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Specificity of monoclonal antibodies generated against arabinoxylans of cereal grains

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Abstract

Xylooligosaccharides substituted by arabinose have been produced by degradation of wheat flour arabinoxylans with an endoxylanase. These oligosaccharides were coupled to carrier proteins (KLH and BSA) and three monoclonal antibodies were isolated. The specificity of antibody recognition was studied using arabino-xylo-oligosaccharides exhibiting different pattern of substitution by arabinose.

ELISA competition tests and molecular modelling suggest that the conformation adopted by beta-(1 → 4) linked xylose residues is an antigenic determinant recognized by the different antibodies. Arabinose was not specifically involved in the interaction of antibody and epitope.

Keywords: Cereal grains; Monoclonal antibodies; Arabinoxylans

1. Introduction

The cell walls from wheat grain and their components have a strong impact on cereal technology and its derived products (Courtin & Delcour, 2002; Fincher & Stone, 1986; Izydorczyk & Biliaderis, 1995). Arabinoxylans (AX) are the main non-starch polysaccharides from grain cell walls in wheat. They have a linear backbone of β-(1 → 4) linked D-xylopyranosyl units to which α-L-arabinofuranosyl units are attached through O-2 and/or O-3. In endosperm AX are partly water extractable (30%), and an arabinose to xylose ratio (A/X) of 0.6 is usually found, but large natural variations are observed (Cleemput et al., 1995; Dervilly, Saulnier, Roger, & Thibault, 2000; Izydorczyk & Biliaderis, 1995) and AX originating from the outer part of the endosperm appear less substituted with arabinose than those from the inner one (Delcour, Van Win, & Grobet, 1999). Besides, AX originating from the aleurone layer and from inner pericarp, testa and nucellar tissue are characterized by lower A/X ratio (0.3–0.4) (Antoine et al., 2003; Bacic & Stone, 1981; Rhodes, Sadek, & Stone, 2002) whereas AX in the outer pericarp have a considerable higher degree of substitution (A/X ~ 1) (Antoine et al., 2003). Although structural variations of AX in cereal grains are well documented, little is known on their real impact on polysaccharide functionalities or cell wall architecture.

In this context the development of antibodies to recognize specific structure of AX such as arabinose substituted areas or un-substituted areas, may help to better understand the molecular architecture of the cell wall, and the functionality of AX. Up to now, few antibodies have been developed against AX (Barry, Prensier, & Grenet, 1991; Migne, Prensier, & Grenet, 1994). We have recently, produced a polyclonal antibody against β(1 → 4) xylan and developed monoclonal antibodies with the aim to recognize areas of xylans substituted by arabinose (Guillon et al., 2004). Our strategy was to produce a mixture of arabino-xylo-oligosaccharide by digestion of AX with an endo-xylanase and to couple this mixture to carrier proteins to generate the antibodies.

In this paper we report on the isolation and characterization of arabino-xylo-oligosaccharide and their use to study the recognition specificity of monoclonal antibodies. Molecular modelling, was used in order to study the influence of the addition of arabinose on the conformation of β-(1 → 4) linked xylobiose.

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2. Results and discussion

2.1. Preparation and characterisation of xylo-oligosaccharides

Digestion of WSAX by an endoxylanase of *Trichoderma viride* produced xylose, xylobiose and a series of arabino-xylo-oligosaccharides as shown on Fig. 1. Eight oligosaccharides were isolated by size-exclusion chromatography on Biogel P2 and semi-preparative HPAEC. Some peaks co-eluting in the conditions of analytical HPAEC were separated in the conditions of the semi-preparative HPAEC. The structure of the different oligomers isolated (Table 1) was determined by MS and ^{1}H NMR spectroscopy from comparison with previously published data (Hoffmann, Geijtenbeek, Kamerling, & Vliegenthart, 1992; Hoffmann, Leeflang, Debarse, Kamerling, & Vliegenthart, 1991; Kormelink et al., 1993). Assignments of anomeric protons are reported in Table 2. We obtained a serie of arabino-xylo-oligosaccharides exhibiting substitution of β-(1 → 4) linked xylose residues by arabinose at O_3 or O_3 and O_2 on a single or on two contiguous xylose residues. In this series, substituted oligomers based on xylotetraose ($A1_3X4$ and $A2_{3d}X4$) and xylopentaose ($A2_{4,3}X5$, $A3_{4,3d}X5$, $A3_{4d,3}X5$, and $A4_{4d,3d}X5$) provided interesting tools to study the influence of mono and di-substitution on the specificity of antibody recognition.

The mixture of arabino-xylo-oligosaccharide (oligoAX) directly obtained from Biogel P2 by collecting fractions Kav 0.3 and 0.6, contained the same set of oligomers than obtained from AX digestion by endoxylanase but was free of xylose and xylobiose (Fig. 2). This mixture of oligosaccharides was coupled to BSA and KLH to generate antibodies against areas of AX substituted by arabinose.

