Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 1047–1060
Carbohydrate RESEARCH

Hydrolysis of *Nothogenia erinacea* xylan by xylanases from families 10 and 11

Wim Nerinckx,[a,*] Anders Broberg,[b,†] Jens Ø. Duus,[b] Patricia Ntarima,[a] Lesley A. S. Parolis,[c] Haralambos Parolis[d] and Marc Claeyssens[a,*]

[a]*Department of Biochemistry, Physiology and Microbiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*
[b]*Department of Chemistry, Carlsberg Laboratory, Valby DK-2500, Denmark*
[c]*Department of Chemical Engineering, University of Cape Town, Rondebosch 7700, South Africa*
[d]*Chemistry Department, University of Cape Town, Rondebosch 7700, South Africa*

Received 19 October 2003; accepted 17 December 2003

Dedicated to the memory of Professor Christian Pedersen, The Technical University of Denmark

Abstract—The structures of several enzymatic hydrolysis products of *Nothogenia erinacea* seaweed xylan, a linear homopolymer with mixed β-(1→3)/β-(1→4) linkages, were analysed by physicochemical and biochemical techniques. With the glycoside hydrolase family 10 β-(1→4)-xylanase from *Cryptococcus adeliae*, hydrolysis proceeds to a final mixture of products containing a mixed linkage-type triose as a major compound, whereas with the family 11 xylanase from *Thermomyces lanuginosus* this is a mixed linkage tetraose. The *Cryptococcus* xylanase is shown to be capable of also catalysing the hydrolysis of β-(1→3) linkages, that is this of a mixed type tetraose intermediary formed, in accordance with the broader substrate specificity of family 10 enzymes. From a partial degradation experiment with the *T. lanuginosus* xylanase, a series of higher mixed oligosaccharides were isolated and identified. The observed oligosaccharide intermediates and splicing pattern indicate an irregular β-(1→3)/β-(1→4) linkage distribution within the linear D-xylose polymer. Similar results were obtained with rhodymenan, the seaweed xylan from *Palmares palmata*.

Keywords: Xylanases; Seaweed; Xylooligosaccharides; Hydrolysis; Rhodymenan

1. Introduction

In the glycoside hydrolase classification,[1,2] β-(1→4)-xylanases (E.C. 3.2.1.8) are mainly found in families 10 and 11, the latter containing low molecular weight enzymes only active on xylooligosaccharides and xylans.[3] On the other hand, family 10 xylanases exhibit a higher molecular mass and are less specific. They can act on aryl cellobiosides[4] and mixed D-glucose–D-xylose derivatives.[5] Although the three-dimensional structures and some catalytic properties are different, both families of xylanases share the same catalytic double displacement mechanism and the same cleft-like active site topology[6] as typical for *endo*-splicing enzymes. Family 10 glycosylases however seem to hydrolyse xylans with greater catalytic efficiencies than those of family 11[3] and their transferring activities could advantageously be exploited in the present study.

Rhodymenan, a seaweed xylan from *Palmares palmata* (formerly *Rhodymenia palmata*), is a linear homopolymer with mixed β-(1→3) and β-(1→4) linkages.[7] This xylan was shown to be hydrolysed by the *Cryptococcus albidus* family 10 xylanase to a triose with

Abbreviations: HPAEC-PAD, High performance anion exchange chromatography with pulsed amperometric detection; DP, degree of polymerisation

* Corresponding authors. Tel.: +32-9-264-5270; fax: +32-9-264-5332; e-mail addresses: wim.nerinckx@ugent.be; marc.claeyssens@ugent.be
† Present address: Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, SE-750 07 Uppsala, Sweden.

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.02.017

the putative structure β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*, whereas with a family 11 xylanase from *Hypocrea jecorina* (formerly *Trichoderma reesei*) a tetrasaccharide was proposed to be the smallest degradation fragment.[3]

We wish to report here the hydrolysis of a xylan from another seaweed, *Nothogenia erinacea* (formerly *Chaetangium erinaceum*), catalysed by *endo*-acting xylanases from both families. Depending on the analytical method used, methylation analysis or periodate oxidation, this xylan has previously been shown[8] to be composed of β-(1→3) and β-(1→4) linkages in the approximate ratios of 2:9 or 1:4.

The hydrolysis experiments of this polymer by either the xylanase from *Thermomyces lanuginosus* (family 11)[9] or that from *Cryptococcus adeliae* (family 10)[10] as described in this paper show the substantial formation of oligosaccharides with mixed linkages. Several were isolated and structural evidences was obtained by high performance anion exchange chromatography, secondary enzymatic degradation experiments and spectroscopic analyses.

2. Results

2.1. Enzymatic hydrolysis of *N. erinacea* xylan

The reaction products of the progressive hydrolyses of *N. erinacea* xylan by the xylanases from *T. lanuginosus* (family 11) and *C. adeliae* (family 10) were analysed by HPAEC-PAD. The formation of oligosaccharides in the β-(1→4) series can clearly be seen in Figure 1, which compares the results after 2 h reaction time for the enzymes from both families. Eluates corresponding to oligosaccharides with putatively mixed β-(1→3) and β-(1→4) linkages (peaks **A**–**F**) could at this stage not be identified because of the lack of standards. Double peaks appear in the case of the *C. adeliae* xylanase (e.g., **B**, **B′**).

At the start of the reaction, xylobiose was observed but not D-xylose. Gradually, the monosaccharide, xylotriose, xylotetraose, xylopentaose, and even a small amount of xylohexaose appeared, as well as a series of oligosaccharides with mixed linkages. At later stages, the reaction profile became less complex as the oligosaccharides all were converted into smaller oligosaccharides. After exhaustive hydrolysis (7 days) the final products were D-xylose, xylobiose and xylotriose (in the β-(1→4)-oligosaccharide series) and, in the case of *T. lanuginosus* xylanase, two further compounds (a minor **A** and a major **B**) had accumulated. In the case of exhaustive hydrolysis by the *C. adeliae* xylanase, only compound **A** was observed next to D-xylose and xylo-oligosaccharides.

Almost identical results (not shown) were found with the seaweed xylan from *R. palmata*.

Before further NMR-spectroscopic proof, a preliminary enzymatic identification of compounds **A** and **B** could be obtained by examining the reaction products obtained by catalytic methanolysis, mediated by the *C. adeliae* xylanase,[10] of the above **A** and **B** containing mixture (Fig. 2a and b). Whereas the concentrations of xylobiose and compound **A** in this reaction mixture remained constant, that of D-xylose increased, and the β-methyl glycoside of β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl*p* appeared, the latter coinciding chromatographically with an authentic reference compound (see Experimental Section). Considering the specificity of the *C. adeliae* xylanase, D-xylose was most probably released from compound **B**, which therefore must contain a reducing terminal β-(1→4)-linked D-xylose residue. The structure of **B** was thus tentatively identified as the tetrasaccharide β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-

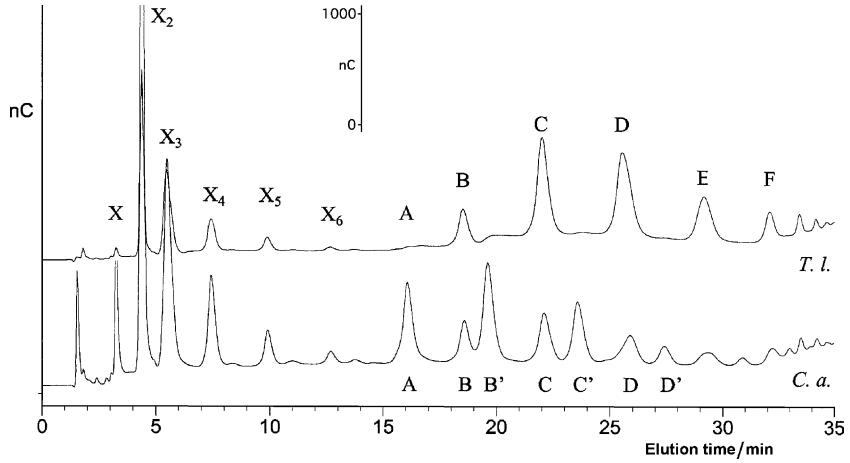

Figure 1. Patterns of products released after 2 h from *N. erinacea* xylan by the hydrolytic action of the xylanase from *T. lanuginosus* and that from *C. adeliae*. HPAEC-PAD analyses of partial enzymatic hydrolysates show the formation of β-(1→4)-xylo-oligosaccharides up to xylohexaose, and of mixed β-(1→4)-/β-(1→3)-xylo-oligosaccharides (**A**–**F**; upper graph with the *T. lanuginosus* xylanase).

