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ORIGINAL CONTRIBUTION

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Enzymic production of a feruloylated oligosaccharide with antioxidant activity from wheat flour arabinoxylan

Summary *Background* Main cereals such as rice, wheat, barley, and corn belong to the family Gramineae and have similar cell-wall composition. Since cereal cell walls are a good source of dietary fibre, meeting one-half of the daily requirement of 30 g of dietary fibre can be achieved by the regular consumption of cereals. Many studies have dealt with the isolation of feruloylated oligosaccharides from Gramineae by treatment with polysaccharide hydrolysing enzymes. *Aim of this study* Therefore, the purpose of this study was to investigate the production of feruloylated oligosaccharides from insoluble wheat flour arabinoxylan (WFAX) by treatment with a *Thermoascus aurantiacus* family 10 endoxylanase (XYLI) and the evaluation of their antioxidant activity. *Methods* The main feruloylated oligosaccharide was purified by anion-exchange and size-exclusion chromatography (SEC). Alkaline saponification and acid hydrolysis were used for product identification. Evaluation of antioxidant activity was performed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction assay and the inhibition of copper-mediated oxidation of low density lipoprotein (LDL). *Results* The optimal conditions for WFAX hydrolysis using the XYLI have been determined to be 100 U g^{-1} of WFAX for 30 min at 50 °C. Saponification of the oligosaccharide released FA and oligosaccharide. The released oligosaccharide consisted of arabinose and xylose in a molar ratio of 1:3 and these results support the identity of the feruloylated oligosaccharide as feruloyl arabinoxylotrisaccharide (FAX_3). FAX_3 showed profound antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction assay exhibiting an antiradical efficiency of 0.035 ($\times 10^{-3}$) and inhibited the copper-mediated oxidation of human low density lipoprotein (LDL) in a dose-dependent manner with almost complete inhibition at 32 μM. *Conclusion* A feruloylated oligosaccharide (FAX_3) was isolated from WFAX after enzymatic treatment with XYLI. We verified antioxidant activity of FAX_3 which may be important in preventing or reducing the progression of atherosclerosis by inhibiting the peroxidation of lipoproteins.

Key words endoxylanase – wheat flour arabinoxylan – antioxidant – low-density lipoprotein – feruloyl-oligosaccharide

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Introduction

Epidemiologic studies have focussed attention on plant phenolic substances present in large quantities in the diet of developed countries, in which atherosclerosis is paradoxically low [1]. These phenolic substances, especially gallic and cinnamic acid derivatives, are potent antioxidants and inhibit LDL oxidation in vivo [2, 3]. The recent discovery of natural antioxidants may lead to replacement of the synthetic antioxidants which are widely used at present. In the graminaceous cell wall, ferulic acid (FA) is located in various tissues and organs [4]. Feruloylation in the Gramineae mainly occurs on arabinoxylan and slightly on xyloglucan [5]. Since cereal cell walls are a good source of dietary fibre, meeting one-half of the daily requirement of 30 g of dietary fibre can be achieved by the regular consumption of cereals [6, 7].

Many studies have dealt with the isolation of feruloylated oligosaccharides from Gramineae by treatment with polysaccharide hydrolysing enzymes [5]. In this work, using a family 10 endoxylanase from *Thermoascus aurantiacus* [8] we have isolated a cinnamoyl-oligosaccharide released from WFAX and studied its antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction assay [9] and copper-catalysed peroxidation of LDL [10].

It has already been reported that xylanases belonging to family 10 exhibit greater catalytic versatility than enzymes of family 11 [11].

Materials and methods

Enzyme

XYLI was produced by *Thermoascus aurantiacus* Mieche, IMI 216529 in a solid state culture with wheat straw as the carbon source and purified as previously described [12]. According to its catalytic properties XYLI can be classified under glycosyl hydrolase family 10 [10].

Arabinoxylan

This arabinoxylan is the water insoluble pentosan fraction from wheat flour and was purchased from Megazyme (Ireland). It was purified to remove starch, β-glucan and protein. Its purity was 95 % with an arabinose to xylose ratio (A/X) of 0.61. The FA content was determined as described below.