2.2. Production and characterisation of the antibodies

Glycoconjugates were prepared by coupling BSA and KLH with oligoAX. The proportion of carbohydrate in the glycoconjugate was 33% (g/100 g of glycoconjugate) for BSA-oligoAX and 25% in KLH-oligoAX. Repeated immunisations with KLH-oligoAX produced a strong humoral response, and 50% of the maximum response was obtained with 1: 25,000 dilution of sera, in indirect ELISA.

The fusion produced eight hybridomas secreting antibodies against oligoAX. Three cloned cell lines, named AX1, AX2 and AX3 stable through cryo-preservation, thawing and re-cloning were obtained. The specificity of the antibodies produced by these three lines was further characterised by indirect competitive ELISA using non- and arabinosylated oligoxylosides as inhibitors and BSA-oligoAX (Table 3).

The three antibodies were similarly inhibited by the oligosaccharides tested for their binding to BSA-oligoAX. Linear (1–4) beta D-oligoxylosides showed an inhibitory effect that increased from xylobiose up to xylotetraose. Oligoxylosides of higher DP (5 and 6) did not show higher inhibition power. Substituted xylotetraose fractions were the best inhibitors. The presence of mono- or di-substitution by arabinose on a single xylose residue did not seem to

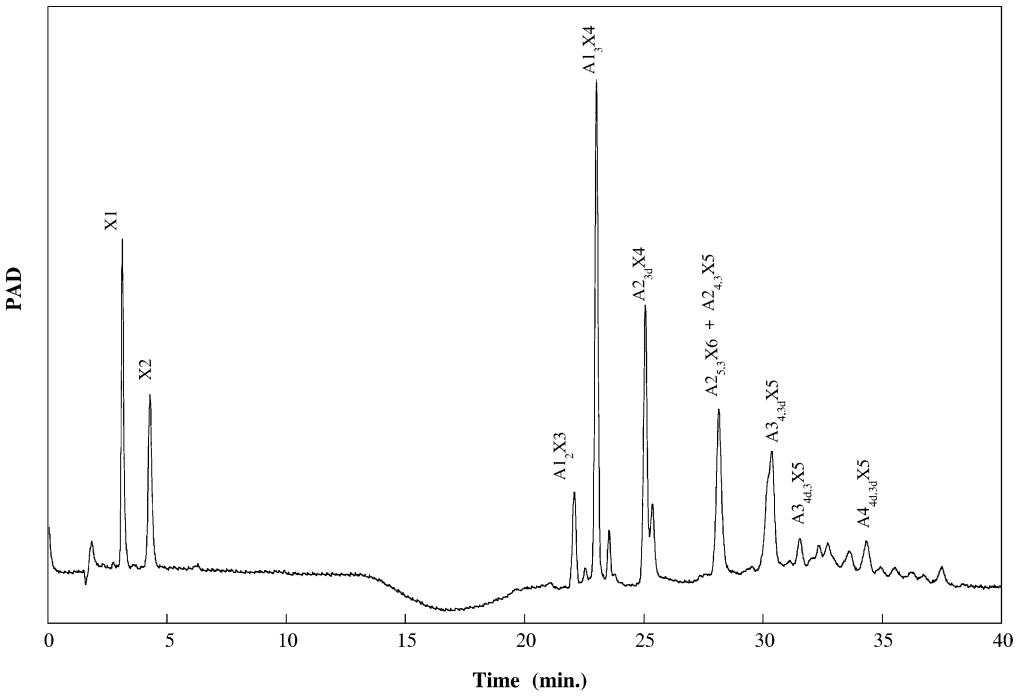
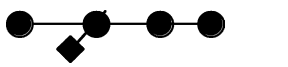
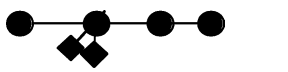
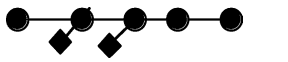
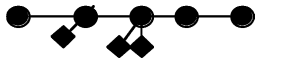
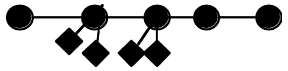
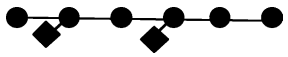


Fig. 1. HPAEC chromatogram of the products of AX degradation by endoxylanase from *Trichoderma viride*.

Table 1
Structure of the eight branched oligosaccharides isolated from wheat AX

Oligomers[a]	Mass (mg)	M/Z	DP	
$A1_2X3$	15[b]	569	4	
$A1_3X4$	77[b]	701	5	
$A2_{3d}X4$	58[b]	833	6	
$A2_{4,3}X5$	0.7[c]	965	7	
$A3_{4,3d}X5$	45[b]	1097	8	
$A3_{4d,3}X5$	0.3[c]	1097	8	
$A4_{4d,3d}X5$	0.9[c]	1229	9	
$A2_{5,3}X6$	0.2[c]	1097	8	

m/z values are measured as $[M + Na]^+$. —●—, β-D-Xyl*p*; ◆, α-L-Ara*f*-(1 → 3); ↓◆α-L-Ara*f*-(1 → 2).