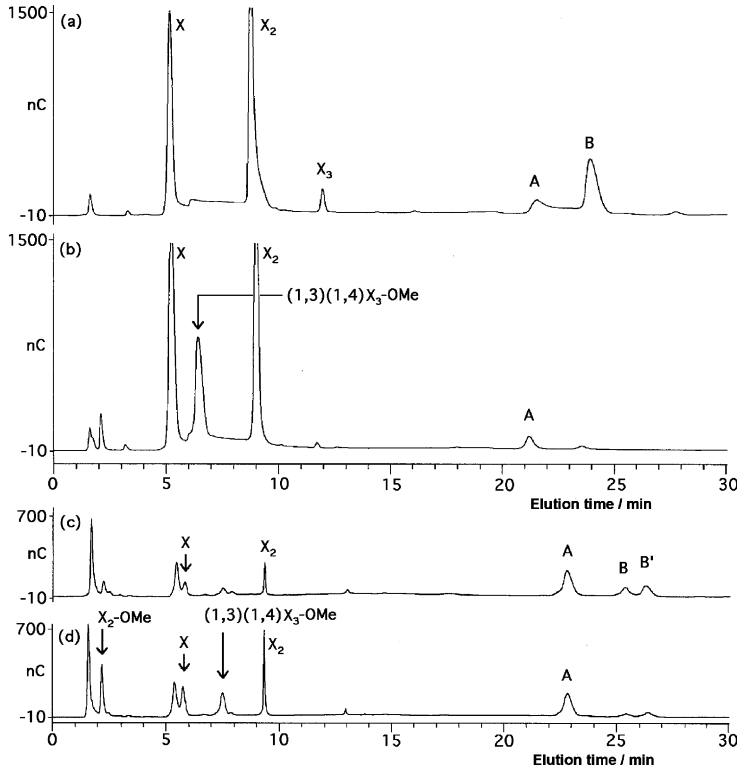

Figure 2. HPAEC-PAD analyses of the formation of β-methyl glycosides by transfer reactions catalysed by the *C. adeliae* xylanase. **(a)** to **(b)**: An enzymatic digest in the presence of methanol (2 M) of the reaction products obtained with the *T. lanuginosus* xylanase; (**a**) before and (**b**) after 48 h incubation with the *C. adeliae* xylanase. **(c)** to **(d)**: The **B**–**B′** fraction obtained with *C. adeliae* xylanase further incubated in the presence of methanol (2 M) and the same enzyme; (**c**) after 10 min and (**d**) after 4 h incubation.

β-D-Xyl*p*-(1 → 4)-α/β-D-Xyl*p*, while that of **A** was tentatively identified as the mixed linkage trisaccharide β-D-Xyl*p*-(1 → 3)-β-D-Xyl*p*-(1 → 4)-α/β-D-Xyl*p*.

2.2. Further identification of the mixed linkage oligosaccharides (A and B) resulting from the exhaustive enzymatic hydrolysis of the *N. erinacea* xylan by the *T. lanuginosus* xylanase

By preparative HPAEC-PAD, the reaction products resulting from the exhaustive enzymatic hydrolysis of the xylan were separated in two fractions, one containing D-xylose, xylobiose and xylotriose, the other a 1:4 mixture of the compounds **A** and **B**. The composition of both fractions was checked by analytical HPAEC-PAD. After dialysis and concentration by lyophilisation, the latter mixture was subjected to spectroscopic analyses.

The ESMS of the **A**–**B** mixture shows the presence of a major ion at m/z 547, which corresponds to the $[M+H]^+$ of a tetrasaccharide, and a minor ion at m/z 415, which corresponds to the $[M+H]^+$ of a trisaccharide.

The ^{1}H NMR spectrum of the **A**–**B** mixture (Fig. 3) shows H-1 signals for three β-linked xylopyranosyl residues at δ 4.670 (1H, $J_{1,2}$ 7.8 Hz), 4.492 (1H, $J_{1,2}$ 7.8 Hz) and 4.480/4.482 (1H, $J_{1,2}$ 7.8/7.6 Hz), and H-1 signals for a reducing xylopyranose residue at δ 4.589 (0.67H, $J_{1,2}$ 8.0 Hz, β-anomer) and at δ 5.189 (0.33H, $J_{1,2}$ 3.6 Hz, α-anomer).

The ^{13}C NMR data of the **A**–**B** mixture (not shown) complement the ^{1}H NMR data showing signals for C-1 of β-linked xylopyranoses at 104.26 and 102.50 (2 × C) ppm and signals for C-1 of the α- and β-anomers of a reducing xylopyranose at 92.86 and 97.35 ppm, respectively. Extra resonances observed in both the ^{1}H and ^{13}C spectra originate from the minor component **A** (see further). The data clearly indicate that the major component **B** in the mixture is a tetrasaccharide.

The linkage carbons and the sequence of the linkages of the xylopyranose residues in the tetrasaccharide **B** were confirmed by 2D NMR spectroscopy. The xylosyl residues of **B** are arbitrarily labelled **a**–**e** in order of decreasing chemical shift of the resonances of their anomeric protons. The ^{1}H resonances for all the residues in **B** were first determined by ^{1}H–^{1}H correlation spectroscopy with DQF-COSY[11] and HOHAHA;[12] the latter TOCSY experiment also allowed confirmation of a 1:4 **A**:**B** ratio present in the mixture. Subsequently, the ^{13}C resonances for each residue of **B** were established by comparison of the assigned ^{1}H resonances with the data obtained from a ^{1}H–^{13}C correlation (gHSQC[13]) experiment. These data are listed in Table 1. From the

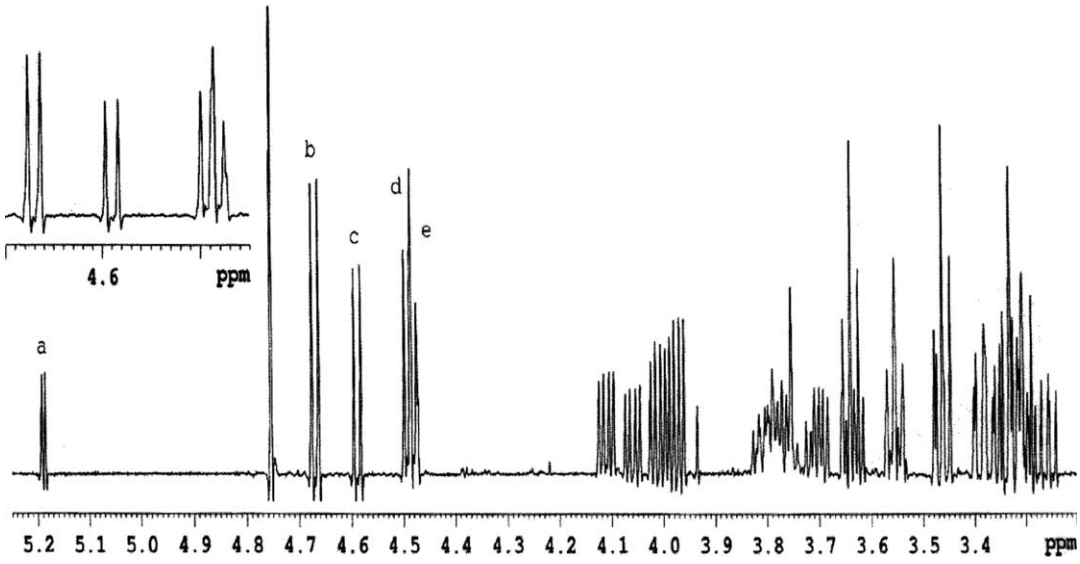

Figure 3. ^{1}H NMR spectrum of the A–B oligosaccharide mixture, isolated by preparative HPAEC-PAD, after exhaustive enzymatic hydrolysis of *N. erinacea* xylan by the *T. lanuginosus* enzyme. The **a**–**e** xylosyl residue labelling of the major compound **B**, arbitrarily by order of decreasing chemical shift of the resonances of the anomeric protons, is indicated.

downfield locations of the resonances compared to those of α- and β-D-xylopyranose,[14] the linkages in the residues can now be identified as C-1 of xylosyl residue **b**, C-1,4 of **e**, C-1,3 of **d** and C-4 of **a/c**.

The sequence of the residues in tetrasaccharide **B** was determined from the through space correlations (dipolar couplings) in a NOESY[15] experiment between the anomeric proton of each residue and the protons attached to the linkage carbons, and from the three-bond connectivities in a gHMBC[16] experiment between the anomeric proton (or carbon) of a residue and the carbon (or proton attached to the carbon) of the residue to which it is glycosidically linked. The inter- and intra-residue NOEs observed for **B** are listed in Table 2 while the three-bond connectivities from the gHMBC experiment are shown in Table 3.