Ferulic acid

The alkali-extractable FA content of WFAX (10 mg) was measured following incubation of substrate with NaOH (40 mL, 0.1 M) for 22 h at room temperature in the dark. After centrifugation at 10000 × g for 10 min, supernatants were neutralised with 1 M HCl and the FA content was assessed by HPLC using a C-18 Nucleosil column (250 × 4.6 mm) (Macherey Nagel) with acetonitrile:water:formic acid (1.3:7:1) as the mobile phase at a flow rate of 1.0 ml min^{-1} and at ambient temperature. FA was detected by a Waters UV detector (Model 440) set at 300 nm.

Preliminary determination of endoxylanase activity

Endoxylanase activity was preliminary determined with soluble arabinoxylan (SA) (Megazyme, Ireland) as reported previously [13]. The release of the reducing sugars was followed spectrophotometrically at 540 nm according to the method of Miller (1959) [14].

Incubation of arabinoxylan with endoxylanase

Hydrolysis of WFAX upon incubation with endoxylanase was assessed according to the following procedure. WFAX (10 mg) was pre-incubated for 1 h in 0.9 mL sodium acetate solution (50 mM, pH 5.0, 50 °C). Suitably diluted enzyme solution (0.1 mL, 0 to 5 U_{SAX}) was added and the test tube incubated under continuous stirring (30 min, 50 °C). After inactivation (10 min, 100 °C) and centrifugation (10000 g, 15 min) the level of total solubilised xylose and arabinose in the supernatant was measured with HPAEC after acid hydrolysis as outlined below.

Enzymic hydrolysis for the production of feruloylated oligosaccharide

Hydrolysis of WFAX 1 % (w/v) (Megazyme, Ireland) in deionised water (100 mL) was performed using endoxylanase (1 U mL^{-1}) for 30 min at 50 °C with constant stirring.

Sugar analysis

Monosaccharide composition was determined by HPAEC. Solubilised fractions were hydrolysed in CF_3COOH (2 N, 1 h, 121 °C, 2 Atm). Samples were diluted 50-fold, filtered, and injected onto a CarboPac PA1 anion-exchange column (4 × 250 mm, Dionex). Neutral monosaccharides were separated in 100 mM NaOH.

Quantitative data of the enzymic hydrolysis pattern were obtained with solvent A: 100 mM NaOH and solvent B: 100 mM NaOH containing 300 mM NaOAc, using the following gradient: t = 0, A = 100 %, t = 10 min, A = 100 %; linear gradient; t = 45 min, B = 100 %; linear gradient; t = 50 min, A = 100 %.

Feruloylated oligosaccharides were separated using HPLC system with NH_2 – Nucleosil 100–5 column (250 × 4.6 mm) (Macherey – Nagel) with acetonitrile: water [75:25] as a mobile phase at a flow rate of 1.0 mL min^{-1}. Feruloylated oligosaccharides were identified using a Waters UV detector (Model 440) at 320 nm.

Chromatographic methods

Chromatography on an AG 1 x 2 anion exchange resin using OH^- as the counter-ion (Bio-Rad Laboratories) was performed on a column (47 × 1.5 cm) equilibrated with distilled water at a flow rate of 60 mL h^{-1}.

Enzymic hydrolysate (70 mL) was loaded onto the column and the gel was washed with distilled water. A two-step elution was performed to remove bound material. First, an ammonium formiate (0.8 M) solution was applied to the column. Then, after extensive washing of the gel with distilled water to remove ammonium salts, the remaining oligosaccharides were eluted with aq EtOH (50 %, v/v). This fraction was further purified by SEC.

SEC was performed on two sequentially connected BioGel P-2 columns (160 × 2.5 cm). A 3 mL of sample was applied and eluted with distilled water at a flow rate of 60 mL h^{-1}; 5 mL fractions were collected and analysed by HPLC as described above. The fractions with the main feruloylated oligosaccharide were combined and freeze dried.

Determination of feruloylated oligosaccharides by spectrophotometry

The concentration of the feruloylated oligosaccharides was determined spectrophotometrically at 286 (free FA) and 323 nm (esterified FA) [15]. The following molar absorption coefficients were determined at pH 6 in a MOPS buffer $\varepsilon_{286} = 14{,}176$ and $\varepsilon_{323} = 10{,}350$ l mol^{-1} cm^{-1} for free FA and $\varepsilon'_{286} = 12{,}465$ and $\varepsilon'_{323} = 19{,}345$ l mol^{-1} cm^{-1} for esterified FA and were used in the calculation.