[a] Subscript figure is related to the position of arabinose substituent on xylo-oligosaccharides. Xylose residues are numbered from the reducing end. Letter d refer to di-substitution.

[b] Purified from BioGel P2.

[c] Purified from Biogel P2 and semi-preparative HPAEC.

change the inhibitory power. The presence of two contiguous xylose residues substituted by arabinose significantly diminished the inhibitory power of the oligosaccharides ($A2_{4,3}X5$, $A3_{4,3d}X5$) or abolished it ($A3_{4,3d}X5$, $A4_{4d,3d}X5$). Obviously, the pattern of mono- and di-substitution on the two contiguous xylose residues drive the recognition of the antibody, and thus affect the possible conformation of the oligosaccharide. Conformation of non-substituted and substituted xylobiose was therefore studied by molecular modelling.

2.3. Molecular mechanics of non-substituted and substituted xylobiose units

The semi-relaxed map of xylobiose is given in Fig. 3. The surface exhibits four low energy regions labelled A, B, C and D with $(\varphi; \psi)$ values of (67; −114), (−114; 70), (−93; −151) and (−90; −85), respectively. All these solutions can be clustered into two types according to the relative orientations of C-O2 and C′-O3′ (unprime and prime annotations refer to non-reducing and reducing six-membered rings, respectively) apart of the glycosidic beta-(1–4) linkage:

On the one hand, A and B solutions have very close distances between O2 and O3′ yielding in both cases to hydrogen bond (0.29 nm). Therefore, these solutions have lowest relative energies ($\Delta E = 0$ and 0.3 kcal/mol, respectively). On the other hand, C and D conformations have opposite directions for C-O2 and C′-O3′ ($d\{O2–O3'\} =$ 0.56 and 0.58 nm, respectively) with higher relative energies ($\Delta E = 1.0$ and 1.5 kcal/mol). Therefore, A or B conformations could potentially be much more influenced by arabinose substitution compared to C and D ones.

In order to further measure the influence of substitution by arabinose in term of steric hindrances of xylobiose flexibility, semi-relaxed energy maps were established for α-L-Araf-(O-2)-β-D-Xylp and α-L-Araf-(O-3)-β-D-Xylp models. Each specific surface exhibited four low energy regions that were then combined to the un-substituted xylobiose map to systematically explore all acceptable solutions for mono or di-substituted rings. The semi-relaxed map computed for di-substituted xylobiose is displayed in Fig. 4. Compared to un-substituted xylobiose map, the location of the four initial regions were kept despite some significant displacements $(\varphi; \psi)$ values are (36; −87), (−81; 81), (−144; −146) and (−64; −72), for A, B, C and D regions, respectively. However, the major fact is the drastic restriction of A region which relative energy is now significantly higher. ($\Delta E = 4.0$, 0.0, 0.2 and 1.0 kcal/mol

Table 2
^{1}H NMR chemical shifts for anomeric proton of oligomers

	$A1_2X3$	$A1_3X4$	$A2_{3d}X4$	$A2_{4,3}X5$	$A3_{4,3d}X5$	$A2_{5,3}X6$	$A3_{4d,3}X5$	$A4_{4d,3d}X5$
β-Xylp-1	5.1845	5.1835	5.184	5.188	5.185	5.184	5.185	5.185
β-Xylp-1	4.5825	4.584	4.5845	4.587	4.584	4.584	4.5795	4.579
β-Xylp-2	4.5085	4.477	4.468	4.471	4.463	4.458	4.467	4.468
β-Xylp-3	4.442	4.5135	4.64	4.513	4.635	4.518	4.4995	4.628
β-Xylp-4	–	4.445	4.4375	4.485	4.4805	4.477	4.5985	4.568
β-Xylp-5	–	–	–	4.435	4.436	4.44	4.4255	4.430
β-Xylp-6	–	–	–	–	–	–	–	–
$\alpha\text{-Araf}_{3\times2}$	5.395	–	–	–	–	–	–	–
$\alpha\text{-Araf}_{3\times3}$	–	5.397	5.274	5.388	4.389	5.391	5.419	5.294
$\alpha\text{-Araf}_{3\times4}$	–	–	–	5.398	4.523		5.274	5.281
$\alpha\text{-Araf}_{3\times5}$	–	–	–	–	–	5.391	–	–
$\alpha\text{-Araf}_{2\times3}$	–	–	5.227	–	4.347	–	–	5.224
$\alpha\text{-Araf}_{2\times4}$	–	–	–	–	–	–	5.233	5.245

Measured at 400 MHz in D_2O at 300°K.