Table 2. NOE data of tetrasaccharide **B** (**b**–**d**–**e**–**a/c**)

Residue	Proton	Correlation to
a →4)-α-D-Xyl*p*	H-1 (5.189)	3.551 (**a**, H-2)
b β-D-Xyl*p*(1→	H-1 (4.670)	3.683 (**d**, H-3); 3.461 (**b**, H-3); 3.305 (**b**, H-5$_{ax}$)
c →4)-β-D-Xyl*p*	H-1 (4.589)	3.552 (**c**, H-3); 3.382 (**c**, H-5$_{ax}$)
d →3)-β-D-Xyl*p*(1→	H-1 (4.492)	3.332 (**d**, H-5$_{ax}$)
e →4)-β-D-Xyl*p*(1→	H-1 (4.480)	3.756 (**a**, H-4)
idem	H-1 (4.482)	3.784 (**c**, H-4)

In the NOESY experiment, NOEs are observed between H-1 of residue **b** and H-3 of residue **d**, and between the H-1s of residue **e** and H-4 of residues **a** and **c**. The NOE from H-1 of residue **e** to H-4 of residue **a**

Table 1. NMR data[a] of tetrasaccharide **B** (**b**–**d**–**e**–**a/c**)

Residue	**a**	**b**	**c**	**d**	**e**
	→4)-α-Xyl	β-Xyl-(1→	→4)-β-Xyl	→3)-β-Xyl-(1→	→4)-β-Xyl-(1→
H-1	5.189	4.670	4.589	4.492	4.482/4.480
C-1	92.86	**104.26**[b]	97.35	**102.50**	**102.51/102.50**
$J_{C\text{-}1,H\text{-}1}$		*164.3*	*162.5*	*164.0*	*163.2*
H-2	3.551	3.330	3.255	3.462	3.304/3.296
C-2	72.23	74.22	74.85	73.31	73.55
H-3	3.758	3.461	3.552	3.638	3.558
C-3	71.80	76.47	74.76	**84.37**	74.50/74.51
H-4	3.756	3.639	3.784	3.703	3.797
C-4	**77.41**	70.07	**77.26**	68.51	**77.26**
H-5$_{eq}$	3.819	3.974	4.060	4.010	4.112
H-5$_{ax}$	3.754	3.305	3.382	3.332	3.380
C-5	59.70	66.02	63.82	65.72	63.83

[a] Chemical shifts in ppm with acetone as standard at δ 2.225 and 31.07 ppm for ^{1}H and ^{13}C, respectively.
[b] Linkage carbons are indicated in bold.

Table 3. Three-bond ^{1}H–^{13}C correlations of tetrasaccharide **B** (**b**–**d**–**e**–**a/c**)

Residue	Proton	Correlation to
a →4)-α-D-Xyl*p*	H-1 (5.189)	71.80 (**a**, C-3); 59.70 (**a**, C-5)
b β-D-Xyl*p*-(1→	H-1 (4.670)	84.37 (**d**, C-3); 76.47 (**b**, C-3); 66.02 (**b**, C-5)
c →4)-β-D-Xyl*p*	H-1 (4.589)	74.76 (**c**, C-3); 63.82 (**c**, C-5)
d →3)-β-D-Xyl*p* (1→	H-1 (4.492)	77.26 (**e**, C-4); 84.37 (**d**, C-3); 65.72 (**d**, C-5)
e →4)β-D-Xyl*p*(1→	H-1 (4.480/ 4.482)	77.26 (**e**, C-4); 74.85 (**e**, C-3); 63.83 (**e**, C-5)

cannot be unambiguously established because the resonances of H-3, H-4 and H-5_{ax} of this residue almost overlap, however, since the linkage carbon for this residue has been established as C-4, the NOE is considered to be to H-4 of **a**. No inter-residue NOE is observed from H-1 of **d**. The NOESY data thus establish the partial sequences **b**-(1 → 3)-**d** and **e**-(1 → 4)-**a/c**.

In the gHMBC experiment, a three-bond correlation is observed between H-1 of **b** and C-3 of **d**. A correlation is also observed between the H-1s of **e** and a carbon with a resonance at 77.26 ppm, which coincides with both C-4 of **e** and **c**. A correlation between H-1 and C-4 of **e** is unlikely, and thus the correlation to the carbon at 77.26 ppm is considered to be a three-bond correlation to C-4 of **c**. This interpretation is consistent with the NOESY data. A three-bond correlation observed between H-1 of unit **d** and a carbon at 77.26 ppm is considered to be from C-4 of **e** as the other carbon resonance at this position was previously assigned to C-4 of **c**. The above data are consistent with the following structures for **B**:

b **d** **e** **a/c**

β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*

Part of the **A**–**B** mixture was further purified by HPAEC, allowing the isolation of approximately 20 nmol of pure trisaccharide **A**. The ^{1}H and ^{13}C resonance signals for **A** can be fully assigned from its ^{1}H spectrum (500 MHz, nanoprobe at 27 °C) and from the gHSQC spectrum of the **A**–**B** mixture; these are listed in Table 4. Comparison of the ^{1}H NMR data of **A** and **B** shows the absence of the signal at δ 4.110 for H-5_{eq} in the spectrum of **A**, indicating the absence of an internal in-chain 4-linked xylopyranose residue. Its structure therefore is

b **d** **a/c**

β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*

This trisaccharide has been isolated previously from a partial acid hydrolysate of the xylan of *N. erinacea*.[7]

2.3. Identification of compound B′, present in the mixture resulting from partial hydrolysis of *N. erinacea* xylan by the *C. adeliae* xylanase

The partial xylan hydrolysis catalysed by the family 10 *C. adeliae* xylanase leads to a series of oligosaccharides (see Fig. 1). By preparative gel filtration, a fraction could be isolated that contained D-xylose, xylobiose, trisaccharide **A**, tetrasaccharide **B** and the compound **B′**; this fraction was devoid of either all-β-(1 → 4)-xylotetraose or higher oligosaccharides. The compound **B′** can be identified by analysis of the reaction products resulting from an enzymatic transfer reaction of this **B**–**B′** containing mixture with methanol as acceptor, in the presence of the same *C. adeliae* xylanase (Fig. 2c and d). This experiment yielded methyl xylobioside and xylobiose at the expense of compound **B′**, next to the

Table 4. NMR data[a] of trisaccharide **A** (**b**–**d**–**a/c**)

Residue	**a**	**b**	**c**	**d**
	→4)-α-Xyl	β-Xyl-(1→	→4)-β-Xyl	→3)-β-Xyl-(1→
H-1	5.190	4.673/4.670	4.589	4.491/4.489
C-1	92.85	**104.28**[b]	97.33	**102.49/102.47**
H-2	3.554	3.331	3.256	3.462
C-2	72.23	74.22		73.31
H-3	3.758	3.463	3.550	3.638
C-3	71.80	76.46		**84.36/84.33**
H-4	3.756	3.639	3.786	
C-4	**77.47**	70.05	**77.30**	68.50
H-5_{eq}	3.819	3.975	4.061	4.013
H-5_{ax}	3.754	3.308	3.380	3.332
C-5	59.78	66.02	63.82	65.70

[a] As for Table 1.
[b] As for Table 1.

β-methyl glycoside of β-D-Xyl*p*-(1→3)-β-D-Xyl*p*(1→4)-D-Xyl*p* resulting from a methanol-transfer reaction with tetrasaccharide **B** (as observed in the analogous experiment in Figure 2a and b). Therefore, it can be concluded that (i) compound **B′** is the centrally β-(1→3)-linked tetraose β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*, and that (ii) the family 10 *C. adeliae* xylanase is capable of hydrolysing a β-(1→3) glycosidic bond flanked by β-(1→4)-linkages.

2.4. Identification of higher mixed oligosaccharides present in the mixture resulting from partial hydrolysis of *N. erinacea* xylan by the *T. lanuginosus* xylanase

The *N. erinacea* xylan was digested as before with the family 11 xylanase from *T. lanuginosus* but for 1 h 30 min after which the products were fractionated on a Bio-Gel P-4 column using water as eluent. The fractions **I**–**V** (Fig. 4, middle insert), which should contain, respectively, tetrasaccharides up to octasaccharides, were further investigated by analytical HPAEC on a CarboPac PA-100 column using PAD (Fig. 4). The NMR-identified D-xylosyl-residue components of the oligosaccharides are given the same index as above, thus **a**: reducing end (1→4)-α-D-Xyl*p*; **b**: nonreducing end β-D-Xyl*p*-(1→3); **c**: reducing end (1→4)-β-D-Xyl*p*; **d**: central (1→3)-β-D-Xyl*p*-(1→4); and **e**: central (1→4)-β-D-Xyl*p*-(1→4). The two extra residues are indexed as **f**: nonreducing end β-D-Xyl*p*-(1→4) and **g**: central (1→4)-β-D-Xyl*p*-(1→3).