Preparation of LDL

The EDTA-containing stock solution human LDL (15–30 mg LDL/mL) was purchased from Sigma. Before oxidation experiments the LDL solution was dialysed in a 500-fold volume of 0.01 M phosphate buffer, pH 7.4, 0.16 M NaCl. The buffer was changed two times. This EDTA-free LDL stock solution was used for all oxidation studies. The stock solution was not stored longer than 24 h at 4 °C.

Free radical scavenging method

The effect of each antioxidant on DPPH radical was estimated according to the procedure described by Brand-Williams et al. [16].

The percentage of the remaining DPPH against the standard concentration was plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50 %. The time needed to reach the steady state to EC_{50} concentration (T_{EC50}) was calculated graphically.

Taking into account that both EC_{50} and T_{EC50} affect the antiradical capacity, a new parameter, antiradical efficiency (AE) [9], which combines these two factors, was defined:

$$AE = 1/EC_{50}\ T_{EC50}$$

Oxidation Experiments

The EDTA-free LDL stock solution was diluted with oxygen-saturated 0.01 phosphate buffer, pH 7.4, 0.16 M NaCl, and the oxidation was initiated by the addition of a freshly prepared aqueous 20 μM copper sulphate solution. For conjugated dienes we measured absorption at 234 nm of a dilute preparation of LDL containing 50 μg of LDL protein/mL for 1h at 30 °C. To study the inhibitory effect, 8 and 32 μM FA and FAX_3 were added .

Results

Ferulic acid content

FA was found to be the only ester-linked cinnamic acid (0.43 % of the dry weight). This value is similar to that found for the water insoluble pentosan from wheat flour isolated by de Vries and co-workers [17].

Effect of xylanase levels on WFAX solubilisation

To determine the optimal parameters, first the hydrolysis of WFAX was studied in a small scale (1 mL reaction mixture). Fig. 1 shows the effect of various levels of xylanase on the solubilisation of WFAX. 25 % of WFAX appears to be easily hydrolysed, even when present at low

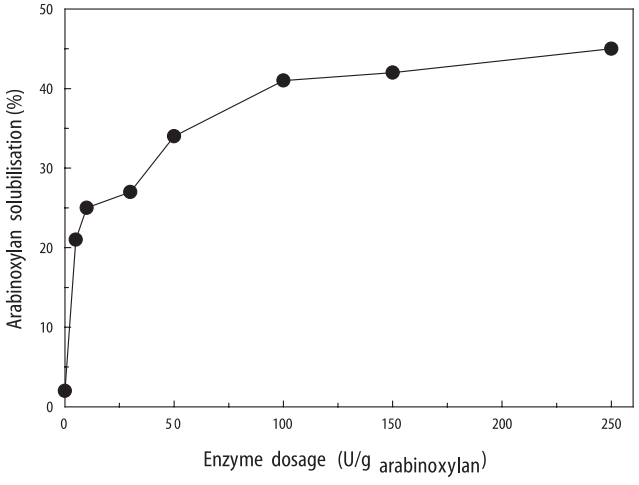

Fig. 1 Influence of *Thermoascus aurantiacus* xylanase XYLI level (0–250 U g^{-1}) on the solubilisation of insoluble wheat flour arabinoxylan after 30 min incubation at 50 °C

levels (10 U g^{-1} of WFAX) while significant increases in XYLI concentrations were required for further solubilisation reaching a maximum (45 %) with 250 U g^{-1} of WFAX. In this condition HPAEC analysis revealed that xylose, xylobiose and xylotriose appear to be the major end-products of xylanase hydrolysis of WFAX. When low xylanase activity was applied (5 U $g^{-1}$of WFAX) significant amounts of xylotetraose were liberated (Fig. 2).

Production and isolation of feruloylated oligosaccharide

From the small scale experiments it can be concluded that 100 U of XYLI should be applied in 100 mL of reaction mixture containing 1 g of WFAX for 30 min at 50 °C. In these conditions feruloylated oligosaccharides were released in significant amounts yielding 6.4 %. The hydrolysate was concentrated by freeze drying to a final volume of 10 mL and applied to an anion exchange AG 1 × 2 (OH^{-1}) column. Initially, all of the oligosaccharides were retained on the column. Application of an ammonium formiate solution (0.8 M) led to the elution of approximately 85 % of the total oligosaccharides. However, no ester bound cinnamic acids were detected in this fraction. After extensive washing of the column with water to remove ammonium salts, the cinnamoyl-oligosaccharides were eluted using aqueous 50 % EtOH. This fraction represented 15 % of the total oligosaccharides and 90 % of the total cinnamic acids. Further purification by SEC led to the isolation of one major oligosaccharide-containing fraction. This fraction was freeze dried and the sugar was further characterised.

