel and subjected to column chromatography over silica gel (60 cm \times 25 mm, 200-300 mesh).

The column was subjected to elution with petroleum ether first, followed by a mixture containing increasing amounts of ethyl acetate. The corresponding fractions eluted at 0% (0.6 L), 2% (0.8 L), 10% (0.8 L), 20% (0.6 L), and 30% (1.0 L) were collected separately and concentrated to obtain fractions 1-15, with a total of four fractions with $\geq 50\%$ inhibitory activity at 0.06 mg/mL (fractions 3, 6, 11, and 13). Fraction 13, which had the highest yield, was selected for further study.

Liquid chromatography/mass spectrometry (LC/MS)

Fraction 13 was analyzed by a thermo two-dimensional linear ion trap LC/MS system (Series LXQ) equipped with a vacuum degasser, quatpump, auto-injector, photo-diode array detector (PDA), and a mass selective detector (MSD). The MSD was operated in atmospheric pressure chemical ionization mode using the following parameters: scan mode, 100-1,200 amu; ionization voltage, 150 V (positive mode); capillary voltage, 2,000 V; corona current, 8.0 A; drying gas flow, 5.0 L/min; drying gas temperature, 345°C; vaporizer temperature, 395°C; and nebulizer pressure, 60 psig. HPLC separation was carried out by reverse phase elution (Agilent XDB (eXtra-Dense Binding)-C18 column, 5 μ m; 250 \times 4.6 mm id), under the following conditions: 100% methanol in 25 min at a flow rate of 1.0 mL/min at 25°C. Ultraviolet (UV) detection was performed with a PDA using a 200-600 nm scan range and a rate of 0.5 scan/s. The LC/MS system was controlled using Xcalibur software.

Kinetics of enzyme inhibition

The inhibition mode of alkylresorcinols (ARs) against α -glucosidase was determined by Michaelis-Menton and double reciprocal plot known as the Lineweaver-Burk plot. The initial rates for α -glucosidase were measured at various *p*NPG concentrations (0.1-0.4 mM) under standard reaction conditions. Enzyme activities were determined in the absence or presence of AR concentrations of 13 and 26 μ g/mL.

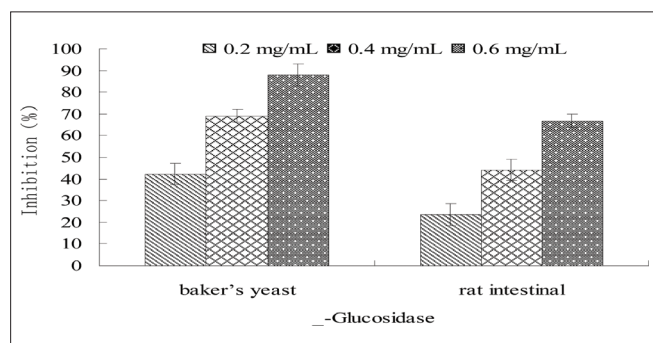


Figure 1: Inhibition of wheat bran ethanolic extracts on α -glucosidase

RESULTS AND DISCUSSION

α -glucosidase inhibitory activity of wheat bran

The extract (WBE) inhibited both baker's yeast and rat intestinal α -glucosidase using *p*NPG as substrate. The inhibitory activity against α -glucosidase from baker's yeast was 42.3-87.9% when the concentration of WBE was 0.2-0.6 mg/mL, whereas that against rat intestinal α -glucosidase was less effective and the inhibition was 23.5-66.8% [Figure 1]. The finding was similar to the α -glucosidase inhibitory activity of extracts from *Syzygium cumini* (Linn.) Skeels seed kernel, and 2,4,6-tribromophenol and 2,4-dibromophenol from the red alga *Grateloupia elliptica*.^[14,15] Hence, the inhibitory activity against mammalian α -glucosidase should be paid more attention in the following separation survey.

