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Food Chemistry 90 (2005) 759–764
Food Chemistry
www.elsevier.com/locate/foodchem

Rapid communication

Antioxidant activity of feruloylated oligosaccharides from wheat bran

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Received 1 March 2004; received in revised form 27 May 2004; accepted 27 May 2004

Abstract

Ferulic acid, the main phenolic acid in wheat bran, is esterified to arabinose residues in the cell wall arabinoxylan. Treatment of wheat bran insoluble dietary fibre with xylanases from *Bacillus subtilis* released feruloylated oligosaccharides, which were purified with Amberlite XAD-2. The antioxidant activity of such oligosaccharides was evaluated using the assay system for erythrocyte hemolysis mediated by peroxyl free radicals generated from 2,2′-azobis-2-amidinopropane dihydrochloride (AAPH) under in vitro conditions. The feruloylated oligosaccharides inhibited hemolysis of erythrocytes in a dose-dependent manner with 91.7% inhibition of erythrocyte hemolysis at 4 mg/ml.

Keywords: Wheat bran; Xylanases; Feruloyl oligosaccharides; Erythrocyte hemolysis; Antioxidant activity

1. Introduction

Epidemiological studies have strongly suggested that diets rich in cereals play a crucial role in the prevention of chronic diseases such as cardiovascular disease and certain types of cancer (Aruoma, 2003; Slavin, 2000). The beneficial health effects derived from the intake of diets rich in cereals have mainly been ascribed to dietary fibre, or to some of the components associated with the fibre, including phenolic acids (Andreasen, Landbo, Christensen, Hansen, & Meyer, 2001b; Zhao, Egashira, & Sanada, 2003). Cereal cell walls contain numerous phenolic compounds, including *p*-coumaric acid, sinapic acid, and ferulic acid predominating (Baublis, Clydesdale, & Decker, 2000). These hydroxycinnamic acids exhibit in vitro chemoprotective and antioxidant properties and it is suspected that they may contribute toward the beneficial effects of a diet rich in cereal bran (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001a).

Wheat bran is a good source of dietary fibre and is produced worldwide in enormous quantities as an important by-product of the cereal industry. Wheat bran contains some 0.4–0.7% w/w cinnamic acids (Clifford, 1999). Ferulic acid, the main phenolic acid in wheat bran cell wall, is covalently bound to the cell wall arabinoxylan via the acid group acetylating the primary hydroxyl at the C5 position of α-L-arabinofuranosyl residues (Hatfield, Ralph, & Grabber, 1999).

Many studies have dealt with the isolation of feruloylated oligosaccharides from Gramineae by treatment with a mixture of polysaccharide hydrolyzing enzymes, such as fungal hydrolase Driselase (Ishii, 1997). The isolation of these feruloyl oligosaccharides has allowed a better understanding of the plant cell wall structures. Furthermore, interest in these oligosaccharides is motivated by their biological activities and their technological applications. The feruloyl oligosaccharides can act as regulators of growth and development in plants (Ishii, 1997). In the food industry, such oligosaccharides are useful, due to their gelation properties induced by oxidative cross-linking (Ishii, 1997; Kroon & Williamson, 1999).

To the best of our knowledge, there is limited literature on the antioxidant activity of feruloylated oligosaccharides from wheat bran insoluble dietary fibre. In the present study, feruloylated oligosaccharides were prepared from wheat bran insoluble dietary fibre by

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doi:10.1016/j.foodchem.2004.05.025

treatment with xylanases from *Bacillus subtilis*, and the antioxidant property of the feruloylated oligosaccharides was evaluated in the assay system for erythrocyte hemolysis induced by peroxyl free radicals generated from 2,2′-azobis-2-amidinopropane dihydrochloride (AAPH) under in vitro conditions.

2. Materials and methods

2.1. Materials

2.1.1. General

Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1 from *B. licheniformis*, 120 KNU/g), protease Alcalase 2.4 L (EC 3.4.21.62, from *B. licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were from Novo Nordisk, Bagsvaerd, Denmark. Amberlite XAD-2 was obtained from Rohm and Haas Company (Philadelphia, USA). Birchwood xylan was purchased from Sigma Company. 2,2′-Azobis-2-amidinopropane dihydrochloride (AAPH) was purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals and solvents used were of analytical grade.