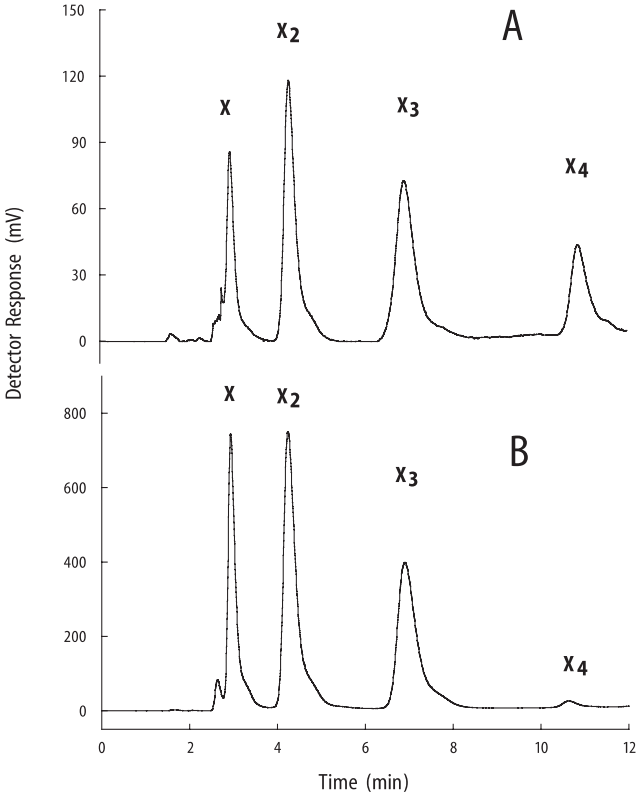

Fig. 2 HPAEC-PAD of the hydrolysis products of insoluble wheat flour arabinoxylan treated with 5 U g^{-1} (**A**) and 150 U g^{-1} (**B**). X:xylose, X_2:xylobiose, X_3:xylotriose, X_4:xylotetraose

Characterization of product

The HPLC analysis of saponified product showed the presence of FA. No other hydrocinnamic acid derivatives, such as coumaric acid, were detected. The glycosyl residue composition of product was arabinose (Ara, A) and xylose (Xyl, X), and the ratio of Ara:Xyl was about 1:3. Thus, since the oligosaccharide moiety of product was composed of AXXX, the product A was identified as feruloylated AXXX (FAX_3), feruloyl arabinoxylotrisaccharide.

Antioxidative activities of FAX_3

Antioxidant activities of FAX_3 were evaluated by comparison with FA. The concentration of antioxidant needed to decrease by 50 % the initial substrate concentration (EC_{50}) is a parameter widely used to measure the antioxidant power [9]. The lower the EC_{50}, the higher the antioxidant power. The values found for FA are shown in Table 1 and they agree with those values reported by Brand-Williams et al. (212) [16] and Sanchez-Moreno et al. (163) [9]. EC_{50} for FAX_3 was found to be 1228.

Sanchez-Moreno et al. [9] defined the parameter T_{EC50} as the time needed to reach a steady state at the concentration corresponding to EC_{50} and classified the kinetic behaviour of the antioxidant compound as follows: < 5 min (rapid); 5–30 min (intermediate) and > 30 min (slow) [9]. FA and FAX_3 exhibit intermediate kinetic behaviour with T_{EC50} values of 16 and 23.5 min respectively (Table 1). The 'Antiradical efficiencies' (AE) of FA and FAX_3 were 292×10^{-6} and 35×10^{-6} respectively.

Table 1 Antioxidant activity of FAX_3 compared with FA in the DPPH oxidation system

	EC_{50} (g Antioxidant kg^{-1} DPPH)	T_{EC50}[a] (min)	Antiradical efficiency[b] ($x10^{-3}$)
FA	214	16.0	0.292
FAX_3	1228	23.5	0.035

[a] Time needed to reach the steady state to EC_{50} concentration
[b] Antiradical efficiency = $\mathbf{1/EC_{50} \cdot T_{EC50}}$

Antioxidative effect against human LDL

The inhibitory effect of FA and FAX_3 on LDL oxidation was examined at various concentrations of these compounds (Fig. 3) (Table 2). Antioxidant activity of FAX_3 was stronger than that of FA and showed a dose-dependent inhibition of cupuric ion-mediated LDL oxidation after 60 min of incubation with almost complete inhibition at 32 µM (Fig. 3).