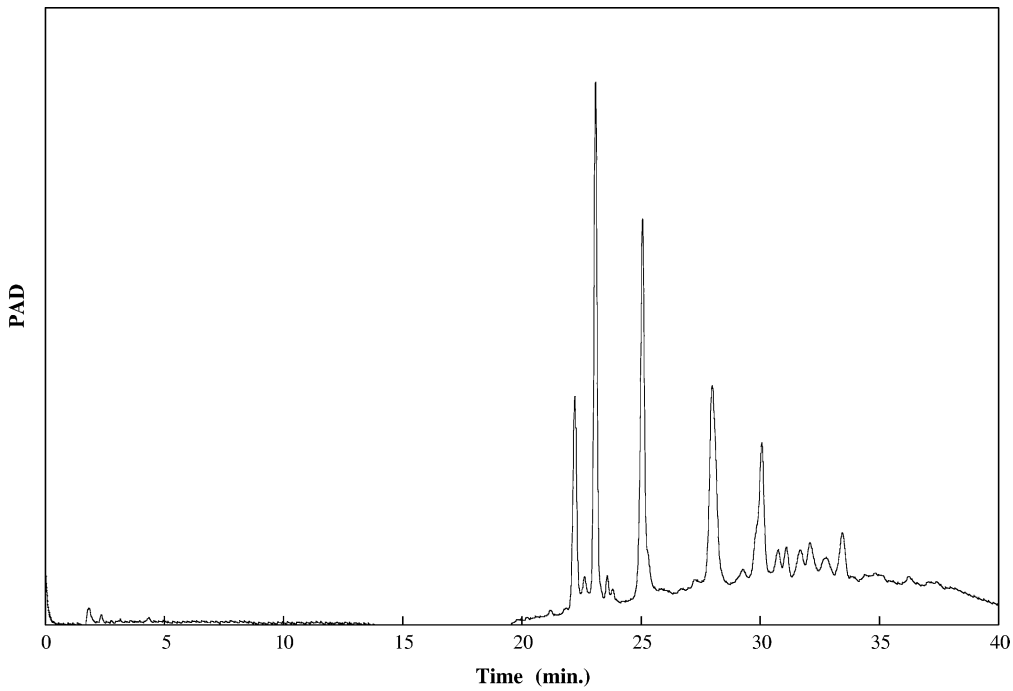


Fig. 2. HPAEC chromatogram of oligo-AX used to generate antibodies.

for A, B, C and D, respectively). Although each ring was di-substituted, these results can mainly be explained by O2 and O3′ substitutions. As expected, C and D conformations are not basically perturbed by these chemical changes because of the large distance between O2 and O3′ (0.48 and 0.54 nm, respectively). But the O2−O3′ short distances for A and B solutions cannot be maintained anymore due to the size of the arabinose ring (d{O2-O3′} = 0.44 and 0.39 nm, respectively). In the A case, the relative orientation of the two arabinose rings is not compatible with a good stacking interaction (highest relative energy), contrary to the B case.

Table 3
Competitive inhibition of monoclonal antibodies against different arabinoxylan derived oligosaccharides

Oligosaccharide	AX1 IC_{50}	AX2 IC_{50}	AX3 IC_{50}
X2	56.2	41.6	1.8
X3	25.6	10.5	0.4
X4	13.7	9.7	0.5
$A1_2X3$	9.8	7.4	0.2
$A1_3X4$	7.0	3.9	0.2
$A2_{3d}X4$	4.9	2.9	1.0
$A2_{4,3}X5$	40.2	33.2	n.d.
$A3_{4,3d}X5$	32.7	20.1	6.2
$A3_{4d,3}X5$	n.i.	n.i.	n.d.
$A4_{4d,3d}X5$	n.i.	n.i.	n.d.
$A2_{5,3}X6$	n.i.	n.i.	n.d.

IC_{50} is defined as the concentration of oligosaccharide (nM/ml) that give 50% inhihibition of the binding of the antibody to the immobilized antigen (BSA-oligoAX), n.i.: no inhition, n.d.: not determined.

Fig. 5 illustrates the evolution of A, B, C and D solutions from un-substituted to di-substituted rings. For sake of clarity, the arabinose rings on O3 and O2′, although presents were not drawn.

Therefore, while the potential energy surfaces retain the same shape and location of wells, differences in energy barrier between wells and depth of them were introduced by di-substitution of xylose residues. Region A is hardly represented because of steric conflicts between arabinose

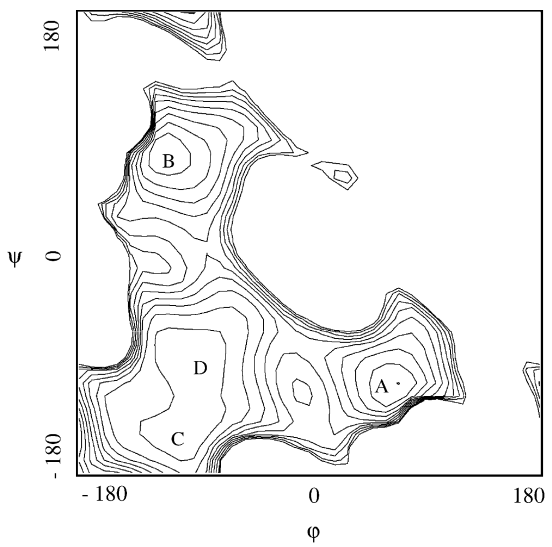


Fig. 3. Potential energy surface computed as a function of φ and ψ for β-(1−4)-xylobiose. The location of the low energy conformers (A−D) is indicated.