2.1.2. Plant material

Wheat bran was obtained from a local milling plant. The bran was milled and passed through a 0.5 mm sieve.

2.1.3. Xylanase

Xylanases from *B. subtilis* were kindly donated by Dr. Tao Liu of the Sunhy Biology Company (Wuhan, the People's Republic of China). Four grammes of xylanases were dissolved in 400 ml of acetate buffer (50 mM, pH 5.0) with continuous stirring for 30 min at 28 °C. The precipitate was removed by centrifugation (10,000*g*, 20 min), whereas the resulting supernatant was used as the enzyme solution.

2.2. Analysis of xylan hydrolysis

Samples (4 ml) of birchwood xylan (2%, w/v) were incubated with 1 ml of 1% xylanase solution for 24 h at 50 °C and pH 5.0. The samples for test were centrifuged at 10,000*g* for 30 min and the supernatant was passed through 0.45 μm filter to remove non-hydrolyzed xylan and other insoluble contaminants. Products of xylan hydrolysis were analyzed by high-pressure liquid chromatography (HPLC), using a Sugar-Pak^{TM}1 column (300×6.5 mm i.d.). The column was maintained at 85 °C. A sample volume of 10 μl was run for 20 min, and the sugars were eluted with water at a flow rate of 0.5 ml/min. The detector signal was electronically monitored with a Waters 2401 refractive index detector.

2.3. Preparation of wheat bran insoluble dietary fibre

Wheat bran (100 g) was autoclaved for 45 min at 121 °C in order to destroy endogenous enzymatic activities (Zilliox & Debeire, 1998) and subsequently swollen at 60 °C for 16 h in water (1000 ml) with continuous stirring. After that, α-amylase (7.5 ml) was added. Beakers were heated in a boiling water bath for 40 min and shaken gently every 5 min. The pH was adjusted to 7.5 with 275 mM NaOH, and samples were incubated with protease (3.0 ml) at 60 °C for 30 min with continuous agitation. After the pH had been adjusted to 4.5 with 325 mM HCl, amyloglucosidase (3.5 ml) was added and the mixture was incubated at 60 °C for 30 min with continuous agitation. The suspension was centrifuged (10,000*g*, 10 min). The residue was stirred in hot distilled water, washed repeatedly by decantation with large volumes of hot water, and then washed with cold distilled water until no cloudiness was evident. Finally, it was washed twice with hot distilled water, 95% (v/v) ethanol and acetone, successively, and then dried at 40 °C overnight in a vacuum oven to get wheat bran insoluble dietary fibre (Bunzel, Ralph, Marita, Hatfield, & Steinhart, 2001).

2.4. Enzymatic degradation of wheat bran insoluble dietary fibre

Ten grammes of wheat bran insoluble dietary fibre were incubated in 200 ml of 1% xylanases (in 50 mM acetate buffer pH 5.0) at 50 °C in the dark for 60 h with constant stirring. After heat inactivation of the enzyme (100 °C, 10 min), the hydrolysate of wheat bran insoluble dietary fibre was obtained by centrifugation (10,000*g*, 20 min). The resulting supernatant solution was passed through 0.45 μm filter and concentrated to 50 ml.

2.5. Fractionation and preparation of feruloyl oligosaccharides

The enzymatic hydrolysate was applied to a column (30×2.5 cm) of Amberlite XAD-2 (previously washed with 95% ethanol and then water). Elution was carried out with 2 column volumes of distilled water, 3 column volumes of 50% (v/v) methanol/water and 2 column volumes of methanol. The fraction eluted by methanol/water was concentrated and freeze-dried with a freeze dry system (AlPHA1-4, Christ, Germany) for further analysis.