Table 2 Antioxidant activity of FAX_3 compared with FA in the LDL oxidation system[a]

	Control	8 µM FAX_3	32 µM FAX_3	8 µM FA	32 µM FA
Maximal concentration (nmol of dienes/mg of LDL)	67.1	51.5	2.7	50.8	43.4
Maximal rate (nmol of dienes/min·mg of LDL)	1.5	1.1	0.06	1.1	1.0

[a] Calculations were based on the molar absorptivity of conjugated lipid hydroperoxides ($\varepsilon_{234} = 29500\ M^{-1}\ cm^{-1}$) [18] and our experimental conditions (50 mg/L)

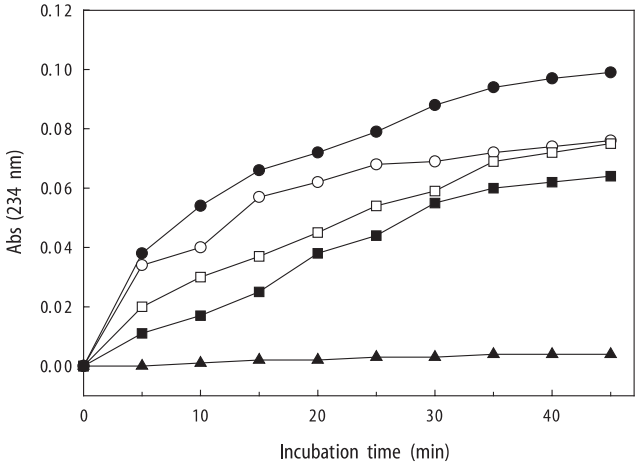

Fig. 3 Antioxidant activity of FAX_3 and FA in the LDL oxidation system. ●, Control; ○, 8 µM FAX_3; ▲, 32 µM FAX_3; □, 8 µM FA; ■, 32 µM FA. The EDTA-free LDL stock solution was diluted with oxygen-saturated 0.01 M phosphate buffer, pH 7.4 and the oxidation was initiated by the addition of a freshly prepared aqueous 20 µM copper sulfate solution. For conjugated dienes we measured absorption at 234 nm of dilute preparation of LDL containing 50 µg of LDL protein/mL for 1 h at 30 °C

These results agreed with those reported from Ohta et al. [10], where chemically hydrolysed corn bran cell-wall fragments showed antioxidant activity in the LDL oxidation system.

Discussion

In this study, we have shown that the Family 10 XYLI hydrolysed WFAX by 45 % and released 95 % of feruloylated oligosaccharides.

Analysis of the hydrolytic products of the XYLI-WFAX showed that xylose, xylobiose and xylotriose are the major end-products. When arabinoxylan was incubated with low levels of xylanase activity significant amount of xylotetraose was also produced. This hydrolysis pattern is typical of an endo-mechanism of enzyme action. The purified xylanase of *Aureobasidium pullulans* was shown to produce xylobiose and higher xylooligomers from arabinoxylan [19].

In order to separate the feruloyl-oligosaccharides we employed anion-exchange chromatography, which allowed the specific separation of feruloylated oligosaccharides and size exclusion chromatography which allowed isolation of the major feruloyl xylooligosaccharide (FAX_3).

The isolated oligosaccharide exhibits a reduced scavenger activity compared to FA. In turn the sugar esterification of FA enhanced its protective acid against copper-catalysed oxidation of LDL.

In the LDL oxidation model, the partition coefficient between the aqueous and the lipophilic phase influences the accessibility of antioxidants to free radicals [20]. FAX_3, which has the hydrophilic sugar moiety, is stronger than FA in the LDL oxidation system [10, 21]. FA acid itself does not associate with the lipid portion of the LDL and exerts its antioxidant properties from the aqueous phase [22]. Chalas et al. [23] showed that ethyl esterification of phenolic acids enhanced their lipophilicity and their protective effect against copper-catalysed peroxidation of LDL.

The enzymic production of feruloylated oligosaccharides from natural sources may thus produce new interesting drugs for the prevention of atherosclerosis.

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