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ARTICLE

Production of Arabinoxylan-oligosaccharides from Flaxseed (*Linum usitatissimum*)

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Flaxseed mucilage from *Linum usitatissimum* L. species was constituted by arabinoxylan (about 75%) and pectin (about 25%). A new procedure was developed to obtain only arabinoxylans which implicated treatment of the pectin fraction by enzymatic hydrolysis with pectinase. Then three processes of depolymerization were evaluated on arabinoxylans. First, a thermic hydrolysis in mild acid conditions was performed and an ultrafiltration process was used as purification method. Second, the potential of xylanases from different glycoside hydrolase families for arabinoxylan-oligosaccharides (AXOS) production was tested, and finally a radical depolymerization was conducted. Average molecular weights were determined by high pressure size exclusion chromatography coupled with multiple angle laser light scattering (MALLS), and carbohydrate compositions were determined by high pH anion exchange chromatography pulse amperometric detector (HPAEC-PAD). Both chemical and enzymatic treatments were inefficient to convert arabinoxylans from flaxseed mucilage into AXOS. Only radical depolymerization process was allowed to obtain arabinoxylan-oligosaccharides presenting different molecular weights (11.9×10^3 to 1.9×10^3 g·mol^{-1}) with satisfactory yields (75% to 35%).

KEYWORDS: Arabinoxylans; oligosaccharides; flaxseed mucilage; *Linum usitatissimum*; oligomers; food industry

INTRODUCTION

In recent years, oligosaccharides have become useful for applications in various fields because of their specific biological activities. In food industries, saccharidic molecules have received increasing attention from industry which wanted to substitute chemical additives for food preservation. Thus some oligosaccharides were described as food additives for their antimicrobial effects against pathogenic bacteria or fungi. A well-known group of oligosaccharides used for their antimicrobial effects are chito-oligosaccharides (*1*). Moreover some antitumoral, antioxidant and radical scavenging activities have been reported (*1*). The second interest of saccharidic substances was to stimulate the growth and development of gastrointestinal microflora described as probiotic bacteria. For example, galacto-oligosaccharides, fructo-oligosaccharides and cyclodextrins were known as prebiotic substances (*2*).

In this context, the large family of xylo-oligosaccharides, which results from the abundant hemicellulosic polymers, has been reported for a range of functional food ingredients. Antimicrobial activities of acidic xylo-oligosaccharides and antioxidant activity of feruloylated oligosaccharides (*3*) were referenced. However, the prebiotic potency is the main biological property of xylo-oligosaccharides (*3*), especially for arabinoxylan-oligosaccharides (AXOS) which result from degradation of arabinoxylans (*4*, *5*).

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Arabinoxylans (AX) were found in the cell wall of cereals such as wheat, corn, rye and barley. They are composed of a linear β-(1→4)-xylopyranose (Xylp) backbone substituted with arabinofuranose (Araf) side chains attached *via* α-(1→3) and/or α-(1→2) linkages (*6*). AX of cereals have been intensely studied contrary to AX of flax species despite seed properties. Flax is an annual plant from the *Linaceae* family. Flaxseeds were known as a functional food source related to their content in high-quality protein and soluble fiber (*7*), and for their medicinal properties like anti-inflammatory effect according to the presence of α-linolenic acid (*8*). AX were present in the mucilaginous cells of the seeds, called mucilage after extraction. AX from flax are much more branched than AX from cereals like wheat and contain L-arabinosyl units at the same *O*-2 and *O*-3 positions along the xylan backbone. Moreover this polysaccharide contains a high amount of D-galactose and L-fucose units which are mostly nonreducing terminal residues (*9*). In our group, flax mucilage from *Linum usitatissimum* L. has been studied only for its interesting physicochemical properties. These rheological properties are dependent on the molecular weight of the polysaccharides and also putative noncovalent interactions with other polysaccharides (*10*).

Oligosaccharides are obtained from chemical, physical or biological degradations of polysaccharides. Acid hydrolysis, thermomechanical and oxidative degradations are the most known. Acid hydrolysis is usually used for analytical purposes or kinetic studies of polymer hydrolysis (*11*). However this

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method produces a large amount of monosaccharides and byproduct by dehydration (*12*).

Thermomechanical degradations include twin-screw extrusion, jet-cooking, ultrasonication (*13*) and microwave irradiation. Ultrasound treatments in alkaline media used on xylan revealed a decrease of the average molecular weight and the viscosity (*14*). However, no oligosaccharides were obtained, and the limiting size of degradation was estimated for instance to 100×10^3 g·mol^{-1} for chitosan (*15*) and 40×10^3 g·mol^{-1} for dextran (*16*). Microwave irradiation was used on hyaluronan in mild acidic conditions as it results that this kind of depolymerization was similar to a thermic degradation by autoclaving (*17*).

Another chemical degradation was the oxidative degradation by free-radical. This reaction could be performed by hydrogen peroxide catalyzed by a metal ion such as the one from Fenton chemistry (*18*). Many polysaccharides were able to be depolymerized by this method, such as sulfated (*19*), acid (*20*), or neutral polysaccharides (*21*). Moreover, it was possible to control the extent of the degradation.

Enzymatic degradation is the other major pathway to obtain oligosaccharides. Enzymes can be isolated from different microorganisms including fungi (*22*) and bacteria (*23*). They can be directly added to the reaction media (*24*) or immobilized in an enzymatic reactor (*25*). Enzymatic hydrolysis allows the production of specific oligosaccharides without undesirable byproducts. Scale-up is easier and does not require special equipment.