Fractionation of wheat bran extract guided by *in vitro* enzyme-inhibition assay

The WBEP fraction displayed significantly higher inhibitory activity on rat intestinal α -glucosidase compared to the other four fractions (WBED, WBEE, WBEB, and WBEW), [Figure 2]. WBEP concentrations of 0.06, 0.3, and 0.6 mg/mL displayed 32.45%, 76.57%, and 100% inhibition against the rat intestinal α -glucosidase, respectively. Specifically, 0.3 mg/mL WBEP had approximately 25 times more inhibitory action against α -glucosidase than that of the same concentration of WBED, and approximately 3, 8, and 13 times more than WBEE, WBEB, and WBEW, respectively. Furthermore, the yield of WBEP was also the highest; hence, the components in WBEP became the focus of further research.

Fractions 3, 6, 11, and 13 from the WBEP were found to have similar activities, which displayed over 50% inhibitory activity at 0.06 mg/mL. Hence, the four fractions might have potent α -glucosidase inhibitory compounds, but fractions 3, 6, and 11 were low in yield, the compositions were relatively complex (from results of mass spectrometry analysis analysis, not reproduced here), and there were difficulties in undertaking further work. Fraction 13, which was flaxen crystal, had the highest yield.

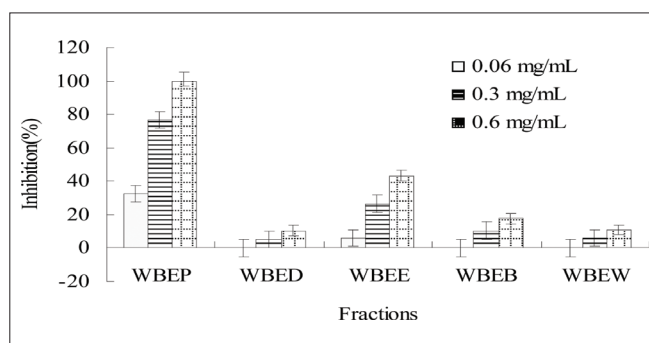


Figure 2: α -Glucosidase inhibitory activity of fractions from wheat bran ethanolic extracts

Fraction 13 was dissolved in methanol, then analyzed with LC/MS.^[16] The results displayed that the molecular ions' m/z of the main compounds in fraction 13 were in a similar range to ARs, such as m/z 347 [M-H]⁻ (5.52 min), m/z 375 [M-H]⁻ (7.07 min), m/z 403 [M-H]⁻ (9.21 min), m/z 431 [M-H]⁻ (12.49 min), m/z 459 [M-H]⁻ (17.18 min), and m/z 487 [M-H]⁻ (23.99 min) [Figure 3].^[17,18] These bioactive phytochemicals were the focus of this research; the major compounds in active fractions 13 were identified as AR homologs with different lengths of the alkyl-side chain (heptadecylresorcinol 17:0, nonadecaneresorcinol 19:0, heneicosylresorcinol 21:0, tricosylresorcinol 23:0, pentacosylresorcinol 25:0, heptacosylresorcinol 27:0) [Table 1]. Feng *et al.* reported^[18] there were twelve chemical compounds in wheat bran ethanolic extract including heptadecylresorcinol (17:0), nonadecylresorcinol (19:0), heneicosylresorcinol (21:0), tricosylresorcinol (23:0), and pentacosylresorcinol (25:0). However, they did not mention the biological activities of the chemical compounds. ARs (1,3-Dihydroxy-5-n-alkylbenzenes), known as 5-ARs, are a group of amphiphilic phenolic lipids, where the alkyl-side chains had predominantly odd numbers of carbons and the alkyl chain lengths ranged from 5 to 27.^[19] ARs were reported for their abundant presence in the bran fraction of rye and wheat, the length of the alkyl-side chain varying from 13 to 27 carbon atoms, and the side chain being usually saturated [Figure 4].^[20,21] C17, C19, and C21 are the three major homologs present in rye, whereas C19 and C21 are the major homologs for wheat bran.^[16] This agrees with our results [Figure 3].