2.4.1. Fraction I. NMR examination (DQF-COSY, HOHAHA, gHSQC, NOESY, gHMBC) of this fraction (Fig. 4-I) indicates that it consists predominantly of a 1:2 mixture of xylotetraose $\mathbf{X_4}$ and oligosaccharide **B**. This was confirmed with HPAEC using authentic samples as internal standards. In addition, samples of $\mathbf{X_4}$ and **B** of fraction **I** were separated by semi-preparative HPAEC, and their ^{1}H NMR spectra (500 MHz,

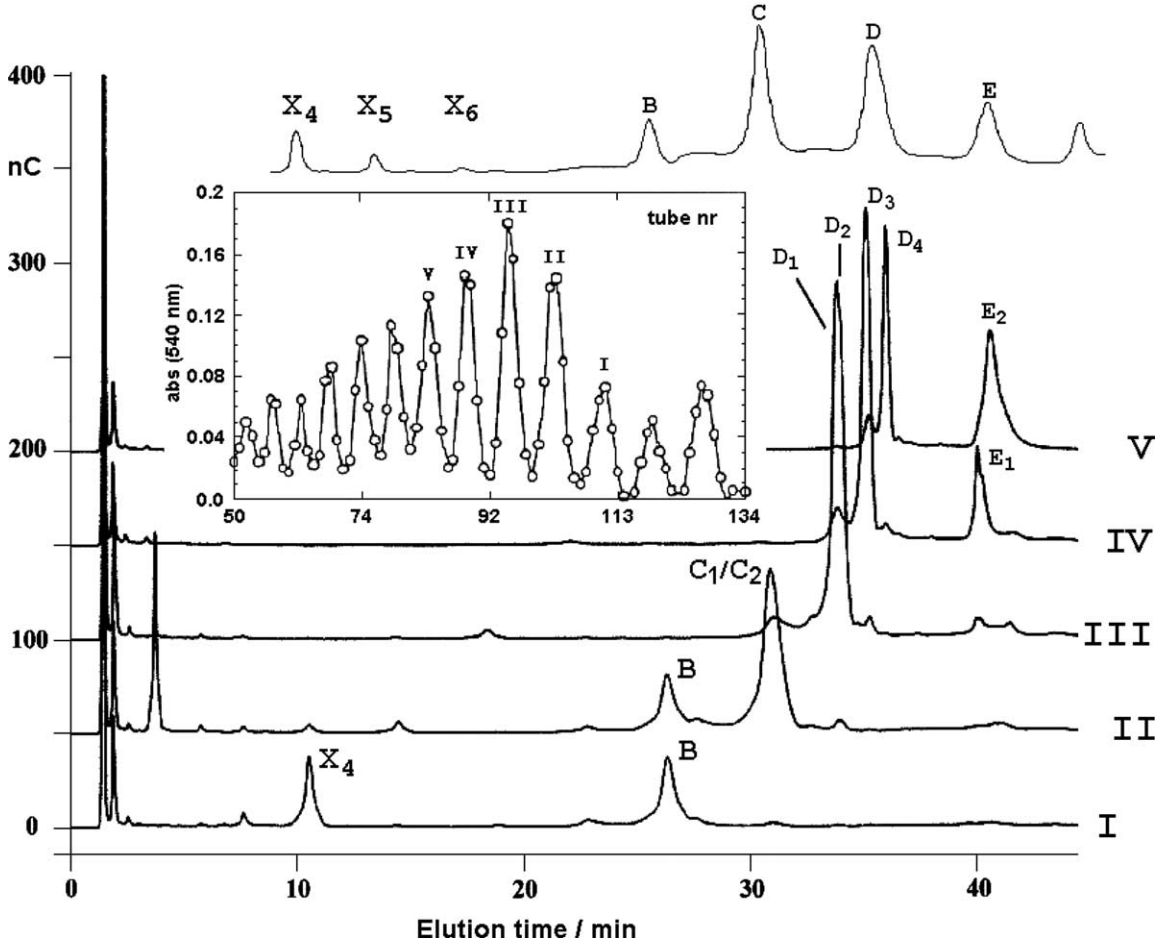

Figure 4. Analytical HPAEC profiles of the oligosaccharide fractions **I**–**V**.The identified compounds are indicated. Top insert: upper graph of Figure 1 for comparison. Middle insert: Bio-Gel P-4 fractionation of the partial (1 h 30) enzymatic hydrolysate of *N. erinacea* xylan by the *T. lanuginosus* xylanase, indicating the analysed oligosaccharide fractions **I**–**V**; total sugar content was determined.[20]

nanoprobe) are indeed identical to those of xylotetraose and oligosaccharide **B**, respectively.

2.4.2. Fraction II. This fraction shows a mixture of the tetrasaccharide **B** and an eluate corresponding to pentasaccharides (Fig. 4-II), and a sample of the latter could be isolated by a separate semi-preparative HPAEC. MALDI-TOF MS of this subfraction shows a major peak at m/z 701.2, which corresponds to a pentasaccharide $[M+Na]^+$ ion, and a very low intensity $[M+Na]^+$ signal of tetrasaccharide **B**. The ^1H NMR spectrum (500 MHz, nanoprobe) of the pentasaccharide subfraction (Fig. 5-II) shows H-1 signals for two nonreducing end β-xylopyranose residues at δ 4.669 (**b**) and 4.464 (**f**) indicating the presence of two pentasaccharides $\mathbf{C_1}$ and $\mathbf{C_2}$ in a 55:45 ratio. The 2D NMR data were performed on fraction **II** (DQF-COSY, HOHAHA, gHSQC, ROESY, gHMBC) and the data for the pentasaccharides are collected in Table 5. The gHMBC three bond connectivity data (not shown) indicate that a residue **b** is linked to **d** while a residue **f** is linked to **g**, which in turn is linked to **d**. Pentasaccharides $\mathbf{C_1}$ and $\mathbf{C_2}$ therefore are:

b **d** **e** **e** **a/c**

β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*

f **g** **d** **e** **a/c**

β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*

2.4.3. Fraction III. Analytical HPAEC indicates that this fraction contains two components (Fig. 4-III), and a sample could be further separated into subfractions $\mathbf{D_1}$ and $\mathbf{D_2}$, each in nanomole quantities. MALDI-TOF MS of both subfractions shows a major peak at m/z 833.3, which corresponds to the $[M+Na]^+$ ion of a hexasaccharide, and a very minor peak at m/z 701.3 of a pentasaccharide. The ^1H NMR spectra (500 MHz, nanoprobe) of $\mathbf{D_1}$ and $\mathbf{D_2}$ are almost identical, therefore all 2D experiments were carried out on the unfractionated **III**. The ^1H NMR spectrum (Fig. 5-III) indicates that unit **f** is the major nonreducing terminal residue and that xylose units **g**, **d**, **e** and **a/c** are present. The 2D NMR data (DQF-COSY, HOHAHA, gHSQC, ROESY, gHMBC) for fraction **III** are reported in Table 6. From the gHMBC and ROESY experiments, the xylosyl residue connectivities **f** to **e**, **f** to **g**, **g** to **d**, **e** to **g**, **d** to **e** and **e** to **a/c** could be established. There is no indication