To our knowledge, no study has been performed to obtain arabinoxylan-oligosaccharides from flaxseed mucilage, excepted one which studied the use of commercially available enzymes to remove flaxseed mucilage and improve extraction of its proteins (*26*). The present study describes the use of three different methods of depolymerization in order to produce oligosaccharides from flaxseed mucilage (*Linum usitatissimum* L.). Thermic in mild acidic medium, enzymatic and radical processes were employed. Reducing sugars, monosaccharide compositions and average molecular weights have been established in order to evaluate the most effective method.

MATERIALS AND METHODS

Flax Origin. Yellow species of flaxseed were provided by CVG (Ets, Dury (Somme), France). CGV was provided with the extraction process and produced flaxseed mucilage at pilot scale. The water-soluble polysaccharides were extracted from seeds with stirring in distilled water (1:8 w/v) during 30 min at 80 °C. Then, the supernatant was collected by centrifugation (4000*g*, 15 min, 25 °C) and precipitated with six volumes of ethanol (80%). After filtration, the solid was recovered before being dried under vacuum. The flaxseed mucilage (FM) was obtained with 4.3% yield.

Enzymes. All enzymes or enzyme preparations were commercially available. Shearzyme 2× (xylanase 1) was an *Aspergillus aculeatus* enzyme preparation (Novozymes Bagsvaerd, Denmark) containing a glycoside hydrolase family (GH) 10 xylanase (1500 XU/mL). Xylanase 2 was a *Trichoderma viride* enzyme preparation (Fluka) containing a xylanase from GH 11 (3.2 U/mg). 1 U corresponds to the amount of enzyme which liberates 1 μM remazol brilliant blue R at pH 6 and 40 °C. Pearex Adex containing pectinase, hemicellulase and arabinase activities was from DSM (Netherlands). An *Aspergillus oryzae* enzyme preparation (8 U/mg) containing β-galactosidase was from SIGMA-ALDRICH (Steinheim, Germany).

Depectinization of Flaxseed Mucilage. Flaxseed mucilage (6 g) in Milli-Q water (1200 mL) was stirred for 2 h at 70 °C. After adjusting the temperature at 50 °C and pH to 4 with acetic acid (10%), 1.5 mL of Pearex Adex was added under continuous stirring. Samples were collected to be used with reducing sugar method, and viscosity was studied with digital viscosimeter model LVTDV II (Brookfield, Middleboro, MA). After a 6 h incubation, reaction was stopped heating the medium at 100 °C during 5 min. The medium was then centrifuged (50000*g*, 40 min, 15 °C) and pH was raised to 7. Finally the solution was purified by tangential ultrafiltration with a Pilot-Scale Crossflow System Sartorius 17521-001 (Sartorius, Goettingen, Germany), equipped with a total membrane area of 0.1 m^2. The membrane in polyethersulfone and manufactured by Sartorius (Goettingen, Germany) has a nominal molecular mass cutoff (NMWCO) of 100000 g/mol. The flow was provided by peristaltic pump Watson Marlow 603S (Cornwall, England). The mix was concentrated down to 0.5 L, and the retentate was further purified by continuous diafiltration with 5 L of Milli-Q water. Retentate was lyophilized, and we obtained the flaxseed mucilage without pectin (FMWP).

Thermic Hydrolysis Of FMWP. Flaxseed mucilage without pectin (1 g/L) was adjusted at different pH (7, 4 and 3) with concentrated sulfuric acid (98%) and was treated by autoclaving at 120 °C for 40 min. The supernatant was raised to pH 7 with NaOH (2 M), and samples were collected to be used with reducing sugar method prior to lyophilization. All solutions were desalinated using same ultrafiltration device with a 5000 Da NMWCO membrane. The solution was concentrated down to 0.4 L, and the retentate was further purified by continuous diafiltration with 4.5 L Milli-Q-water. The concentrate was recovered and freeze-dried.

Enzymatic Hydrolysis Of FMWP. Flaxseed mucilage without pectin was incubated with different commercial enzymes. FMWP (2 g/L) was dissolved in Milli-Q water for 30 min at room temperature. The pH and temperature were adjusted according to enzyme. Incubation with xylanase 1 was at pH 7 and 37 °C. Incubation with xylanase 2 was at pH 6 and 40 °C. Incubation with β-galactosidase was at pH 5 and 40 °C. Dual incubations with β-galactosidase and xylanase 2 were carried out at pH 5 and 40 °C.

After 6 h of incubation, reaction was stopped by heating the medium at 100 °C during 5 min. Samples were collected for reducing sugar evaluation.

Radical Depolymerization of FMWP. All reactions were performed using 0.5 g of FMWP in Milli-Q water (1 g/L) and 30 mL of 1.6×10^{-3} M cupric acetate monohydrate. Temperature was kept at 50 °C, and pH was maintained at 7 by automatic addition of 0.1 M NaOH.

In a first experiment, a 10% (v/v) hydrogen peroxide solution was added with a peristaltic pump during 2 h with a flow rate of 0.5 mL/min. Samples collected for reducing sugar method were injected through Chelex 100 resin column (Biorad, Marnes-la-Coquette, France) to stop reaction.

In a second experiment, variable hydrogen peroxide quantities (1.50, 2.50, 3.75, 5.25, 7.50 mL) were added during 15 min. The reaction was stopped one hour after the end of the hydrogen peroxide runoff by the Chelex100 resin.

All solutions were desalinated using the same ultrafiltration device with a 1000 Da NMWCO membrane. The solution was concentrated down to 0.3 L, and the retentate was further purified by continuous diafiltration with 2 L Milli-Q water. The concentrate was recovered and freeze-dried.

Reducing Sugar Method. Depolymerization was determined by measuring the release of reducing sugar using DNS (radical depolymerization) (*27*) or Nelson (enzymatic and thermic hydrolysis) (*28*) methods.