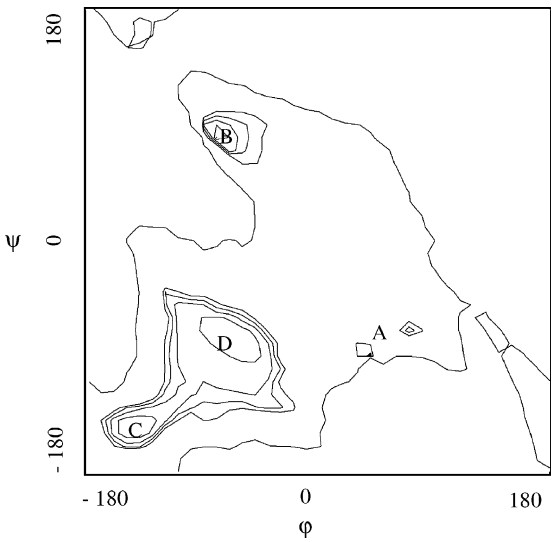


Fig. 4. Potential energy surface computed as a function of φ and ψ for di-substituted xylobiose. The location of the low energy conformers (A–D) is indicated.

residues linked at O-2 at the non-reducing side and the arabinose residue linked at O-3 at the reducing side. Exploration of other pattern of substitution indicated that the only parameter that induce a steric hindrance was the presence of both an arabinose residue linked in O-2 at the non-reducing side and an arabinose residue linked in O-3 at the reducing side, whereas other pattern had no significant effect on the conformational behavior of the β-(1–4) glycosidic linkage between xylose residues. It was noteworthy that oligomers $A2_{4d,3}X5$, $A2_{4d,3d}X5$ that presented this specific pattern of substitution were not recognized by the different antibodies.

3. Conclusion

Oligoxylosides substituted by arabinose have been produced by degradation of wheat flour AX with an endoxylanase. These haptens have been coupled to carrier proteins (KLH and BSA) with the aim to produce antibodies specifically directed against substituted areas of AX. The clone lines selected from hybridoma supernatants generated with KLH-oligo-AX recognised specifically xylan and AX polysaccharides in different plant tissues (Guillon et al., 2004). However, the interaction of the different antibodies with various AX cannot be explained simply on the basis of AX structure, and especially by the presence of arabinose as side-chains. As a matter of fact, amongst polysaccharides tested, heteroxylans (Chanliaud, Saulnier, & Thibault, 1995) exhibited the highest inhibitory potency (IC_{50} 35 μg/ml, AX1 antibody) followed by beta-(1 → 3)(1 → 4) xylan from *Palmaria palmata* (Deniaud, Quemener, Fleurence, Lahaye, 2003) (IC_{50} 45 μg/ml, AX1 antibody). Arabinoxylan from wheat flour exhibiting different extent of substitution by arabinose (Dervilly-Pinel, Thibault, & Saulnier, 2001) had a more limited inhibition power (<20%, at a concentration of 100 μg/ml), although oligo-AX haptens used to produce the antibodies were generated from their degradation by an endoxylanase. In this paper, the contribution of arabinose in the interaction with the antibodies was therefore studied using a range of arabino-xylo-oligosaccharides exhibiting different pattern of substitution by arabinose.

Compared to xylotetraose, $A1_3X4$ and $A2_{3d}X4$ had a slightly better inhibitory potency, but the presence of one or two arabinose on a single xylose residue had no specific effect. This absence of effect of mono or di-substitution on