2.6. Analysis of feruloyl oligosaccharides by paper chromatography

The feruloyl oligosaccharides were analyzed by paper chromatography, which was performed on Whatman

No.1 filter paper by the descending method with *n*-butanol/acetic acid/water (12:3:5) as the mobile phase (Wende & Fry, 1997). The separated feruloyl oligosaccharides were located by UV radiation (before and after exposure to NH_3) (Smith & Harris, 2001), and the spots were visualized with an oxalate/aniline reagent (2 volumes of 2% aniline in ethanol and 3 volumes of 2.5% oxalic acid) by heating in an oven at 105 °C for 10 to 20 min for sugar detection (Lequart, Nuzillard, Kurek, & Debeire, 1999).

2.7. Antioxidant activity assay

The in vitro antioxidant activity of the feruloylated oligosaccharides from wheat bran insoluble dietary fibre was measured as the inhibition of erythrocyte hemolysis, according to the procedures described by Ng, Liu, and Wang (2000) with some modifications. The erythrocyte hemolysis was performed by the thermolabile azo-compound AAPH as the free radical initiator. The advantages of this method were that the AAPH decomposes thermally to generate radicals without biotransformations or enzymes and the rate of radical generation was easily controlled by adjusting the concentration of initiator (Hseu et al., 2002; May, Qu, & Mendiratta, 1998).

Blood samples were obtained from Male Kunming rats of body weight 30 ± 2 g by heart puncture. Blood was collected in heparinized tubes and centrifuged (1500*g*, 10 min) at 4 °C using a refrigerated centrifuge (20PR-52D, Hitachi, Japan). Erythrocytes separated from the plasma and the buffy coat were washed three times by centrifugation(1500*g*, 5 min) in 10 volumes of 10 mM phosphate buffer saline (PBS), which consisted of 125 mM NaCl and 10 mM NaH_2PO_4 and Na_2HPO_4 in deionized water, adjusted to pH 7.4. The supernatant and buffy coat of white cells was carefully removed with each wash. During the last washing, the erythrocytes were obtained by centrifugation(1500*g*, 10 min). Washed erythrocytes were finally suspended using the same buffer to the desired hematocrit level and stored at 4 °C and used within 6 h of sample preparation.

First, the hemolysis of 5% rat erythrocyte induced by AAPH with different concentrations was performed. A volume of 5% suspension of erythrocytes was incubated under air atmosphere at 37 °C for 5 min. A similar volume of AAPH with various concentrations (0–100 mM, in PBS pH 7.4) was added to initiate hemolysis. The reaction mixture was shaken gently while being incubated at 37 °C. At specific intervals, a volume of 400 μl of the reaction mixture was removed and diluted with 8 ml of PBS and centrifuged at 2000*g* for 10 min. The absorbance (*A*) of the resulting supernatant was measured at 540 nm by a spectrophotometer. Similarly, the reaction mixture was treated with 8 ml of distilled water to obtain complete hemolysis and the absorbance (*B*) of its supernatant in the same conditions was measured at 540 nm. The percentage hemolysis was calculated from the ratio of the readings $(A/B) \times 100$. Secondly, a volume 100 μl of 5% suspension of erythrocytes in PBS was added to 200 μl of 100 mM AAPH solution (in PBS pH 7.4) and 100 μl of feruloyl oligosaccharides with different concentrations (1–5 mg/ml, in PBS pH 7.4). The reaction mixture was shaken gently, while being incubated at 37C for 3 h, to choose a desired dose of feruloyl oligosaccharides with the strongest inhibitory effect on erythrocyte hemolysis. The inhibition ratio (%) was calculated using the following formula

$$\text{Inhibition ratio } (\%) = 100 - \text{Percentage hemolysis.}$$

Finally, a volume of the desired dose of feruloyl oligosaccharides was added to a similar volume of 5% suspension of erythrocytes and two volumes of 100 mM AAPH solution (in PBS, pH 7.4). The reaction mixture was shaken gently while being incubated at 37 °C for 4.5 h. At specific intervals, a volume of 400 μl of the reaction mixture was removed for determining the percentage hemolysis that gave effective inhibition time of feruloyl oligosaccharides on erythrocyte hemolysis. Each test was performed at least three times and the results were reproducible within 10% deviation. Data represent the mean ± standard deviation ($n = 3$) of one representative experiment.