All absorbance measurements were determined by a spectrophotometer UVIKON 930 (KONTRON Instrument, Yvelines, France) at 540 nm for the DNS method and 700 nm for the Nelson method.

The results were expressed in xylose equivalents (xylose moles/mucilage mass (mmol/g)).

Protein Determination. The concentration of protein was determined by using the method described by Bradford (*29*).

All absorbance measurements were determined by a spectrophotometer UVIKON 930 (KONTRON Instrument, Yvelines, France) at 595 nm.

Size Exclusion Chromatography (SEC-MALLS). Samples were filtered through a 0.2 μm, 10 mm membrane filter (Anotop 10, Whatman, Maidstone, England) and were manually injected through a 100 μL full loop. The SEC line consisted of an SB-G guard column as protection and three columns in series (SB-806 HQ, SB-804 HQ and SB-803 HQ). The elution was performed with a flow rate of 0.5 mL·min^{-1} (LC-20AD, Shimadzu, Duisburg, Germany). $NaNO_3$, 0.1 M, and NaN_3, 2.5 mM, used as carrier, was filtered through a 0.02 μm, 47 mm membrane filter (Anotop 47, Whatman, Maidstone, England), carefully degassed. Detection was achieved with a light scattering detector (MiniDAWN, Wyatt Technology Corporation, Santa Barbara, CA) and a refractive index detector (RID-10 A, Shimadzu, Duisburg, Germany). Data acquisition was performed using ASTRA software.

Table 1. Monosaccharide Content (Expressed as Percentage (%) Dry Matter) and D-Ara/D-Xyl Ratio (A/X) of Flaxseed Mucilage, Evaluated by HPAEC Studies

	FM	FMWP	permeate
D-Xyl	21.7 ± 0.2	31.4 ± 0.6	5.0 ± 0.1
D-Gal	8.1 ± 0.1	10.2 ± 0.3	1.4 ± 0.1
D-Ara	7.9 ± 0.1	9.0 ± 0.1	1.4 ± 0.1
L-Fuc	3.4 ± 0.1	4.9 ± 0.2	0.1 ± 0.1
L-Rha	8.4 ± 0.1	0.9 ± 0.2	4.7 ± 0.1
D-GalA	6.3 ± 0.1	0.7 ± 0.1	3.2 ± 0.1
D-Glc	1.7 ± 0.1	0.3 ± 0.1	1.5 ± 0.1
A/X	0.36	0.28	0.27

Composition Analysis. An aliquot (6 mg) of each sample was hydrolyzed 4 h at 100 °C with 3 mL of trifluoroacetic acid (4 M). The composition was determined using high pH anion-exchange chromatography on a Carbopac PA-1 analytical column (4 mm × 250 mm). The elution was performed with a flow rate of 1 mL·min^{-1} by a gradient mode. The gradient for neutral sugars (eluent A, 160 mM NaOH, and eluent B, 200 mM NaOH) was 10% of A during 25 min, 100% of B for 10 min and finally equilibration step with 10% of A (25 min). The gradient for uronic acid (eluent A, 160 mM NaOH, and eluent B, 160 mM NaOH + 600 mM AcONa) was 0% of B during 5 min, 30 min of linear gradient from 0 to 100% of B, 100% of B during 5 min and finally equilibration step with 0% of B (10 min). Detection was performed with a pulsed amperometric ED50 detector [Dionex Corporation, Sunnyvale, CA]. Samples (20 μL) were injected with an autosampler. Carbohydrate concentrations were determined after integration of respective areas [Chromeleon management system (Dionex)] and their comparison with standard curves obtained with L-rhamnose, D-xylose, D-galactose, D-arabinose, D-fucose and D-glucose (Sigma).

RESULTS AND DISCUSSION

Extraction of Water-Soluble Polysaccharides from Flaxseeds. The flaxseed mucilage was extracted by an aqueous process. Various constitutive polysaccharides were commonly characterized consisting of acidic polysaccharides (25%), which are pectin-like and neutral polysaccharides (75%), which are composed of arabinoxylans (*30*).

Determination of monosaccharide content by HPAEC (**Table 1**) revealed that xylose was the most abundant saccharide (21.7%). Galactose (8.1%), arabinose (7.9%) and rhamnose (8.4%) were present at comparable lower levels. Minor monosaccharides were glucose (1.7%), fucose (3.4%) and galacturonic acid (6.3%). So, neutral polysaccharides of flaxseed mucilage were estimated to 72% with xylose, arabinose, galactose and fucose. Acidic polysaccharides close to rhamnogalacturonans were estimated to 28% with rhamnose, galacturonic acid and a little part of galactose and arabinose (*31*). The proportion between acidic and neutral polysaccharides was found similar as previously reported by Warrand et al. (*30*) on the flaxseed mucilage. The presence of glucose was due to extraction of β-glucans during flaxseed mucilage production. Intermolecular interactions with arabinoxylans are well-known for this cell-wall polymer (*6*).

In these conditions, the flaxseed mucilage corresponded to 2.1% of the seeds weight and contained 4.7% proteins. These results were in agreement with the data of other authors who reported mass yield of 2.1% for mucilage extraction at 40 °C for 2 h and 1% protein contamination (*30*).

Purification of Arabinoxylans. A large-scale purification procedure using anion exchange chromatography was able to separate the neutral and the acidic fractions (*30*). For preparative separation, conventional chromatography was complex due to the high viscosity of mucilage solution so chromatographic support was mixed with mucilage. Neutral polysaccharides corresponding to unretained molecules were collected by centrifugation. The acidic polysaccharides like rhamnogalacturonans, or ferulic acids were responsible for the high mucilage viscosity by cross-links with arabinoxylan. In our process, we used enzymatic degradation of acidic backbone to obtain the neutral fraction of flaxseed mucilage.