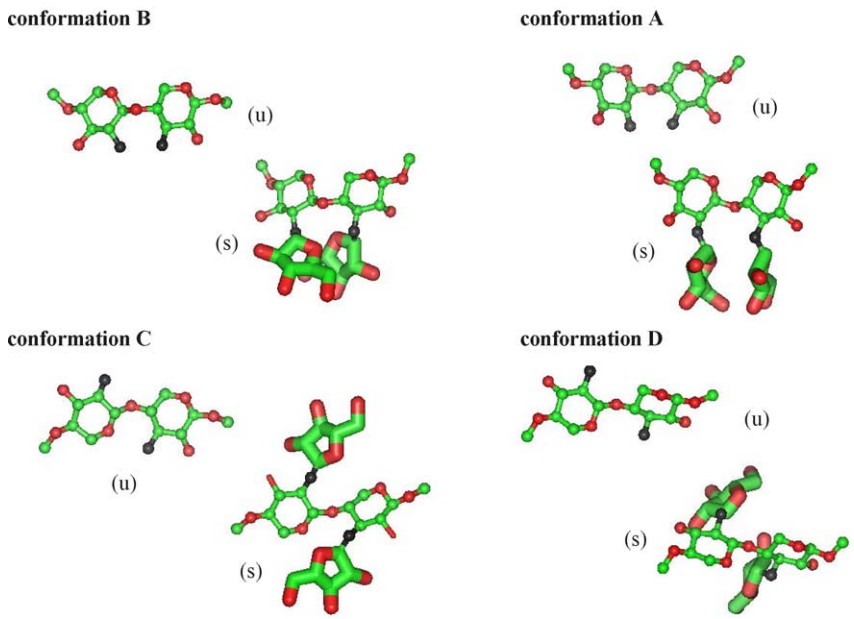


Fig. 5. Ball and stick representation of low energy conformers of un-substituted (u) and di-substituted (s) xylobiose. In thick stick: arabinose rings attached on O2 and O3′ positions (those in O3 and O2′, although taken into account, are not drawn here) Hydrogen atoms are omitted for clarity.

the binding was further confirmed with xylopentaose oligomers with two contiguous xylose residues bearing arabinose. As a matter of fact $A2_{4,3}X5$ and $A3_{4,3d}X5$ inhibited antibodies to the same extent. These results suggest that arabinose is not specifically involved in the recognized epitope. The comparison of inhibitory potency of $A3_{4,3d}X5$ (inhibitor) and $A3_{4d,3}X5$ (not inhibitor) or $A4_{4d,3d}X5$ (not inhibitor) bring further evidence that arabinose is not specifically involved in the recognition. Molecular modelling of xylobiose and fully substituted xylobiose have shown that only the presence of both an arabinose residue linked in O-2 at the non-reducing side and an arabinose residue linked in O-3 at the reducing side (case of $A3_{4d,3}X5$ and $A4_{4d,3d}X5$) had an effect on the conformational behaviour of the β-(1–4) glycosidic linkage between xylose residues. In conclusion, the results suggest that the conformation adopted by beta-(1 → 4) linked xylose residues is the antigenic determinant recognized by the different antibodies. Arabinose is not specifically involved in the interaction between antibody and epitope but its presence can change the possible conformation adopted by the xylan chain and consequently its recognition by the antibody. Although the actual specificity of the monoclonal antibodies characterized in this study, differed from the expected specificity, they are excellent tools to recognize xylan type polysaccharides in plant tissues.

Anti-α-L-arabinofuranose antibody reacting against arabinofuranose-containing polysaccharides, such as rice endosperm AX and some plant arabinogalactan has been generated (Kaku, Shibata, Satsuma, Sone, & Misaki, 1986). It is therefore, likely that the oligosaccharides used in this study to produce antibodies had too long un-substituted xylose sequence, and clone lines reacting against this structure were preferentially retained. We anticipate that $A4_{4d,3d}X5$ that is not an inhibitor of the antibodies generated in this study, might be an excellent hapten to produce antibodies against substituted areas of AX.

4. Experimental

4.1. Polysaccharides

Water-soluble AX fractions with different arabinose to xylose ratio (A/X: 0.37 for F30, 0.75 for F60 and 1.21 for F70) were sub-fractions isolated by size exclusion chromatography from graded ethanol fractionation (saturation level of ethanol of 30, 60 and 70%) of water soluble AX (WSAX) (Dervilly-Pinel et al., 2001). Heteroxylans were isolated from maize bran as previously described (Chanliaud et al., 1995). Beta-(1 → 3) (1 → 4) xylan was extracted from seaweeds (*Palmaria palmata*) as previously described (Deniaud et al., 2003).

4.2. Preparation of arabinoxylan oligosaccharides

Oligoxylosides (DP 2–6) were purchased from Megazyme (Bray, Ireland). Oligoxylosides substituted by arabinose were prepared from WSAX (Faurot et al., 1995). 2.5 g of WSAX were dissolved in 0.02 M NaOAc buffer, pH 4.6 (500 ml) and incubated for 32 h at 50 °C with an endo-xylanase (1 U/mg of WSAX) from *Trichoderma viride* (Megazyme-Bray, Ireland). The reaction was stopped by boiling the solution for 10 min. The solution was then filtered through 3 μm filters (Millipore), and resistant polysaccharides were precipitated with ethanol 95% at 4 °C for 2 h. The supernatant was recovered after centrifugation at 11 200 g for 20 min, ethanol was evaporated and the residue re-dissolved in water and finally freeze-dried.

