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# Mode of action of (1 → 4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan

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## ABSTRACT

Arabinoxylan-derived oligosaccharides were treated with (1 → 4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and two types of α-L-arabinofuranosidase, A and B. Analysis of reaction products by high performance anion-exchange chromatography indicated the removal of arabinofuranosyl groups from singly substituted xylopyranosyl residues. In addition, differences in the specificity of these enzymes towards the various differently substituted oligosaccharides were observed. $^1$H NMR spectroscopy and methylation analysis of alkali-extractable wheat-flour arabinoxylan treated with AXH confirmed the specificity of AXH towards (1 → 3)-linked arabinofuranosyl groups on singly substituted xylopyranosyl residues. With these techniques, α-L-arabinofuranosidase B was found to cause minor changes in (1 → 2)- and (1 → 3)-linked arabinofuranosyl groups on doubly substituted xylopyranosyl residues.

## INTRODUCTION

Some endo-(1 → 4)-β-D-xylanases (endo-xylanases; EC 3.2.1.8) and α-L-arabinofuranosidases (arabinofuranosidases; EC 3.2.1.55) have been described which were able to hydrolyse the α-(1 → 3) linkages between arabinofuranosyl and xylopyranosyl residues in arabinoxylans[1,2]. However, no detailed studies have been carried out on the specificity towards the various arabinofuranosyl groups in arabinoxylan and arabinoxylan-derived oligosaccharides.

In earlier papers, we reported on the purification and characterisation of a (1 → 4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) from *Aspergillus awamori*[3] as well as α-L-arabinofuranosidases A and B from *Aspergillus niger*[4]

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(Arafur A and Arafur B, respectively). AXH was found to release α-(1 → 3)-linked arabinofuranosyl groups from arabinoxylans only and not from other arabinose-containing substrates such as sugar-beet arabinan, arabinan-rich apple pectin fragments (MHR)[5], arabinogalactans, and stractan[3]. In all these cases, arabinofuranosyl groups were α-(1 → 2)-, α-(1 → 3)- or α-(1 → 5)-linked. Arafur A was only active towards low oligomeric arabinofuranosides. Arafur B was preferentially active towards L-arabinofuranosyl groups of arabinan, arabinogalactan, and arabinoxylan[4].

In this paper, specific data are presented concerning the mode of action of AXH, Arafur A, and Arafur B towards arabinoxylan and arabinoxylan-derived oligosaccharides.

## EXPERIMENTAL

*Materials.*—The preparation and characterisation of alkali-extractable wheat-flour arabinoxylan (BE1-U) is described elsewhere[6]. Arabinoxylan-derived oligosaccharides were purified by Gruppen et al.[7]. The tetrameric fragment $2^3$-α-Ara*f*-$Xyl_3$ was isolated from a barley arabinoxylan digest[8]. (1 → 4)-β-D-Arabinoxylan arabinofuranohydrolase (AXH) and endo-(1 → 4)-β-D-xylanase III (Endo III) were purified from *A. awamori* CMI 142717 by Kormelink et al.[3,9]. α-L-Arabinofuranosidases A and B (Arafur A and Arafur B, respectively) were purified from *A. niger* by Rombouts et al.[4].

*Degradation of arabinoxylan-derived oligosaccharides.*—Arabinoxylan-derived oligosaccharides (5–10 μg) were dissolved in sodium acetate buffer (200 μL, 50 mM, pH 5.0) and incubated with AXH (1.0 μg/mL, 4.4 mU), Arafur A (1.0 μg/mL, 13.4 mU), or Arafur B (1.0 μg/mL; 29.4 mU) for 6 h at 30°C. After incubation, enzymes were inactivated at 100°C for 10 min.