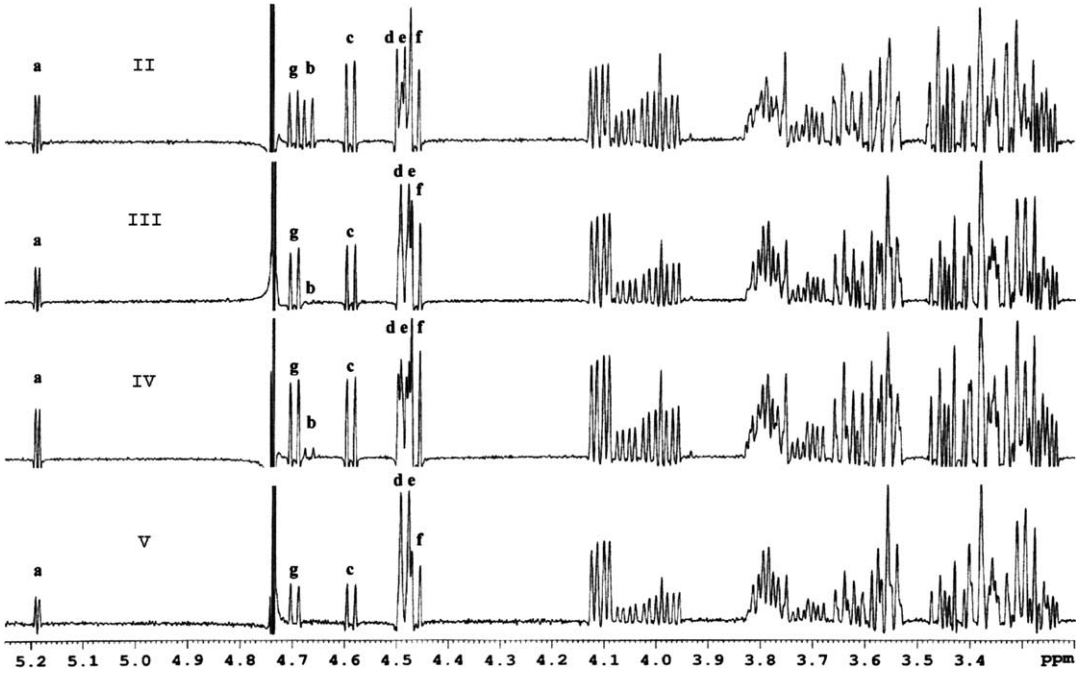

Figure 5. ^1H NMR spectra of the subfraction-containing pentasaccharides $\mathbf{C_1}$ and $\mathbf{C_2}$ in fraction **II**, and of the oligosaccharide fractions **III**–**V**.

Table 5. NMR data[a] of the pentasaccharides $\mathbf{C_1}$ and $\mathbf{C_2}$ (**b–d–e–e–a/c** and **f–g–d–e–a/c**) in fraction **II**

Residue	**a**	**g**	**b**	**c**	**d**	**e′**	**e**	**f**
	→4)-α-Xyl	→4)-β-Xyl-(1→	β-Xyl-(1→	→4)-β-Xyl	→3)-β-Xyl-(1→	→4)-β-Xyl-(1→	→4)-β-Xyl-(1→	β-Xyl-(1→
H-1	5.188	4.698	4.669	4.589	4.491	4.486	4.483/4.480	4.464
C-1	92.84	**104.10**[b]	**104.29**	97.32	**102.55**	**102.55**	**102.55**	**102.69**
H-2	3.550	3.366	3.329	3.254	3.460	3.297	3.299	3.262
C-2	72.24	74.14	74.21	74.85	73.35	73.58	73.58	73.63
H-3	3.756	3.589	3.462	3.552	3.642	3.559	3.560	3.432
C-3	71.78	74.51	76.51	74.70	**84.31**	74.51	74.51	76.47
H-4	3.754	3.797	3.637	3.783	3.706	3.789	3.792	3.632
C-4	**77.37**	**77.30**	70.02	**77.38**	68.47	**77.30**	**77.30**	70.02
H-5_{eq}	3.815	4.109	3.973	4.060	4.009	4.110	4.110	3.977
H-5_{ax}	3.750	3.374	3.307	3.382	3.330	3.380	3.378	3.311
C-5	59.69	63.78	66.05	63.79	65.72	63.78	63.78	66.05

[a] As for Table 1.
[b] As for Table 1.

of a **d** to **a/c** connectivity, ruling out the presence of the hexasaccharide **f–e–e–g–d–a/c**. Therefore, fraction **III** contains the following two major hexasaccharides $\mathbf{D_1}$ and $\mathbf{D_2}$:

f **e** **g** **d** **e** **a/c**

β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*

f **g** **d** **e** **e** **a/c**

β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*

The NMR data also show the presence of a minor amount of residue **b**, a nonreducing terminal D-xylopyranoside that is β-(1→3)-linked (thus to a residue **d**). These data together with the minor $[M+Na]^+$ ion at m/z 701.3 are consistent with the presence of a small amount of the earlier observed pentasaccharide $\mathbf{C_1}$.

2.4.4. Fraction IV. The ^{1}H NMR spectrum of this fraction (Figs. 4-IV and 5-IV) indicates the presence of residues **a**, **c–g** and also some **b**. Semi-preparative HPAEC allowed the isolation of subfractions $\mathbf{D_3}$ and $\mathbf{E_1}$, which were examined by ^{1}H NMR spectroscopy (500 MHz, nanoprobe) and MALDI-TOF MS. Subfraction $\mathbf{D_3}$ gave a major ion at m/z 965.4, which corresponds to the $[M+Na]^+$ ion of a heptasaccharide, while subfraction $\mathbf{E_1}$ gave prominent $[M+Na]^+$ and $[M+K]^+$ ions at m/z 965.2 and 981.2, respectively. Subfraction $\mathbf{D_3}$ does not contain a residue **b**, and the chemical shifts for the ^{1}H resonances of the residues were established from a DQF-COSY experiment and are reported in Table 7. These data indicate that subfraction $\mathbf{D_3}$ contains one or more heptasaccharide(s) with the

Table 6. NMR data[a] of the hexasaccharides $\mathbf{D_1}$ and $\mathbf{D_2}$ (**f–e–g–d–e–a/c** and **f–g–d–e–e–a/c**) in fraction **III**

Residue	**a**	**g**	**b**	**c**	**d**	**e′**	**e**	**f**
	→4)-α-Xyl	→4)-β-Xyl-(1→	β-Xyl-(1→	→4)-β-Xyl	→3)-β-Xyl-(1→	→4)-β-Xyl-(1→	→4)-β-Xyl-(1→	β-Xyl-(1→
H-1	5.188	4.698	4.670	4.589	4.489	4.486	4.483/4.480	4.463
C-1	92.83	**104.21**[b]	**104.28**	**97.30**	**102.55**	**102.55**	**102.55**	**102.73**
H-2	3.536	3.365	3.329	3.253	3.460	3.297	3.304	3.262
C-2	72.23	74.13		74.88		73.57	73.57	73.64
H-3	3.756	3.588	3.459	3.552	3.639	3.553	3.554	3.431
C-3	71.78	74.54	76.50		**84.27**	74.53	74.53	76.50
H-4	3.753	3.802	3.638	3.783	3.706			3.627
C-4	**77.38**			**77.41**	68.47	**77.27**	**77.27**	70.04
H-5_{eq}	3.820		3.976	4.058	4.010	4.110	4.110	3.976
H-5_{ax}	3.753	3.377	3.311	3.379	3.332	3.380	3.378	3.311
C-5	59.65	63.82	66.09	63.82	65.78	63.82	63.82	66.09

[a] As for Table 1.
[b] As for Table 1.

Table 7. 500 MHz ^{1}H NMR data[a] of the heptasaccharide(s) **f–e$_m$–g–d–e$_n$–a/c** in subfraction **D$_3$**

Residue	**a**	**g**	**c**	**d**	**e**	**f**
	→4)-α-Xyl	→4)-β-Xyl-(1→	→4)-β-Xyl	→3)-β-Xyl-(1→	→4)-β-Xyl-(1→	β-Xyl-(1→
H-1	5.188	4.699	4.590	4.490	4.484	4.464
H-2	3.551	3.367	3.255	3.461	3.299	3.261
H-3	3.763	3.588	3.552	3.639	3.561	3.432
H-4		3.799	3.794	3.709	3.797	3.632
H-5$_{eq}$			4.064	4.013	4.113	3.978
H-5$_{ax}$			3.381	3.332	3.382	3.311

[a]As for Table 1, nanoprobe.

following structures, each having only one β-(1→3) bond:

f **e$_m$** **g** **d** **e$_n$** **a/c**

β-D-Xyl*p*-[(1→4)-β-D-Xyl*p*]$_m$-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-[(1→4)-β-D-Xyl*p*]$_n$-(1→4)-α/β-D-Xyl*p*

b **d** **g** **d** **e** **e** **a/c**

β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*

in which subscript **m** can have a value of 0, 1 or 2 when subscript **n** has a value of 3, 2 or 1, respectively.