3. Results and discussion

3.1. Xylan hydrolysis

Birchwood xylan hydrolyzed by *B. subtilis* xylanases was studied at 50 °C and pH 5.0. The hydrolysis products were analyzed by HPLC (Fig. 1). It can be observed

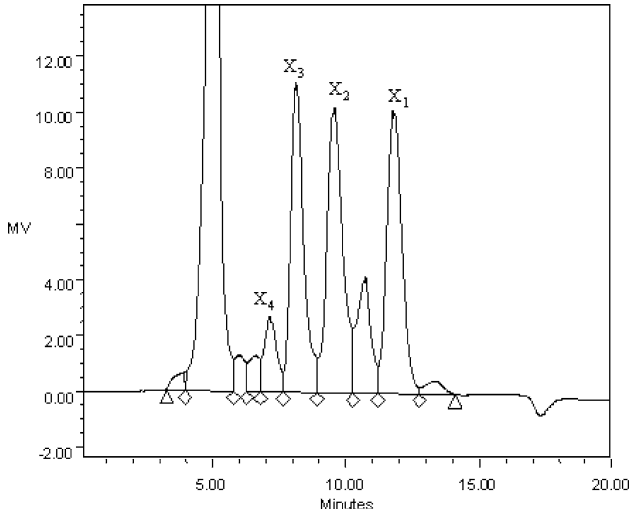

Fig. 1. HPLC of the birchwood xylan hydrolysate by *B. subtilis* xylanases (X_1: xylose; X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose).

that the main products of xylan hydrolysis were xylose, xylobiose, xylotriose and higher xylooligosaccharides. At the same time, there were other monosaccharides produced from xylan by treatment with xylanases. These results showed that the xylanases from *B. subtilis* could cleave the backbone of xylan at random into small oligomers.

3.2. Fractionation of feruloyl oligosaccharides

The enzymatic degradation of wheat bran insoluble dietary fibre was performed at 50 °C and pH 5.0. The hydrolysate was centrifuged and passed through 0.45 μm filter to remove non-hydrolyzed wheat bran insoluble dietary fibre and other insoluble contaminants. The resulting solution was concentrated to a final volume of 50 ml and applied to a column (30×2.5 cm) of Amberlite XAD-2, which is a polymeric adsorbent binding aromatic compounds (Saulnier, Vigouroux, & Thibault, 1995). Initially, all of the oligosaccharides were retained on the column. Application of distilled water to the column led to the elution of oligosaccharides that do not contain ester-linked ferulic acid. The feruloyl oligosaccharides were eluted using aqueous 50% methanol. The fraction eluted by methanol/water was subjected to paper chromatography. These separated compounds fluoresced blue under UV radiation and their colour turned green on exposure to NH_3, indicating they were feruloyl oligosaccharides (Harris & Hartley, 1976). These spots with fluorescence occurred with a reddish colour when they were stained with an oxalate/aniline reagent (Fig. 2).

Fig. 2. Paper chromatography of feruloylated oligosaccharides. The sugars were stained with an oxalate/aniline reagent.

3.3. Antioxidant activity

The in vitro antioxidant activity of the feruloylated oligosaccharides from wheat bran insoluble dietary fibre was measured as the inhibition of erythrocyte hemolysis mediated by peroxyl free radicals. A water soluble azo compound, AAPH is a peroxyl radical initiator that generates free radicals by its decomposition at physiological temperature and attack the erythrocytes to induce the chain oxidation of lipid and protein, disturbing the membrane organization and leading to eventually to hemolysis (Miki, Tamai, Mino, Yamamoto, & Niki, 1987).

As shown in Fig. 3, the rat erythrocytes are stable and little hemolysis took place within 4 h in the absence of AAPH. Following the addition of AAPH, the hemolysis occurred quickly. Also, it can be seen in Fig. 3, the rate of hemolysis is correlated, dose-dependently, with the concentration of AAPH.