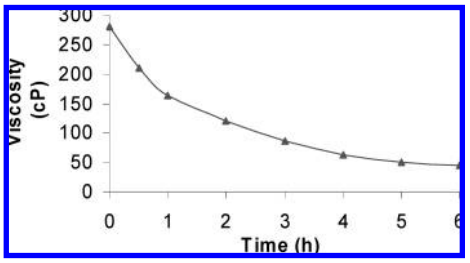

Figure 1. Evolution of viscosity during enzymatic reaction with pectinase.

Flaxseed mucilage was incubated 6 h at 50 °C with a commercially available enzyme preparation called Pearex Adex which contained pectinase, hemicellulase and arabinase activities. The viscosity of flaxseed mucilage solution (5 g·L^{-1}) was measured during the incubation. **Figure 1** shows a decrease from 280 cP to 45 cP after 6 h incubation. In order to eliminate the pectin residues, the medium was purified by tangential ultrafiltration (membrane with nominal molecular mass cutoff of 100 × 10^3 g·mol^{-1}) leading to flaxseed mucilage without pectin (FMWP). FMWP was freeze-dried and corresponded to 59.2% (±5.2) of the weight of the flaxseed mucilage.

The monosaccharide content of FMWP and ultrafiltration permeate were determined by HPAEC (**Table 1**). We noticed an important decrease of rhamnose concentration to 0.9%, galacturonic acid concentration to 0.7% and glucose concentration to 0.3% in FMWP compared to flaxseed mucilage (respectively 8.4%, 6.3% and 1.7%). Simultaneously, concentrations of these monosaccharides were increased in the ultrafiltration permeate (rhamnose (4.7%), galacturonic acid (3.2%) and glucose (1.5%)). So enzymatic depolymerization with pectinase preparation allowed removal of acidic polysaccharides of flaxseed mucilage and β-glucans. The A/X ratios were close to 0.28 and corresponded to those obtained by chromatographic preparation (*32*).

Permeate contained xylose (5%) and arabinose (1.4%), which indicated the presence of neutral polysaccharides. This result was in agreement with the 59.2% yield which implicated that neutral fractions with molecular weight lower to 100 × 10^3 g·mol^{-1} were eliminated by ultrafiltration. The concentration of protein in FMWP was determined at 0.9%. This value was lower than in flaxseed mucilage.

The number-average molecular weight of FMWP was evaluated by SEC-MALLS to 10.0 (±0.1) × 10^5 g·mol^{-1} (**Table 2**). More investigations were achieved on average molecular weight of FMWP (**Figure 2**). Three populations were detected, a high molecular weight fraction (M_w = 43.4 × 10^5 g·mol^{-1}), a medium molecular weight fraction (M_w = 13.7 × 10^5 g·mol^{-1}) and a low molecular weight fraction (M_w = 7.8 × 10^5 g·mol^{-1}). These three distinct families in neutral polysaccharide from flaxseed mucilage are noted in the literature with similar tendencies (57.0 × 10^5 g·mol^{-1}, 9.3 × 10^5 g·mol^{-1}, 3.2 × 10^5 g·mol^{-1}) (*32*). Obtained low molecular weight fraction was higher than those from Warrand and al (*32*). The purification process on 100000 g·mol^{-1} membrane, which eliminates a major part of low molecular weight molecules, may explain this difference.

Warrand et al. showed a large-scale purification procedure to isolate neutral and anionic polymers from flaxseed mucilage and determinated the presence of three distinct populations in neutral

Table 2. Molecular Weights ($g \cdot mol^{-1}$) of Flaxseed Mucilage and FMWP Analyzed by SEC-MALLS

	M_n ($\times 10^5$)	M_w ($\times 10^5$)	M_n/M_w
FMWP[a]	10.01 ± 0.12	17.07 ± 0.25	1.71
detail of FMWP			
peak 1	35.14 ± 0.14	43.41 ± 1.34	1.23
peak 2	12.97 ± 0.45	13.66 ± 0.48	1.05
peak 3	6.57 ± 1.41	7.81 ± 1.88	1.19

[a] FMWP: Flaxseed mucilage without pectin.

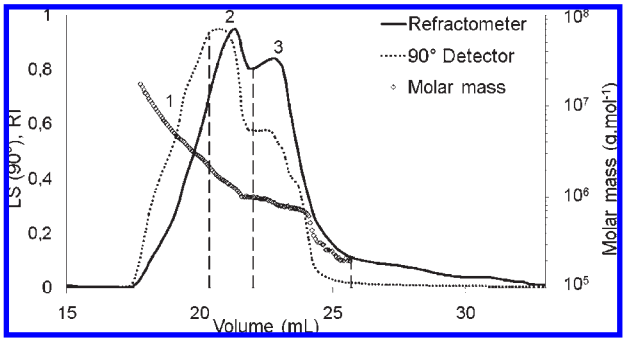

Figure 2. 90° MALLS, molar mass and refractometer signals of flaxseed mucilage without pectin after chromatography on OHPAK SB 803, 804, and 806 HQ columns.

polysaccharide (*30*). We obtained similar results using an enzymatic treatment with a commercial pectinase, which is simpler and implied fewer efforts.