A sample of the endoxylanase digestion (600 mg) was dissolved in water (8 ml) and applied to a column (2.6 × 100 cm) of Biogel P2, eluted with water at 50 °C at a flow rate of 25 ml/h. Fractions (3 ml) were collected and analyzed for total carbohydrate by the automated orcinol method (Tollier & Robin, 1979). The different fractions collected were analyzed by high performance anion-exchange chromatography (HPAEC-PAD) to check purity. Finally four pure oligomers with degree of polymerisation (DP) 4, 5, 6 and 8 were isolated.

Some of the BioGel P2 fractions constituted of a mixture of oligomers were further purified using a semi-preparative Carbopac PA-100 column (9 × 250 mm) with a HPAEC-PAD system (Dionex 4500i). Elution was carried out at a flow rate of 1 ml/min at room temperature and gradient elution (0–1 min 80% A, 10% B, 10% C; 1–30 min 70% A, 20% B, 10% C; 30–40 min 65% A, 25% B, 10% C; 40–41 min 80% A, 10% B, 10% C; where A, Water; B, 1 M NaOAc; C, 0.5 M NaOH), the detection PAD comprised the following pulse potentials $E_1 = +0.1$, $E_2 = +0.6$, and $E_3 = -0.6$ V. Fractions were immediately neutralized by 0.75 M H_2SO_4 using a Carbohydrate Membrane Desalter (Dionex) with a flow rate of 7–9 ml/min and freeze-dried. Four oligomers were isolated, one of DP 7, two of DP 8 and one of DP 9.

Another sample of endoxylanase digestion was injected on the BioGel P2 column, and oligomers eluted between Kav 0.3 and 0.6 were collected and freeze-dried. This mixture (oligoAX) was used for preparation of BSA and KLH conjugates.

Analytical HPAEC was performed on a Waters quaternary gradient module, using a Carbopac PA-1 analytical column (4 × 250 mm) at 25 °C with a flow rate of 1 ml/min and gradient elution (0–5 min 80% A, 0% B, 20% C; 5–20 min 75% A, 5% B, 20% C; 20–30 min 70% A, 10% B, 20% C; 30–40 min 60% A, 20% B, 20% C; 41–60 min 80% A, 0% B, 20% C where A, Water; B, 1 M NaOAc and C, 0.5 M NaOH) equipped with a model PAD detector with $E_1 = +0.05$, $E_2 = +0.6$, and $E_3 = -0.6$ V.

4.3. Structural characterization of oligosaccharides

The molecular mass of oligosaccharides were determined on a LCQ Advantage Ion-Trap Mass Spectrometer (ThermoFinnigan, USA), equipped with an electrospray source. Samples were dissolved in water (15 and 60 μg/ml) and mixed with one volume of methanol 50%, and then run at a flow rate of 2.5 μl/min. MS analysis was carried out in the positive mode using a spray voltage of 4 kV and a capillary temperature of 200 °C. Nitrogen was used as sheath gas (20 arbitrary units). The capillary voltage was set a 45 kV and the tube lens voltage at 35 kV.

The purified oligomers were analysed by ^{1}H NMR on a BRUKER ARX 400 spectrometer. Samples were dissolved in D_2O (5–10 mg/ml) and spectra were recorded at 300 K. Approximately 128 pulses were collected, pulse repetition time was 240 μs and pulse angle was 54.7°.

4.4. Synthesis of protein-hapten-conjugates

OligoAX were conjugated to bovine serum albumin BSA (Sigma A7030) and keyhole limpet hemocyanin KLH (Pierce, Rockford, IL) by reductive amination as previously described (Roy, Katzenellenbogen, & Jennings, 1984). The relative proportion of carbohydrate to protein in the conjugate was determined using the orcinol method (Tollier & Robin, 1979) for the carbohydrate determination and the Bradford procedure (Bradford, 1976) for protein determination using xylose and BSA, respectively as standards. The protein determination of KLH-oligoAX conjugates cannot be performed by the Bradford method because of the occurrence of cupric ion in KLH.

4.5. Production and characterization of monoclonal antibodies

Female Balb/c mice were immunized with KLH-oligoAX. Four subcutaneous injections were carried out at 3-week intervals with a mixture of KLH-oligoAX (50 μg) dissolved in PBS (50 μl) and synthetic adjuvant (50 μl) (Titermax Gold, CytRx corporation, GA). 14 days after each injection, sera reactivity was evaluated in indirect ELISA test against BSA-oligoAX. Four days before fusion, the best immunized mouse was given a pre-fusion intravenous boost with 10 μg of KLH-oligoAX in PBS.

Spleen was removed and lymphocytes were isolated and fused with SP2/0 myeloma cell line using standard hybridoma preparation and limiting dilution cloning procedures. Hybridoma supernatants resulting from the fusion were screened for the presence of anti-oligoAX antibodies using indirect ELISA test. 3 hybridomas were cloned by limiting dilution and expand to produce supernatant for characterisation and immuno-fluorescence labelling.