Fig. 4 shows that the inhibitory effect of different concentrations of feruloyl oligosaccharides (1–5 mg/ml) on AAPH-induced hemolysis of 5% rat erythrocytes for 3 h. The feruloyl oligosaccharides inhibited hemolysis of erythrocytes in a dose-dependent manner with 91.7% of inhibition of erythrocytes hemolysis at 4 mg/ml. As shown in Fig. 5, the addition of feruloyl oligosaccharides (at 4 mg/ml) to the AAPH-initiated hemolysis system generated additional inhibition over of 120 min.

The results obtained clearly demonstrate that the feruloylated oligosaccharides could efficiently protect normal rat erythrocytes against hemolysis induced by free radicals under in vitro conditions.

The main mechanism of action of phenolic antioxidants is considered to be the scavenging of free radicals

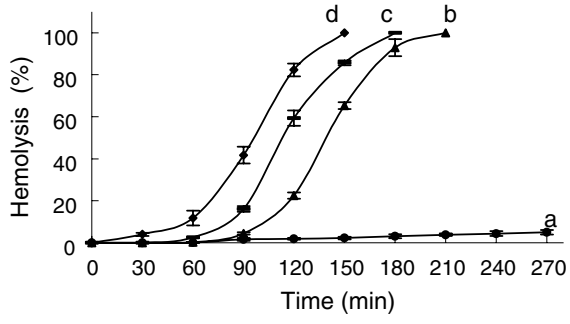

Fig. 3. Hemolysis curves of 5% rat erythrocytes initiated by AAPH in 10 mM PBS (pH 7.4) under air atmosphere at 37 °C. The initial concentrations of AAPH were: (a) 0, (b) 40, (c) 70, (d) 100 mM. Data represent the mean ± SD ($n = 3$) of one representative experiment.

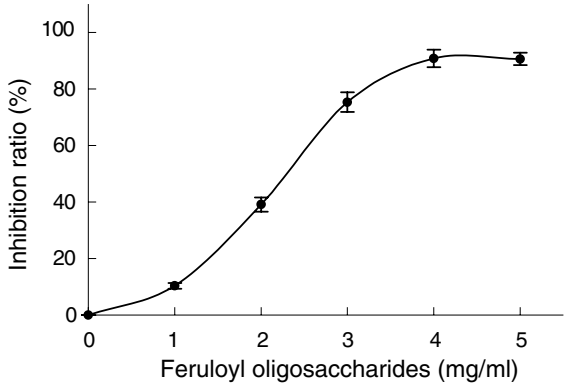

Fig. 4. Inhibitory effect of different concentrations of feruloyl oligosaccharides (1–5 mg/ml) on AAPH-induced hemolysis of 5% rat erythrocytes induced by 100 mM AAPH for 3 h at 37 °C. Data represent the mean ± SD ($n = 3$) of one representative experiment.

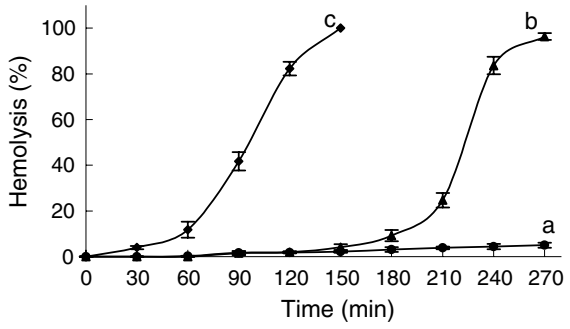

Fig. 5. Time course of hemolysis of rat erythrocytes induced by AAPH. Rat erythrocyte suspensions (5%) were incubated with 10 mM PBS (pH 7.4) under air atmosphere at 37 °C (a) in the absence of AAPH. Rat erythrocyte suspensions (5%) were incubated with 100 mM AAPH alone (c) or plus the presence of 4 mg/ml feruloyl oligosaccharides (b) at 37 °C. Data represent the mean ± SD ($n = 3$) of one representative experiment.