Thermic Hydrolysis of FMWP In Mild Acidic Conditions. Flaxseed mucilage without pectin was subjected to thermic hydrolysis in mild acidic conditions at different pH (7, 4 and 3) during 40 min at 120 °C. As shown in **Table 3**, when the pH was decreased from 7 to 3, xylose equivalents in the supernatant increased from 1.0×10^{-2} to 29.3×10^{-2} mmol/g. A/X ratio decreased from 0.29 (pH 7) to 0.22 (pH 3). This suggests the hydrolysis of arabinose substituents from the xylan backbone may have decreased A/X ratio values. Recently, similar changes in the A/X ratio at low pH and high temperature were obtained by acidic hydrolysis of psyllium seed husk (*33*). Moreover, mass yields decreased from 90 to 75% of the mucilage weight with decreasing pH. The purification process by tangential ultrafiltration, which eliminated low molecular weight molecules, explained the recovery yield decreased by the release of substituents in permeate.

As expected, molecular weights decreased with decreasing pH (**Table 3**). M_n of pH 7 fraction was divided by four in comparison with M_n of FMWP (respectively 2.60×10^5 $g \cdot mol^{-1}$ and 10.01×10^5 $g \cdot mol^{-1}$) and was strongly polydisperse (M_w/M_n to 3.09). A slow depolymerization occurred during autohydrolysis at the temperature we used in this study. Autohydrolysis at mild temperature produces high-molar mass arabinoxylan; more efficient hydrolysis was conducted at higher temperature (150−240 °C) (*34*). Then, M_n decreased progressively according to pH from 0.78×10^5 $g \cdot mol^{-1}$ (pH 4) to 0.32×10^5 $g \cdot mol^{-1}$ (pH 3). The polydispersity also decreased from 2.08 (pH 4) to 1.59 (pH 3) but remained higher than FMWP. The lowest molecular weight values were obtained at the lowest pH (3). Both low pH values and high temperatures were needed to depolymerize the xylose backbone into smaller fragments.

Simms et al. (*35*) have obtained oligosaccharides with this procedure on an acid polysaccharide. We have shown that thermic hydrolysis in mild acidic was partial and no oligosaccharides could be obtained in these conditions. Moreover, this method would not be interesting because the carbohydrate composition change.

Table 3. Mass Yield (%), Xylose Equivalents (Xylose Moles/Mucilage Mass (mmol/g)), D-Ara/D-Xyl Ratio and Molecular Weights ($g \cdot mol^{-1}$) Analyzed by SEC-MALLS of Flaxseed Mucilage without Pectin after Thermic Depolymerization at Different pH

	pH 7	pH 4	pH 3
mass yield (%)	91	77	74
Xyl equiv ($\times 10^{-2}$)	1.0 ± 0.2	6.0 ± 0.1	29.3 ± 0.1
M_n ($\times 10^5$)	2.60 ± 0.07	0.78 ± 0.01	0.32 ± 0.01
M_w ($\times 10^5$)	8.03 ± 0.27	1.63 ± 0.02	0.52 ± 0.01
M_n/M_w	3.09	2.08	1.59
A/X	0.29	0.27	0.22

Table 4. Xylose Equivalents (Xylose Moles/Mucilage Mass (mmol/g)) from FMWP after 6 h of Enzymatic Reaction

	E/FMWP ratio[a]		Xyl equiv ($\times 10^{-2}$)
xylanase 1	0.30		2.4 ± 0.1
	1.00		10.0 ± 0.1
xylanase 2	0.13		2.3 ± 0.1
β-galactosidase	0.08		12.7 ± 0.1
β-galactosidase + xylanase 2	0.08	0.28	42.0 ± 0.1

[a] E/FMWP: Enzyme/flaxseed mucilage without pectin ratio (w/w).

Enzymatic Hydrolysis. In order to obtain arabinoxylan-oligosaccharides, a degradation was conducted by using xylanase. Studies have been performed on flaxseed mucilage without pectin. FMWP was incubated at the concentration of 2 $g \cdot L^{-1}$ with a set of commercially available enzyme. Two essays were performed in the same incubation conditions with a different ratio of enzyme/FMWP (w/w) for xylanase 1 (family 10 endoxylanase). After 6 h of incubation, xylose equivalent was estimated to 2.4×10^{-2} $mmol \cdot g^{-1}$ and 10.0×10^{-2} $mmol \cdot g^{-1}$ for an E/FMWP ratio to 0.3 and 1.0 respectively (**Table 4**). As expected, a higher E/FMWP ratio enhanced the reducing sugar assay. However, xylose equivalent values were comparable to those obtained with thermic hydrolysis, therefore no oligosaccharides were obtained with xylanase 1.

A treatment with Xylanase 2 (family 11 endoxylanase) at 40 °C was thus performed. Xylose equivalents were estimated in same range (2.3×10^{-2} $mmol \cdot g^{-1}$) with a lower E/FMWP ratio. However no evolution was noticed with higher E/FMWP ratio (data not shown).

Xylanases were referenced as family 10 and family 11 of the glycosyl hydrolase enzyme families according to CAZY classification (http://www.cazy.org/). It was shown that family 10 endoxylanase has relatively higher molecular weights and a more complex structure than family 11 endoxylanase. Moreover family 10 endoxylanase has substrate binding sites smaller than those of family 11 endoxylanase. Thus, family 10 endoxylanase has greater catalytic versatility or lower substrate specificity than family 11 endoxylanase (*36*). The results were in accordance with a higher hydrolysis of AX by xylanase 1. Consequently choice of enzyme family (10 or 11 endoxylanases) has an influence on efficiency of FMWP depolymerization.