The major heptasaccharide in subfraction **E$_1$** was further purified by semi-preparative HPAEC, and its ^{1}H NMR spectrum recorded at 800 MHz in a 3-mm probe is shown in Figure 6. The ^{1}H chemical shift data for the component residues that were obtained from a DQF-COSY experiment (800 MHz, 3-mm probe) are collected in Table 8. The data confirm the presence of D-xylosyl units **a**, **b**, **c**, **d** (twice), **e** (twice) and **g**. These units can only be arranged into three different heptasaccharides that contain two β-(1→3) linkages, each starting with this bond connection: (i) **b–d–e–e–g–d–a/c** with the second β-(1→3) bond in penultimate position; (ii) **b–d–e–g–d–e–a/c** with the second β-(1→3) bond in the fourth position, or (iii) **b–d–g–d–e–e–a/c** with the second β-(1→3) bond in the third position. Close examination of the H-1/H-2 cross-peak of a residue **e** in the DQF-COSY spectrum reveals anomeric (mutarotational) splitting, indicating that an **e**: (1→4)-β-D-Xyl*p*-(1→4) residue is adjacent to the reducing end **a/c**, which rules out structure (i). In order to distinguish between structures (ii) and (iii), the **E$_1$** heptasaccharide was partially hydrolysed (20 nmol, 10 min, 0.1 M TFA, 100 °C) and the hydrolysate was examined by HPAEC and compared with reference compounds. Peaks with retention times identical to those of xylotetraose and compounds **A**, **B**, **C$_1$** and **D$_1$/D$_2$** were observed. This is consistent with arrangement (iii) only. Heptasaccharide **E$_1$** therefore has the following structure:

f **e$_m$** **g** **d** **e$_n$** **a/c**

β-D-Xyl*p*-[(1→4)-β-D-Xyl*p*]$_m$-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-[(1→4)-β-D-Xyl*p*]$_n$-(1→4)-α/β-D-Xyl*p*

in which subscript **m** can have a value of 0, 1, 2 or 3 when subscript **n** has a value of 4, 3, 2 or 1, respectively.

2.4.5. Fraction V. This fraction (Fig. 4-V) was further separated by semi-preparative HPAEC into subfractions **D$_4$** and **E$_2$**, which were examined by MALDI-TOF MS. **D$_4$** gave major ions at *m/z* 1097.4 and 1113.4, which correspond to the $[M+Na]^+$ and $[M+K]^+$ ions of an octasaccharide, while subfraction **E$_2$** gave major $[M+Na]^+$ and $[M+K]^+$ ions at *m/z* 1097.2 and 1113.2, respectively, and minor but significant $[M+Na]^+$ and $[M+K]^+$ ions for a heptasaccharide at *m/z* 965.2 and 981.2. The latter subfraction was not further investigated. The ^{1}H NMR spectrum (500 MHz, nanoprobe) of **D$_4$** is shown in Figure 7 while the chemical shifts for residues **a** and **c–g** obtained from a DQF-COSY experiment are listed in Table 9. The data indicate that subfraction **D$_4$** consists of one or more octasaccharide(s) with only one β-(1→3) bond, of the following general structure:

f **e$_m$** **g** **d** **e$_n$** **a/c**

β-D-Xyl*p*-[(1→4)-β-D-Xyl*p*]$_m$-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-[(1→4)-β-D-Xyl*p*]$_n$-(1→4)-α/β-D-Xyl*p*

3. Discussion

In the course of the enzymatic hydrolysis of *N. erinacea* xylan, considerable amounts of different oligosaccharides are formed, most of them containing only one β-(1→3) glycosidic bond. Moreover, substantial amounts of several all-β-(1→4)-xylooligosaccharides

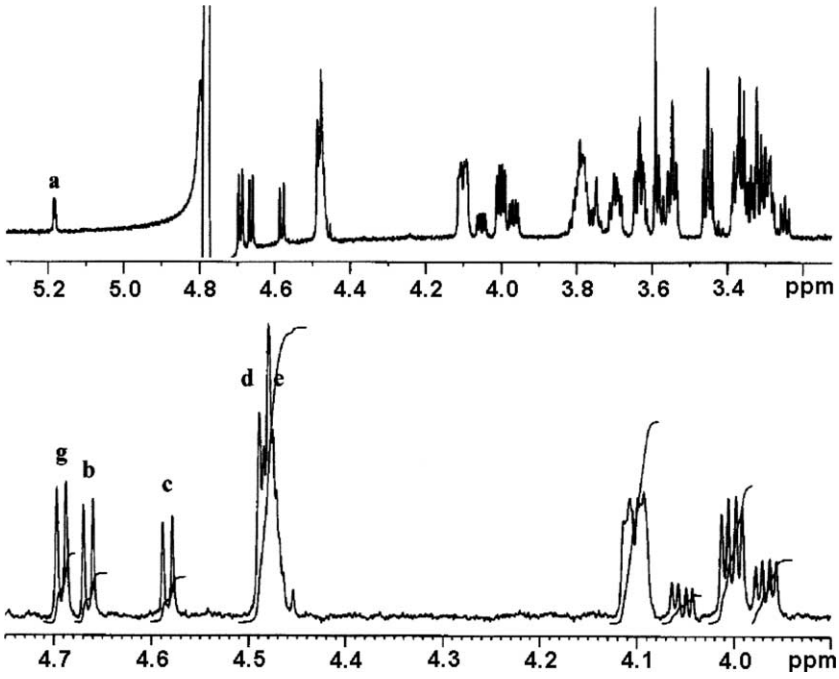

Figure 6. ^{1}H NMR spectrum of the bis-β-(1 → 3)-linked heptasaccharide $\mathbf{E_1}$.

Table 8. 800 MHz ^{1}H NMR data[a] of heptasaccharide $\mathbf{E_1}$ (**b–d–g–d–e–e–a/c**)

Residue	a	g	b	c	d	e
	→4)-α-Xyl	→4)-β-Xyl-(1→	β-Xyl-(1→	→4)-β-Xyl	3)-β-Xyl-(1→	→4)-β-Xyl-(1→
H-1	5.188	4.699	4.672	4.592	4.491	4.484
H-2	3.551	3.365	3.327	3.254	3.458	3.297
H-3	3.760	3.588	3.461	3.553	3.640	3.555
H-4		3.801	3.640	3.765	3.703	3.791
H-5_{eq}		4.115	3.975	4.065	4.005	4.115
H-5_{ax}		3.375	3.305	3.385	3.325	3.375

[a]As for Table 1, 3-mm probe.

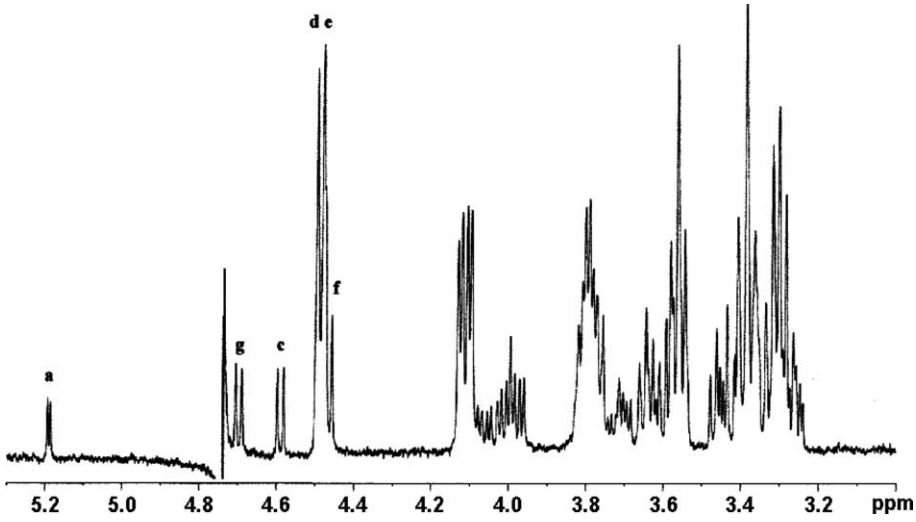

Figure 7. ^{1}H NMR spectrum of the octasaccharide(s) in subfraction $\mathbf{D_4}$.

Table 9. 500 MHz ^{1}H NMR data[a] of the octasaccharide(s) **f–e_m–g–d–e_n–a/c** in subfraction $\mathbf{D_4}$

Residue	a	g	c	d	e	f
	→4)-α-Xyl	→4)-β-Xyl(1→	→4)-β-Xyl	→3)-β-Xyl-(1→	→4)-β-Xyl-(1→	β-Xyl-(1→
H-1	5.188	4.698	4.589	4.489	4.484	4.464
H-2	3.551	3.367	3.255	3.461	3.299	3.262
H-3	3.763	3.589	3.555	3.643	3.562	3.433
H-4		3.799	3.784	3.709	3.795	3.633
H-5_{eq}		4.113	4.063	4.010	4.113	3.978
H-5_{ax}		3.382	3.383	3.334	3.382	3.312

[a]As for Table 1, nanoprobe.

are produced, the longest detectable at any stage being xylohexaose. No oligosaccharides were detected that contain two (or more) successive β-(1→3) linkages. However, a substantial amount of heptasaccharide $\mathbf{E_1}$ was isolated, which contains two β-(1→3) linkages interspaced by one β-(1→4)-linked D-xylopyranoside unit. The conditions of the partial enzymatic hydrolysis experiments, that is, only 2 h incubation at substrate concentrations near the K_M value for xylooligosaccharides,[9,10] make it unlikely that these oligosaccharides have originated from self-transfer reactions. The combined observations indicate that the xylan structure consists of an irregular distribution of solitary β-(1→3) linkages within β-(1→4)-linked sequences of variable length; and this with a β-(1→3) to β-(1→4) linkage ratio, as previously estimated by chemical methods,[8] of 2:9 (methylation analysis) or 1:4 (periodate oxidation).