ARs possess multiple biological activities, including antimicrobial, anti-parasitic, antioxidant, and antimutagenic

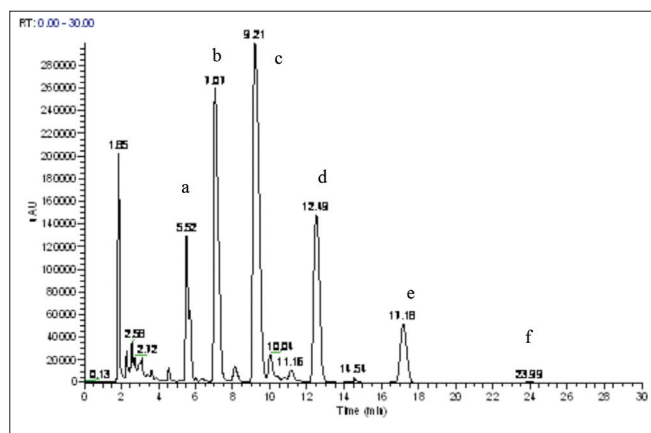


Figure 3: High performance liquid chromatography chromatogram of alk(en)ylresorcinols of petroleum ether fraction of wheat bran at 275 nm. Peak assignments: (a) heptadecylresorcinol (C17:0); (b) non-adecaneresorcinol (C19:0); (c) heneicosylresorcinol (C21:0); (d) tricosylresorcinol (C23:0); (e) pentacosylresorcinol (C25:0); (f) heptacosylresorcinol (C27:0)

activities.^[22,23] Although the potential health benefits and biological activities of ARs had generated significant scientific interest, α -glucosidase inhibitory activity of ARs from wheat bran had not, so far, been reported. Guided by the inhibition assay against the rat intestinal α -glucosidase, we found ARs were bioactive phytochemicals for postprandial glycemic control of type 2 diabetes.

Enzyme inhibitory activity

ARs inhibited α -glucosidase activity in a dose-dependent manner. The IC₅₀ values were determined to be 37.58 μ g/mL. At a concentration of 60 μ g/mL, α -glucosidase inhibition was 78.6%. A significant biological role of ARs was reported as direct modulation of enzymatic activities such as the inhibition on acetylcholinesterase, and the inhibitory activity was effected by the length of the alkyl side chain.^[24,25] However, factors affecting inhibition of ARs on α -glucosidase still require further studies.

Kinetics of enzyme inhibition

In this study, the initial velocity ' v ' of the hydrolysis reactions catalyzed by α -glucosidase was measured at 0.1-0.4 mmol/L *p*-NPG concentrations [*S*] in the absence and presence of 13 μ g/mL and 26 μ g/mL ARs [*I*], as shown in Figure 5. The double reciprocal plots showing

Table 1: Compounds of wheat bran petroleum ether fraction identified by atmospheric pressure chemical ionization-mass spectrometry

| Compound | [M-H] ⁻ (m/z) |
|------------------------------|--------------------------|
| Heptadecylresorcinol (C17:0) | 347.40 |
| Nonadecaneresorcinol (C19:0) | 375.42 |
| Heneicosylresorcinol (C21:0) | 403.51 |
| Tricosylresorcinol (C23:0) | 431.52 |
| Pentacosylresorcinol (C25:0) | 459.57 |
| Heptacosylresorcinol (C27:0) | 487.63 |