*High performance anion-exchange chromatography (HPAEC).*—Enzyme digests were analysed by HPAEC using a Dionex Bio-LC GPM-II quaternary gradient module (Dionex Corporation, Sunnyvale, CA, USA) equipped with a Dionex CarboPac PA-1 column (250 × 4 mm) and a Dionex PED detector in the pulsed amperometric detection (PAD) mode, as described by Gruppen et al.[7]. Elution (1 mL/min) involved linear gradients of sodium acetate in 0.1 M NaOH from 0–100 mM during 5 min, then 100–400 mM during 35 min at 20°C.

*$^1H$ NMR spectroscopy.*—In order to allow NMR spectroscopy, the viscosity of alkali-extractable wheat-flour arabinoxylan was reduced by dissolving 60 mg in 50 mM sodium acetate (12 mL, pH 5.0) with subsequent treatment with Endo III (0.25 mU/mL) for 80 min at 30°C followed by enzyme inactivation (10 min, 100°C). After enzyme action, the apparent molecular weight, as determined by high performance gel permeation chromatography[9], was 50 000–55 000. The columns were calibrated by using dextrans T500 (mol wt 500 000), T70 (mol wt 70 000), T40 (mol wt 40 000), and T10 (mol wt 10 000), maltoheptaose (mol wt 1152), maltopentaose (mol wt 828), and raffinose (mol wt 504). Fractions (2 mL) containing 10 mg

of the partially degraded arabinoxylan were incubated with AXH (1 μg/mL, 44 mU) and/or Arafur B (5 μg/mL, 1470 mU) for 8 h at 30°C. After inactivation at 100°C for 10 min, samples were dialysed extensively against distilled water and the retentates were freeze-dried. Samples were analysed by a 600-MHz Bruker AM 600 spectrometer as described previously[10]. A decrease in the intensity of the H-1 signals representing terminal α-(1 → 2)- and/or α-(1 → 3)-linked arabinofuranosyl groups was studied qualitatively by comparing these signals with the intensity of the signal at 4.50 ppm.

*Methylation analysis.*—Methylation was carried out according to a modified Hakomori method[11]. After methylation, samples were dialysed and dried in a stream of air. The methylated samples were subsequently hydrolysed using 2 M $CF_3CO_2H$ (121°C, 1 h) and derivatised[12]. The methylated alditol acetates were analysed on a fused-silica capillary column (30 m × 0.32 mm; wall coated with DB1701; 0.25 μm), using a Carlo Erba Fractovap 4160 GC equipped with an FID. Glycosidic linkage composition was calculated using effective carbon response (ECR) factors[13]. The identity of compounds was confirmed by GC–MS (Hewlett–Packard, MSD 5970-B coupled to an HP 5890), equipped with a fused-silica column (CPSIL 19CB, 26 m × 0.22 mm; 0.18 μm). As 2-*O*- and 3-*O*-methylated xylitol acetates co-eluted, their relative amounts were calculated from the relative abundance of the ions at *m/z* 117 and 129.

*Protein determination.*—The protein content was determined according to Sedmak and Grossberg[14], using bovine serum albumin as standard.

## RESULTS AND DISCUSSION

AXH, Arafur A, and Arafur B all act on arabinoxylan oligosaccharides. In combination with previous published[3,4] data on polymeric arabinoxylan, remarkable similarities as well as differences can be observed in their specificity towards oligomeric and polymeric arabinoxylans with different arabinofuranosyl substitution. AXH and Arafur B showed activity on arabinoxylan as well as arabinoxylan-derived oligosaccharides. Arafur A, however, only seemed to be active on arabinoxylan-derived oligosaccharides.

In order to study the differences in specificity towards differently located arabinofuranosyl groups, arabinoxylan-derived oligosaccharides of known structure were incubated with AXH, Arafur A, and Arafur B, and analysed by HPAEC. The results are summarised in Table I. As can be seen, arabinofuranosyl groups were removed only from singly substituted xylopyranosyl residues by AXH regardless of whether the substituted xylopyranosyl residue was in a terminal or a nonterminal position. AXH is also able to hydrolyse the α-(1 → 2)-linked arabinofuranosyl groups from $2^3$-α-Ara*f*-$Xyl_3$ (ref 8).