The enzymatic hydrolysis modes in a hypothetical structure of the linear *N. erinacea* xylan are represented in Figure 8. Arrows indicate the possible scission sites by the enzymes from family 10 and 11, giving rise to the identified oligosaccharides. This representation readily explains our results with partial hydrolysis experiments. Indeed, if a regular distribution of β-(1→3)/β-(1→4) linkages would be present, the sequence should repeat itself already after the fifth successive linkage, in which case partial hydrolysis with the family 11 enzyme would almost exclusively yield D-xylose and xylobiose (and xylotriose if the repeat would occur after the sixth linkage), but would never yield any higher all-β-(1→4)-xylooligosaccharides. It would also solely yield the mixed-linkage compounds **A** and **B**, or mixed-linkage oligomers of at least DP 8 in which each β-(1→3) linkage must be interspaced by four consecutive β-(1→4) linkages, that is coming from twice (or more) the repeating unit. This is not supported by experimental evidence.

Under the same enzymatic hydrolysis conditions, the *P. palmata* seaweed xylan (rhodymenan) shows essentially the same hydrolysis pattern, also indicating an analogous irregular β-(1→3) linkage distribution within β-(1→4)-linked sequences of variable length (and with a previously estimated 1:5 ratio of β-(1→3) to β-(1→4) linkages[7]).

Some conclusions can also be drawn regarding the specificities of both *endo*-splicing enzymes. Neither is capable of catalysing the hydrolysis of the β-(1→4) linkage that immediately follows a β-(1→3)-glycosidic bond. In terms of ligand/substrate recognition, this implies that for both enzymes the presence of a rhodymenabiose moiety [-β-D-xylopyranosyl-β-(1→3)-D-xylopyranosyl-] within their -2/-1 subsites is not allowed. The *T. lanuginosus* family 11 enzyme also cannot hydrolyse β-(1→3)-glycosidic bonds, and therefore also

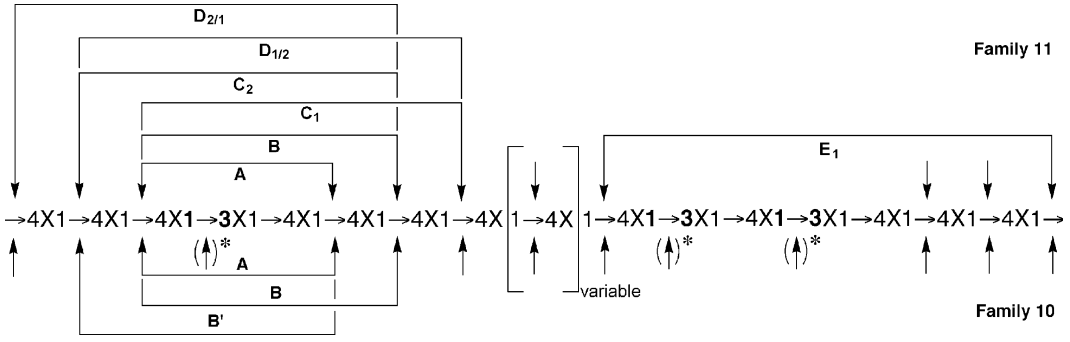

Figure 8. *N. erinacea* xylan and its degradation by family 10 and 11 enzymes. The linear chain of the seaweed xylan is represented as an irregular repetition of β-(1→3)- and β-(1→4)-linked D-xylopyranosyl units. The enzymatic scission sites and the isolated hydrolysis fragments are indicated. Upper splicing pattern: from partial and exhaustive enzymatic hydrolysis with the *T. lanuginosus* xylanase; lower pattern: from exhaustive hydrolysis with the *C. adeliae* enzyme.

does not accept this disaccharyl moiety within its −1/+1 subsites. The *C. adeliae* family 10 enzyme however can hydrolyse β-(1→3) linkages that are flanked on both sides by β-(1→4) bonds (indicated with asterisk) as evidenced with the mixed linkage tetraose **B′**. This corroborates the results of Biely et al.[3] and proves the more relaxed substrate specificity of family 10 xylanases.[17]

It can be noted that specific β-(1→3)-xylanases belonging to other glycoside hydrolase families have been isolated, for example, Ref. 18.

In conclusion, our results from enzymatic hydrolysis experiments and identification of oligosaccharides formed with the *N. erinacea* and *R. palmata* seaweed xylans point to an irregular distribution of solitary β-(1→3)- to contiguous β-(1→4)-glycosidic bonds in these homopolymers. It should be mentioned that linear all-β-(1→4)-xylans show a twisted ribbon conformation;[19] a variable presence of β-(1→3) linkages may severely disrupt this secondary structure.

4. Experimental section

4.1. Materials

The xylan from *N. erinacea* was isolated and purified as described.[8] Rhodymenan[7] was a gift from Dr. Peter Biely (Slovak National Academy, Bratislava, Slovakia). The xylanases from *T. lanuginosus*[9] and *C. adeliae*[10] were obtained as indicated. D-Xylose and β-(1→4)-xylo-oligosaccharides (DP 2–6) were commercial products (Megazyme, Ireland). Samples of the β-methyl glycosides of xylobiose and β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl*p* were kindly donated by Dr. P. Kovac (NIH, Bethesda, USA). Total sugar content (D-xylose) was measured by the phenol–sulfuric acid method.[20]

4.2. Enzymatic digests

To *N. erinacea* xylan (1 mL of a 1% w/v soln in 50 mM phosphate buffer pH 6.4) pre-incubated at 37 °C, was added a solution of *T. lanuginosus* xylanase (50 μL, 0.1 mg/mL). The soln was kept at the same temperature for partial (2 h) or exhaustive (7 days) hydrolysis. Sodium azide (0.01%) was added to the reaction mixtures to prevent microbial growth. Reactions were stopped by boiling (5 min). Digests with *C. adeliae* xylanase were similarly performed but at 25 °C.

4.3. Analytical and preparative separation of the hydrolysis products

Hydrolysis products were analysed with an HPAEC-PAD system (Dionex, Sunnyvale, USA) using a CarboPac PA-100 column (4×250 mm), a gradient pump (GP 40) and electrochemical detection (ED 40). The oligosaccharides were eluted with a linear gradient of sodium acetate (from 20 to 200 mM) in a sodium hydroxide solution (60 mM) during 45 min at a flow rate of 1 mL/min. Peak identification was done by comparing retention times with those of appropriate standards (D-xylose, xylooligosaccharides and their methylglycosides).

Unknown compounds were isolated by the same technique, using a preparative CarboPac PA-100 column (22×250 mm). Between 250 and 300 μL of the sample (10 mg/mL) was injected onto the column. Elution was performed with a nonlinear gradient of sodium acetate (from 80 to 200 mM) in sodium hydroxide (60 mM), at a flow rate of 9 mL/min for 38 min. Sodium hydroxide was removed from selected fractions with a carbohydrate membrane desalter (Dionex CMD system) using sulfuric acid (50 mM); alternatively, neutralisation with AcOH and desalting with an AG 50W-X8 cation exchanger (Bio-Rad Laboratories, Hercules, CA, USA) was used. The desalted samples were concentrated by freeze-drying and their purity was checked by analytical HPAEC-PAD as described above.

Alternatively, preparative gelfiltration was used to isolate oligosaccharide fractions. The sample (1 mL, 10 mg) resulting from partial hydrolysis was applied to two water-jacketed columns (1 m×2.5 cm) in series, packed with BioGel P-4 (<400 mesh). The elution (50 °C) was performed with deionised water (Millipore, USA) at a flow rate of 0.3 mL/min, and 4.5 mL fractions were collected. Total sugar content was measured in each tube and selected fractions containing oligomers of given DP were analysed by HPAEC-PAD and concentrated by freeze-drying for further purification and analysis. The selected dried fractions were fractionated by semi-preparative HPAEC-PAD (CarboPac PA-100, 4×250 mm, 60 mM sodium hydroxide, 20–40 mM sodium acetate in 27 min, 40–100 mM sodium acetate in 10 min, 100 mM sodium acetate for 8 min, 1 mL/min). Fractions were manually collected and desalted by a cation exchanger as described above. One HPAEC fraction containing a heptasaccharide was further purified by the same technique, but with 48 mM sodium hydroxide and a 70–90 mM gradient of sodium acetate in 30 min.