Indirect ELISA tests were carried out on plates (Nunc 442404, F96 Maxisorp) coated, overnight at +4 °C, with the BSA-oligoAX (0.5 μg/well) diluted in carbonate buffer (0.05 M) pH = 9.5. Remaining sites were blocked with 300 μl of 4% skimmed milk powder in PBS for 2 hours at room temperature. Each well was incubated for two hours at room temperature with 100 μl of the hybridoma culture supernatants at appropriate dilution in PBS-skimmed milk 0.1%. Secondary antibody (goat anti-mouse IgG peroxydase conjugate, BioRad) (100 μl, dilution: 1/3000)) was incubated for 1 h at room temperature. After each step, reagents were washed three times with 300 μl of PBS-Tween 20 0.05%. Coloration was performed with 100 μl of *O*-phenylenediamine (Sigma) at 0.4 mg/ml and 0.03% H_2O_2 in 0.05 M sodium citrate pH 5.5 for 20 min at room temperature and stopped with 100 μl of 2M H_2SO_4. Then the absorbance was read at 490 nm.

Competitive ELISA tests were carried out as above except for the following modifications. Each well was incubated for 2 h with 50 μl of the hybridoma culture supernatants diluted 1:250 in PBS containing 0.1% of skimmed milk together with the same buffer containing various concentration of oligosaccharides inhibitor at room temperature. The plates were washed three times with PBS-Tween 20 and developed with the goat anti-mouse IgG-peroxidase conjugate as described.

Competitive inhibition of antibody binding to the immobilised oligoAX is reported as a percentage of signal obtained with antibody in the absence of competitors. Concentrations of competitors resulting in 50% inhibition (IC_{50}) of antibody binding were determined by plotting competitor concentration against absorbance. Values from controls with no competitors were taken as 0% inhibition of binding, and values from controls with no antibody represented 100% inhibition of binding.

4.6. Molecular modelling

The conformational behaviour of xylobiose, with or without α-L-arabinofuranose residues in position O-2 and O-3 were investigated using the molecular mechanics package from MSI (Discover, Insight II). All calculations were performed with the CFF91 force field. Although mainly designed for protein molecules, this robust force field has been successfully employed for saccharide molecules (Andre et al., 1996). Reducing and non-reducing ends of the disaccharide were artificially 'methylated' in order to avoid creation of spurious internal hydrogen bonds.

The relative orientation of the contiguous carbohydrate units a part from the glycosidic linkages are mainly described by the values of the two torsional angle φ and ψ. Possible conformations can be evaluated by contour maps of the variation of potential energy of the disaccharide subunit as a function of these torsional angles. Rigid energy map were established by varying the torsional angles (φ; ψ), in 5° increments from −180 to 180°. The term 'rigid map' refers to the protocol used and means that all other internal parameters (lengths, valence angle, dihedral angle) were

fixed. This procedure allowed to locate the zones of low energy in the space (φ; ψ). Starting from these minima, conformations were refined without any constraint on the internal conformational parameters of the rings or of the glycosidic linkage. In this study, relaxation was achieved only in the minima regions found on the rigid maps and the conformation of the rings was assumed to remain more or less unchanged in the vicinity of these regions. This procedure led to a slight variation of the values of φ and ψ, and allowed the adjustment of conformational parameters of the disaccharides so that they could adapt to their environment and decrease as much as possible contacts between atoms of adjacent rings. Various orientations of side groups (primary alcohol groups) have been studied and conformers with the lowest calculated energy for each of the minima regions have been kept.

For xylobiose, four minima corresponding to four conformations which are likely to exist were found out. Each of the four conformations was minimised to obtain four local rigid maps which were finally merged into a semi-relaxed map of xylobiose. Rigid maps of α-L-arabinofuranose-(O-2)-β-D-xylopyranose were performed to locate the low energy zones in the space ($\varphi 2$; $\psi 2$). Four minima corresponding to four conformations were found out. A semi-relaxed map of α-L-arabinofuranose-(O-2)-β-D-xylopyranose was then built as previously described for xylobiose. Exactly in the same way, semi-relaxed map of α-L-arabinofuranose-(O-3)-β-D-xylopyranose was obtained. Four low energy regions were located.

Systematic exploration of the allowed conformations for α-L-arabinofuranose-(O-3) (O-2)-β-D-xylopyranose was performed by combining the solutions obtained for α-L-Ara*f*-(O-2)-β-D-Xyl*p* and α-L-Ara*f*-(O-3)-β-D-Xyl*p*. Sixteen di-substituted conformations were then constructed. Finally, systematic exploration of conformational space of di-substituted xylobiose was performed by combining the four possible conformations of xylobiose with the sixteen possible conformations of di-substituted xylose: 64 combinations were constructed. Most of them were discarded according to their instant energy, the other ones were minimised. Finally, for each of the four possible conformations of the xylobiose, the most probable di-substituted one was kept. Partially rigid maps were performed for each of these four conformations and then merged into a semi-relaxed map of di-substituted xylobiose.

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