by donating the phenolic hydrogen atom (Bakalbassis, Lithoxoidous, & Vafiadis, 2003). It is suggested that the inhibitory effect of feruloyl oligosaccharides on the oxidative hemolysis of rat erythrocyte might be related to their scavenging activity against peroxyl radicals, thereby inhibiting the peroxidation of erythrocytes in vitro. The membrane of erythrocytes is rich in polyunsaturated fatty acids that are very susceptible to free radical-mediated peroxidation. The generating free radicals (R·) by AAPH thermal decomposition, can attack the polyunsaturated fatty acids (LH) in the membrane of erythrocytes to induce lipid peroxidation (Eqs. (1)–(6)). The lipid peroxidation is a free radical chain reaction and one initiating radical could induce more twenty propagation reactions (Liu, Yu, & Liu, 1999), the membrane of erythrocytes is rapidly damaged, leading to hemolysis. The lipid peroxidation of erythrocytes, initiated by the azo compound AAPH, can be represented by the following simplified reactions.

Initiation

$$R - N = N - R(AAPH) \rightarrow 2eR^{\cdot} + N_2 + (1 - e)R - R \quad (1)$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \quad (2)$$

$$ROO^{\cdot} + LH \rightarrow ROOH + L^{\cdot} \quad (3)$$

Propagation

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (4)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (5)$$

Termination

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{molecular products} \quad (6)$$

However, the presence of electron-donating groups on the benzene ring (3-methoxy, and more importantly, 4-hydroxyl) of the ferulic acid moiety of the feruloyl oligosaccharides gave the additional resonance structures of the resulting phenoxyl radical (Ishii, 1997) (Fig. 6), contributing to the stability of this intermediate or even terminating free radical chain reactions. Therefore, in the presence of feruloyl oligosaccharides (FH), the peroxyl radical can be trapped and a new radical, F·, produced (Eq. (7)). Since the new radical F· is a stabilized radical, it can promote the rate-limiting hydrogen abstraction reaction (Eq. (7)) and undergo a fast termination reaction (Eq. (8)), and subsequently inhibit lipid peroxidation.

$$LOO^{\cdot} + FH \rightarrow LOOH + F^{\cdot} \quad (7)$$

Termination

$$LOO^{\cdot} + F^{\cdot} \rightarrow LOOF \quad (8)$$

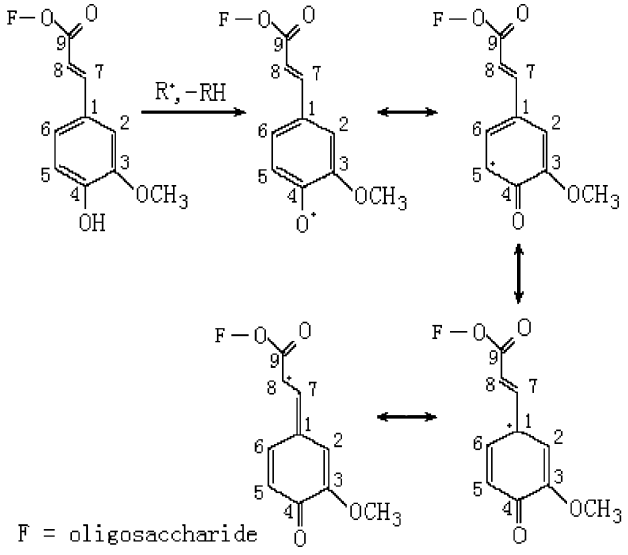

Fig. 6. Possible structures of mesomeric radicals of feruloyl oligosaccharides. Free radical initiation occurs at the 4-hydroxyl group by abstraction of hydroxyl H-atom.

4. Conclusions

The *B. subtilis* xylanases catalyzed the hydrolysis of birchwood xylan to xylooligosaccharides and xylose, and was able to hydrolyze wheat bran insoluble dietary fibre, producing feruloyl oligosaccharides. The feruloyl oligosaccharides could efficiently protect normal rat erythrocytes against hemolysis induced by free radicals under in vitro conditions, indicating that the feruloyl oligosaccharides from wheat bran insoluble dietary fibre have antioxidative potential. Free radical scavenging ability of the feruloylated oligosaccharides in relation to their structural characteristics will be further investigated.

Acknowledgements

This project is financially supported by the Graduate Innovation Fund administered by Jiangsu Education Committee of the People's Republic of China.

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