Enzymatic hydrolysis was a suitable method to obtain oligosaccharides from AX of wheat (*24*) and rye (*37*). These polysaccharides were mainly composed of arabinoxylan backbone with small amounts of uronic and ferulic acids, covalently linked through ester linkages to the arabinose. They were less branched than flaxseed mucilage polysaccharides, enabling more efficient enzymatic hydrolysis. We suggested the high ramification of AX polysaccharides could decrease efficiency of enzyme. After xylose, galactose was the most abundant monosaccharide in FMWP

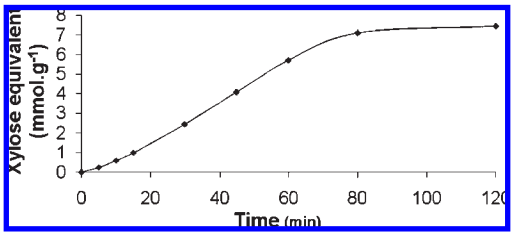

Figure 3. Evolution in xylose equivalents (mmol·g^{-1}) of radical depolymerization of flaxseed mucilage without pectin (FMWP).

(**Table 1**). We have chosen to remove galactose ramification to allow xylanase access to the backbone of arabinoxylan.

In this purpose, FMWP was incubated with β-galactosidase at 40 °C for an E/FMWP ratio of 0.08. Reducing sugar was estimated to 12.7×10^{-2} mmol·g^{-1} which confirmed a significant efficacy to remove some branched galactose unit (**Table 4**). So FMWP was incubated with two enzymes, β-galactosidase and xylanase 2. Xylose equivalents were increased to 42.0×10^{-2} mmol·g^{-1}, which was much higher than for other hydrolyses (**Table 4**). After 6 h of incubation with β-galactosidase and xylanase 2, average molecular weights were determined by SEC-MALLS. M_n and M_w were evaluated to 4.12 ($\pm$0.34) $\times$ 10^4 g·mol^{-1} and 7.94 ($\pm$0.16) $\times$ 10^4 g·mol^{-1} respectively and with polydispersity coefficient close to 2. These molecular weights were lower than FMWP (M_w to 10.01 ($\pm$0.12) $\times$ 10^5 g·mol^{-1}), but this molecular weight decrease remained too low.

Enzymatic hydrolysis including a synergy between xylanases and β-galactosidase allowed the FMWP to be cut and confirmed that the high branched rate of arabinoxylans obstructed the access to the enzymatic site. These enzymatic ways were inefficient to obtain arabinoxylan-oligosaccharides of flaxseed mucilage.

Radical Depolymerization. Flaxseed mucilage without pectin (FMWP) was depolymerized by a radical process. The radical depolymerization proceeds through the catalytic action of copper and formation of free radicals from hydrogen peroxide. The active principle is $OH^{\bullet}$ produced by Fenton reaction in the presence of Cu^{2+} ions. The relative amount of H_2O_2 is one of the keys to control depolymerization.

A preliminary study was carried out on FMWP (1 g/L solution) with cupric acetate monohydrate (30 mL, 1.6×10^{-3} M) without interruption of flow rate of hydrogen peroxide (**Figure 3**). Xylose equivalent was estimated to be 744.0×10^{-2} mmol·g^{-1} at 2 h, which was extremely higher than the enzymatic hydrolysis and revealed a very important depolymerization. After 80 min, xylose equivalent was stable and reaction was considered as finished.

In a second experiment, radical depolymerization was performed with variations of hydrogen peroxide quantities expressed in a H_2O_2/FMWP ratio. The reaction was conducted one hour after the end of the hydrogen peroxide runoff (15 min) in order to consume all hydrogen peroxide. Continual addition of hydrogen peroxide was an important step in the radical process. Studies had demonstrated that reproducibility of depolymerization was much better with a continual addition of hydrogen peroxide than a one-step initial addition at the beginning of the reaction (*38*). Then after the chelation of copper, an ultrafiltration with a 1000 g·mol^{-1} NMWCO membrane was performed, and the medium was freeze-dried and weighed.

For a H_2O_2/FMWP ratio of 0.3, mass yield of the end of the reaction corresponded to 75% whereas a ratio of 1.5 conducted to 34% of the FMWP weight (**Table 5**). As expected, mass yields decreased with increasing of H_2O_2/FMWP ratio (*39*). M_n of these fractions varied from 1.9×10^3 g·mol^{-1} to 11.9×10^3 g·mol^{-1} for 1.5 and 0.3 H_2O_2 /FMWP ratio respectively (**Table 5**). As can be seen, data specified that these fractions were low-molecular-weight polysaccharides and confirmed the high amount of reducing sugar obtained in the preliminary study suggesting an important depolymerization. Moreover, as previously demonstrated, average molecular mass decreased with increasing of hydrogen peroxide quantity which is a more important factor than time (*40*). During the radical process, the polydispersity coefficients were ranging from 1.30 to 1.45, respectively with 1.05 and 0.75 H_2O_2 /FMWP ratio (**Table 5**). Average molecular weights decreased linearly between H_2O_2/FMWP ratio up to 0.5.

Table 5. Mass Yield (%), Molecular Weights (g·mol^{-1}), D-Ara/D-Xyl Ratio and Polydispersity of Flaxseed Mucilage without Pectin (FMWP) Analyzed by SEC-MALLS According to Different H_2O_2/FMWP Ratio (w/w)

H_2O_2/FMWP ratio	mass yield	A/X	M_n ($\times 10^3$)	M_w ($\times 10^3$)	M_n/M_w
0	–	0.28	1001 ± 12	1707 ± 25	1.71
0.30	75.4	0.30	11.95 ± 2.77	15.90 ± 2.34	1.33
0.50	70.5	0.30	6.41 ± 1.07	9.04 ± 0.86	1.41
0.75	64.3	0.30	5.14 ± 0.32	7.46 ± 0.34	1.45
1.05	36.5	0.30	3.76 ± 0.27	4.87 ± 0.26	1.30
1.50	34.2	0.30	1.87 ± 0.21	2.47 ± 0.34	1.32

Composition and A/X ratio of these low-molecular-weight polysaccharides were comparable and very close to FMWP. Radical depolymerization had no influence on the degree of substitution of xylan backbone by arabinose.