4.4. Formation of methyl glycosides by enzymatic transfer reactions

MeOH (2 M final concentration) and *C. adeliae* xylanase (50 μL, 0.16 mg/mL) were added to 1 mL of a soln containing approximately 1 mg xylooligosaccharides resulting from partial or exhaustive hydrolysis of *N. erinacea* xylan by the xylanases as described above. The mixtures were incubated at 25 °C and aliquots (20 μL) were taken at several time intervals and analysed by HPAEC-PAD. Retention times of methanolysis

products were compared with those of the β-methyl glycosides of xylobiose and β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-α/β-D-Xyl*p*.

4.5. Partial TFA hydrolysis

The heptasaccharide (approximately 2 nmol) was treated with TFA (60 μL of a 0.1 M soln) at 100 °C for 10 min. The resulting solution was transferred to an eppendorf tube, cooled and evaporated on a speed-vac. The resulting oligosaccharide mixture was analysed by HPAEC-PAD.

4.6. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-600 spectrometer in D_2O at 27 °C with acetone as standard (δ 2.225 for ^{1}H and 31.07 ppm for ^{13}C). ^{1}H–^{1}H correlated experiments (DQF-COSY,[11] HOHAHA,[12] NOESY[15]) were performed using 2K×512 data points. Data matrices were zero filled to 1024 points in the F1 dimension, and a shifted squared sine window function was applied in both dimensions prior to Fourier transformation. The mixing times in the HOHAHA and NOESY experiments were 85 and 800 ms, respectively. The ^{1}H–^{13}C correlated experiments were performed using pulsed field gradients, with 2K×512 data points (gHSQC)[13] and 2K×256 data points (gHMBC).[16]

Nanoprobe NMR analyses were performed at 500 MHz on a Varian Unity Inova NMR spectrometer (Varian, Palo Alto, CA, USA) equipped with a ^{1}H-observe nanoprobe. Samples were dissolved in D_2O (40 μL) and transferred to the nanotube. During all experiments the samples were spun at approximately 2000 Hz and the temperature was set to 27 °C. For each sample, 96–256 scans were recorded and the residual HDO signal was pre-saturated (1.5 s) during the repetition delay (in total 12 s). A sweep width of 8000 Hz and an acquisition time of 2.0 s was used for all one-dimensional ^{1}H NMR experiments. Two-dimensional DQF-COSY experiments were performed on samples **D$_4$** and **E$_2$** using sweep widths of 4000 and 8000 Hz, respectively (32 scans, 4000×900 data points).

NMR analysis at 800 MHz was performed on a Varian Unity Inova NMR spectrometer using a 3-mm ^{1}H{^{13}C, ^{15}N} triple resonance pulsed field gradient probe. Sample **E$_1$** was dissolved in D_2O (120 μL) and transferred, together with a small amount of acetone, to a 3 mm NMR tube. A one-dimensional ^{1}H NMR spectrum was recorded with a sweep width of 6000 Hz and an acquisition time of 2.6 s. The temperature was 23 °C and the spectrum was recorded with pre-saturation (1.5 s) of the residual HDO signal during the repetition delay (in total 4.5 s). Two-dimensional ^{1}H NMR experiments (DQF-COSY and TOCSY) were performed with sweep widths of 6000 Hz in the F1 and F2 dimensions. The residual HDO signal was pre-saturated during the repetition delay. The DQF-COSY experiment was recorded with 32 scans for each increment (4000×800 data points). The TOCSY experiment was recorded with eight scans for each increment (2000×512 data points).

4.7. Mass spectrometry

Mass spectrometry analyses were performed on a Bruker Reflex III MALDI-TOF MS (Bruker-Daltonik, Germany), operating in the delayed extraction mode. An accelerating voltage of 20 kV and a reflectron voltage of 22.8 kV was used in the measurements. 2,5-Dihydroxybenzoic acid was used as matrix (20 mg/mL; 3:2 water/MeCN) and approximately 10–100 pmol of each oligosaccharide was deposited as a mixture together with the matrix on a stainless steel target, and subsequently dried under reduced pressure. During the experiments, the laser power was adjusted to a level just above the threshold for formation of observable ions. The results from 20 to 100 laser shots were summed for each sample. A mixture of maltooligosaccharides (DP 3–7) was used as external calibrant.

The ESMS analysis was carried out on a triple quadrupole mass spectrometer (Bio-Q upgraded to the Quattro II version, Micromass Ltd., Manchester, UK) equipped with a pneumatically assisted electrospray source. Optimisation and calibration have been described elsewhere.[21]

Acknowledgements

We are indebted to Prof. Dr. C. Gerday (University of Liège, Belgium) and Prof. Dr. M. K. Bhat (I.F.R. Norwich, UK) for generous gifts of enzymes, and to Prof. Dr. P. Kovac (N.I.H., Bethesda, USA) and Prof. Dr. P. Biely (Slovak Academy of Sciences, Bratislava, Slovak Republic) for gifts of carbohydrate samples. The ES-MS spectrum was recorded at the Laboratory for Protein Chemistry of Prof. Dr. J. Van Beeumen (Ghent University, Belgium). The 800 MHz spectra were obtained using the Varian Unity Inova spectrometer of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules. P.N. wishes to thank the Burundese government for financial support.

References

1. Henrissat, B. *Biochem. J.* **1991**, *280*, 309–316.
2. Gilkes, N. R.; Henrissat, B.; Kilburn, D. G.; Miller, R. R.; Warren, R. A. J. *Microbiol. Rev.* **1991**, *55*, 303–315.
3. Biely, P.; Vrsanská, M.; Tenkanen, M.; Kluepfel, D. *J. Biotechnol.* **1997**, *57*, 151–166.
4. Grépinet, O.; Chebrou, M.-C.; Béguin, P. *J. Bacteriol.* **1988**, *170*, 4576–4581.

5. Christakopoulos, P.; Nerinckx, W.; Kekos, D.; Macris, B. J.; Claeyssens, M. *J. Biotechnol.* **1996**, *51*, 181–189.
6. Rye, C. S.; Withers, S. G. *J. Curr. Opin. Chem. Biol.* **2000**, *4*, 573–580.
7. Howard, B. H. *Biochem. J.* **1957**, *67*, 643–651.
8. Nunn, J. R.; Parolis, H.; Russell, I. *Carbohydr. Res.* **1973**, *26*, 169–180.
9. Bennett, N. A.; Ryan, J.; Biely, P.; Vrsanská, M.; Kremnicky, L.; Macris, B. J.; Kekos, D.; Christakopoulos, P.; Katapodis, P.; Claeyssens, M.; Nerinckx, M.; Ntarima, P.; Bhat, M. K. *Carbohydr. Res.* **1998**, *306*, 445–455.
10. Petrescu, I.; Lamotte-Brasseur, J.; Chessa, J. P.; Ntarima, P.; Claeyssens, M.; Devreese, B.; Marino, G.; Gerday, C. *Extremophiles* **2000**, *4*, 137–144.
11. Piantini, U.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800–6801.
12. Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355–360.
13. Schleucher, J.; Schwendinger, M.; Sattler, M.; Schmidt, P.; Schedletzky, O.; Glaser, S. J.; Sørensen, O. W.; Griesinger, C. *J. Biomol. NMR* **1994**, *4*, 301–306.
14. Bock, K.; Thøgersen, H. *Annu. Rep. NMR Spectr.* **1982**, *13*, 1–57.
15. Baumann, R.; Wider, G.; Ernst, R. R.; Wüthrich, K. *J. Magn. Reson.* **1981**, *44*, 402–406.
16. Wilker, W.; Leibfritz, D.; Kerssebaum, R.; Bermel, W. *Magn. Res. Chem.* **1993**, *31*, 287–292.
17. Andrews, S. R.; Charnock, S. J.; Lakey, J. H.; Davies, G. J.; Claeyssens, M.; Nerinckx, W.; Underwood, M.; Sinnott, M. L.; Warren, R. A. J.; Gilbert, H. J. *J. Biol. Chem.* **2000**, *275*, 23027–23033.
18. Araki, T.; Inoue, N.; Morishita, T. *J. Gen. Appl. Microbiol.* **1998**, *44*, 269–274.
19. Rees, D. A. In *Polysaccharide Shapes. Outline Studies in Biology*; Ashworth, J. M., Ed.; Chapman and Hall, John Wiley & Sons: London, New York, 1977.
20. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Biochem.* **1956**, *28*, 350–356.
21. Klarskov, K.; Roecklin, D.; Bouchon, B.; Sabatié, J.; Van Dorsselaer, A.; Bischoff, R. *Anal. Biochem.* **1994**, *216*, 127–134.