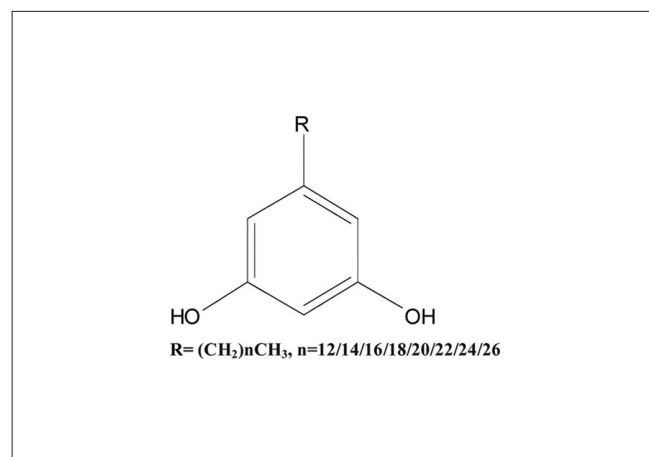


Figure 4: Structures of 5-n-alkylresorcinols present in cereals

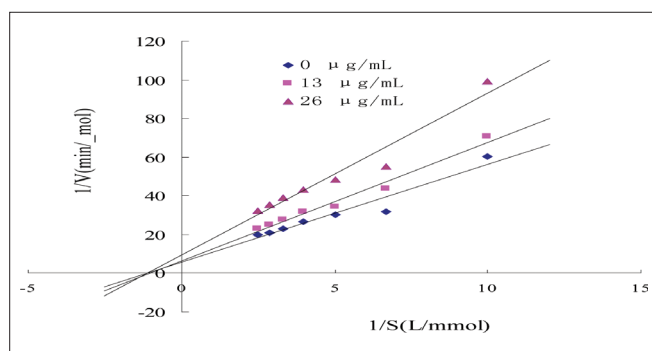


Figure 5: Lineweaver-Burk plots of α -glucosidase inhibitory effects by alkylresorcinols

straight lines were obtained with *p*NPG substrates for α -glucosidase. With increasing ARs concentration, both the slope 's' and vertical axis intercept 'i' increased. These results indicated that adding ARs affected the velocity of the reaction catalyzed by α -glucosidase, proportionate to the concentration of ARs in the reaction mixture, without affecting the K_m . These results suggest that the inhibition of ARs on α -glucosidase was non-competitive.

Kinetic constants for the inhibition of α -glucosidase were determined according to the effect of either inhibitor or substrate concentration on the slope and vertical axis intercept of the corresponding plots. α -Glucosidase had a Michaelis-Menton constant (K_m) of 0.91 mmol/L for *p*NPG as substrate and V_{max} value of 178 mmol/min. Apparent V_{max} values in the presence of 13 and 26 μ g of ARs were found to be 153 and 107 mmol/min, respectively. The inhibitory constant (K_i) was 17.24 μ g/mL of ARs.

The inhibitory activity of ARs against α -glucosidase to date has not been described in the literature. The inhibition mode displayed in this study was similar to acarbose, phenolic compounds extracted from millet seed coat and chebulagic acid from *Terminalia chebula*^[26-28] which were all non-competitive inhibitors. ARs has a high inhibition activity ($IC_{50} = 37.58 \mu$ g/mL, $K_i = 17.24 \mu$ g/mL) *in vitro*; further studies need to be conducted *in vivo* by considering the degradation of ARs in the human body by acid in the stomach or digestive enzymes. There is a substantial yield of ARs in wheat bran.^[20] The observed biological activities of ARs in general are in line with the low-glycemic index character of wheat bran. Additionally, dietary fiber, phenolic compounds, and phosphatidic acids may be the other anti-diabetic bioactive phytochemicals in wheat bran.^[7,29,30] Hence, wheat bran might be useful as a medicinal food or as a source of natural α -glucosidase inhibitor for use in suppressing postprandial hyperglycemia in the management of Type 2 diabetes.

CONCLUSIONS

In the presented study, we fractionated the ethanolic extract of wheat bran guided by the inhibitory activity assay against rat intestinal and yeast α -glucosidase. The results indicated that wheat bran contained several kinds of inhibitors and saturated AR homologs (ARs), including heptadecylresorcinol (C17:0), nonadecaneresorcinol (C19:0), heneicosylresorcinol (C21:0), tricosylresorcinol (C23:0), pentacosylresorcinol (C25:0), and heptacosylresorcinol (C27:0). These ARs are effective non-competitive inhibitors of carbohydrate-hydrolyzing enzymes. Furthermore, ARs may interfere or delay the absorption of dietary carbohydrates in the small intestine, leading to the suppression of postprandial blood glucose surges. ARs are involved in multiple biological activities, and suggested as biomarkers of whole grain wheat- and rye-rich foods.^[21] The food-grade ARs from wheat bran are potentially safe. This information will help in the use of wheat bran as a cost-effective functional food component to control the uptake of postprandial blood glucose in type 2 diabetes.

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