Radical depolymerization with variations of H_2O_2/FMWP ratio allowed various low molecular weight fractions to be obtained. This chemical way was the most efficient to obtain arabinoxylan-oligosaccharides from flaxseed mucilage. Furthermore the carbohydrate composition did not change.

Conclusion. The production of arabinoxylan-oligosaccharides from flaxseed mucilage was studied with three different methods. Processes required pure arabinoxylan in order to perform the depolymerization. In this study, we demonstrated that enzymatic hydrolysis with pectinase and an ultrafiltration step were able to purify arabinoxylans from flaxseed mucilage by removing acidic fractions.

Although the molecular weight of FMWP decreased during thermic hydrolysis under mild acidic conditions, these conditions did not allow release of oligosaccharides. Moreover, A/X ratio changed according to pH which implicated a modification of the degree of substitution by arabinose.

Enzymatic processes with xylanases were achieved with endoxylanase families 10 and 11. Limited degradation was noticed, probably due to the highly branched backbone of arabinoxylan from flaxseed mucilage.

Finally, a significant depolymerization was found using a radical process. The depolymerization rate slightly depended on the H_2O_2/polysaccharide ratio. Thus, we demonstrated in this study that it is possible to produce using a radical depolymerization a desired mixture of arabinoxylan-oligosaccharides in a short time, with a high yield, and without variation of A/X ratio.

ABBREVIATIONS

AX, arabinoxylans; AXOS, arabinoxylan-oligosaccharides; FM, flaxseed mucilage; FMWP, flaxseed mucilage without pectin; Xylp, xylopyranose; Araf, arabinofuranose; CVG, Centre de valorisation des glucides; XU, Xylose unity; U, unit; NMWCO, nominal molecular mass cutoff.

LITERATURE CITED

(1) Kim, S.-K.; Rajapakse, N. Enzymatic production and biological activities or chitosan oligosaccharides (COS): a review. *Carbohydr. Polym.* **2005**, *62*, 357–368.

(2) Mussatto, S. I.; Mancilha, I. M. Non-digestible oligosaccharides: A review. *Carbohydr. Polym.* **2007**, *68*, 587–597.

(3) Moure, A.; Gullon, P.; Dominguez, H.; Parajo, J. C. Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. *Process Biochem.* **2006**, *41*, 1913–1923.

(4) Grootaert, C.; Delcour, J. A.; Courtin, C. M.; Broekaert, W. F.; Verstraete, W.; Van de Wiele, T. Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine. *Trends Food Sci. Technol.* **2007**, *18*, 64–71.

(5) Van Craeyveld, V.; Swennen, K.; Dornez, E.; Van de Wiele, T.; Marzorati, M.; Verstraete, W.; Delaedt, Y.; Onagbesan, O.; Decuypere, E.; Buyse, J.; De Ketelaere, B.; Broekaert, W. F.; Delcour, J. A.; Courtin, C. M. Structurally different wheat-derived arabinoxylooligosaccharides have different prebiotic and fermentation properties in rats. *J. Nutr.* **2008**, *138*, 2348–2355.

(6) Izydorczyk, M. S.; MacGregor, A. W. Evidence of intermolecular interactions of β-glucans and arabinoxylans. *Carbohydr. Polym.* **2000**, *41*, 417–420.

(7) Oomah, B. D. Flaxseed as a functional food source. *J. Sci. Food Agric.* **2001**, *81*, 889–894.

(8) Clark, W. F.; Parbtani, A.; Murray, W.; Spanner, E.; De Salis, H.; Chin-Yee, I.; Philbrick, D. J.; Holub, B. J. Flaxseed: a potential treatment for lupus nephritis. *Kidney Int.* **1995**, *48*, 475–780.

(9) Naran, R.; Chen, G.; Carpita, N. Novel Rhamnogalacturonan I and Arabinoxylan Polysaccharides of Flax Seed Mucilage. *Plant Physiol.* **2008**, *148*, 132–141.

(10) Warrand, J.; Michaud, P.; Picton, L.; Muller, G.; Courtois, B.; Ralainirina, R.; Courtois, J. Contributions of Intermolecular Interactions between Constitutive Arabinoxylans to the Flaxseed Mucilage Properties. *Biomacromolecules* **2005**, *9*, 1871–1876.

(11) Jinglin, Y.; Shijun, W.; Fengmin, J.; Sun, J.; Yu, J. The structure of C-type Rhizoma Dioscorea starch granule revealed by acid hydrolysis method. *Food Chem.* **2009**, *113*, 585–591.

(12) Akpinar, O.; Erdogan, K.; Bostanci, S. Production of Xylooligosaccharides by Controlled Acid Hydrolysis of Lignocellulosic Materials. *Carbohydr. Res.* **2009**.

(13) Cote, G. L.; Willet, J. L. Thermomechanical depolymerization of dextran. *Carbohydr. Polym.* **1999**, *39*, 119–126.

(14) Chang, Y.-H.; Yang, J.-C. Molecular mass distribution and degradation rate of xylan sonicated in acid and alkaline media. *Food Hydrocolloids* **2006**, *20*, 348–356.

(15) Czechowska-Biskup, R.; Bozena, R.; Lotfy, S.; Ulanski, P.; Rosiak, J. M. Degradation of chitosan and starch by 360-kHz ultrasound. *Carbohydr. Polym.* **2005**, *60*, 175–184.

(16) Portenlänger, G.; Heusinger, H. The influence of frequency on the mechanical and radical effects for the ultrasonic degradation of dextranes. *Ultrason. Sonochem.* **1997**, *4*, 127–130.

(17) Bezáková, Z.; Hermannová, M.; Dřímalová, E.; Malovíková, A.; Ebringerová, A.; Velebný, V. Effect of microwave irradiation on the molecular and structural properties of hyaluronan. *Carbohydr. Polym.* **2008**, *73*, 640–646.

(18) Sweikert, C.; Liszkay, A.; Schopfer, P. Polysaccharide degradation by Fenton reaction- or peroxydase-generated hydroxyl radicals in isolated plant cell walls. *Phytochemistry* **2002**, *61*, 31–35.

(19) Rota, C.; Liverani, L.; Spelta, F.; Mascellani, G.; Tomasi, A.; Iannone, A.; Vismara, E. Free radical generation during chemical depolymerization of heparin. *Anal. Biochem.* **2005**, *344*, 193–203.

(20) Jahn, M.; Baynes, J. W.; Spiteller, G. The reaction of hyaluronic acid and its monomers, glucuronic acid and N-acetylglucosamine, with reactive oxygen species. *Carbohydr. Res.* **1999**, *321*, 228–234.

(21) Hjerde, T.; Stokke, B. T.; Smidsrød, O.; Christensen, B. E. Free-radical degradation of triple-stranded scleroglucan by hydrogen peroxide and ferrous ions. *Carbohydr. Polym.* **1998**, *37*, 41–48.

(22) Haltrich, D.; Nidetzky, B.; Kulbe, K. D.; Steiner, W.; Župančič, S. Production of fungal xylanase. *Bioresour. Technol.* **1996**, *58*, 137–161.

(23) Choi, Y. J.; Kim, E. J.; Piao, Z.; Yun, Y. C.; Shin, Y. C. Purification and Characterization of Chitosanase from Bacillus sp. Strain KCTC 0377BP and Its Application for the Production of Chitosan Oligosaccharides. *Appl. Environ. Microbiol.* **2004**, *70* (8), 4522–4531.

(24) Akpinar, O.; Erdogan, K.; Bostanci, S. Enzymatic production of Xylooligosaccharide from selected agricultural wastes. *Food Bioprod. Process.* **2008**.

(25) Ai, Z.; Jiang, Z.; Li, L.; Deng, W.; Kusakabe, I.; Li, H. Immobilization of Streptomyces olivaceoviridis E-86 xylanase on Eudragit S-100 for xylooligosaccharide production. *Process Biochem.* **2005**, *40*, 2707–2714.

(26) Wanasundara, P.K.J.P.D.; Shahidi, F. Removal of flaxseed mucilage by chemical and enzymatical treatments. *Food Chem.* **1997**, *59*, 47–55.

(27) Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428.

(28) Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **1944**, *153*, 375–380.

(29) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(30) Warrand, J.; Michaud, P.; Picton, L.; Muller, G.; Courtois, B.; Ralainirina, R.; Courtois, J. Large-Scale Purification of Water-Soluble Polysaccharides from Flaxseed Mucilage, and Isolation of a New Anionic Polymer. *Chromatographia* **2003**, *58*, 331–335.

(31) Mohnen, D. Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.* **2008**, *11*, 266–277.

(32) Warrand, J.; Michaud, P.; Picton, L.; Muller, G.; Courtois, B.; Ralainirina, R.; Courtois, J. Structural investigation of the neutral polysaccharide of *Linum usitatissimum* L. seeds mucilage. *Int. J. Biol. Macromol.* **2005**, *35*, 121–125.

(33) Van Craeyveld, V.; Delcour, J. A.; Courtin, C. M. Extractability and chemical and enzymic degradation of psyllium (Plantago ovata Forsk) seed husk arabinoxylans. *Food Chem.* **2009**, *112*, 812–819.

(34) Carvalheiro, F.; Silva-Fernandes, T.; Duarte, L. C.; Girio, F. M. Wheat Straw Autohydrolysis: Process Optimization and Products Characterization. *Appl. Biochem. Biotechnol.* **2009**, *153*, 84–93.

(35) Simms, J. P.; Hotchkiss, A. T., Jr; Irwin, P. L.; Hicks, K. B. High-performance liquid chromatographic separation of oligogalacturonic acids on a cyclomaltoheptaose (β-cyclodextrin) bonded-phase column. *Carbohydr. Res.* **1995**, *278*, 1–9.

(36) Biely, P.; Vršanská, M.; Tenkanen, M.; Kluepfel, D. Endo-β-1,4-xylanase families: differences in catalytic properties. *J. Biotechnol.* **1997**, *57*, 151–166.

(37) Rantanen, H.; Virkki, L.; Tuomainen, P.; Kabel, M.; Schols, H.; Tenkanen, M. Preparation of arabinoxylobiose from rye xylan using family 10 Aspergillus aculeatus endo-1,4-β-D-xylanase. *Carbohydr. Polym.* **2007**, *68*, 350–359.

(38) Petit, A.-C.; Noiret, N.; Sinquin, C.; Ratiskol, J.; Guézennec, J.; Colliec-Jouault, S. Free-radical depolymerization with metallic catalysts of an exopolysaccharide produced by a bacterium isolated from a deep-sea hydrothermal vent polychaete annelid. *Carbohydr. Polym.* **2006**, *64*, 597–602.

(39) Yang, Z.; Li, J.-P.; Guan, H.-S. Preparation and characterization of oligomannuronates from alginate degraded by hydrogen peroxide. *Carbohydr. Polym.* **2004**, *58*, 115–121.

(40) Thimma Reddy, T.; Tammishetti, S. Free radical degradation of guar gum. *Polym. Degrad. Stab.* **2004**, *86*, 455–459.

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