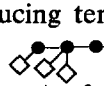
Arafur A, which acted only on low $M_w$ arabinoxylans, could remove all α-(1 → 3)-linked arabinofuranosyl groups from singly substituted xylopyranosyl residues, and therefore acts similarly as AXH.

TABLE I

Structure of arabinoxylan-derived oligosaccharides and their degradation products by AXH, Arafur A, and Arafur B

| Structure [a] | Enzyme | Product |
|---|---|---|
| 3.2  | AXH |  |
| | Arafur A |  |
| | Arafur B |  |
| 4.1  | AXH |  |
| | Arafur A |  |
| | Arafur B | N.D. [b] |
| 4.2.  | AXH |  |
| | Arafur A | n.d. [c] |
| | Arafur B | n.d. |
| 5.2.  | AXH |  |
| | Arafur A |  |
| | Arafur B |  |
| 6.2.  | AXH |  |
| | Arafur A |  |
| | Arafur B | N.D. |
| 7.1  | AXH |  |
| | Arafur A |  |
| | Arafur B |  |
| 8.1  | AXH |  |
| | Arafur A |  |
| | Arafur B | N.D. |

[a] Structures derived from Gruppen et al.[7]; ●, Xyl*p*; ◇, α-Ara*f*; ●—●, β-Xyl*p*-(1 → 4)-Xyl*p*;  , α-Ara*f*-(1 → 2)-β-Xyl*p*;  , α-Ara*f*-(1 → 3)-β-Xyl*p*. [b] No degradation. [c] Not determined.

Arafur B on the other hand could only remove arabinofuranosyl groups from singly substituted, non-reducing terminal xylopyranosyl residues (Table I: structures  ,  , and  ). An unsubstituted, nonreducing terminal xylopyranosyl residue hinders Arafur B activity.

In most cases, no complete debranching of arabinoxylan-derived oligosaccharides was observed, because of the presence of doubly substituted xylopyranosyl residues. However, doubly substituted xylopyranosyl residues do not inhibit the removal of arabinofuranosyl groups from the adjacent, singly substituted xylopyranosyl residues by AXH, Arafur A, or Arafur B (Table I: structures  and

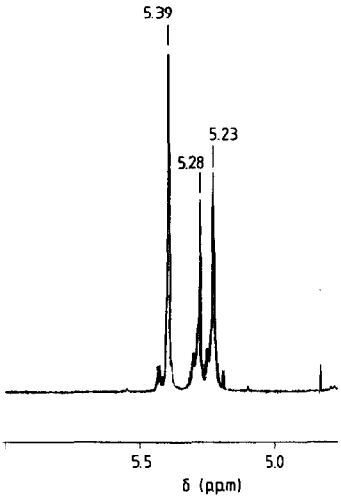



Fig. 1. 600.1-MHz $^1$H NMR spectrum of endo-xylanase-III-pretreated wheat alkali-extractable arabinoxylan (blank).

 ). Structures with only doubly substituted xylopyranosyl residues were not attacked by any of the three enzymes.

$^1$H NMR spectroscopy of the endo-xylanase pre-treated arabinoxylan showed H-1 signals of terminal α-L-arabinofuranosyl groups at 5.23, 5.28, and 5.39 ppm (Fig. 1). The signal at 5.39 ppm was assigned to anomeric protons of terminal arabinofuranosyl groups linked to O-3 of singly substituted xylopyranosyl residues. The signals at 5.23 and 5.28 ppm were assigned to anomeric protons of terminal arabinofuranosyl groups linked to O-2 and O-3 of doubly substituted xylopyranosyl residues[15–17].

Incubation with AXH removed most of the α-(1 → 3)-linked arabinofuranosyl groups from singly substituted xylopyranosyl residues, as indicated by the almost complete disappearance of the $^1$H NMR signal at 5.39 ppm (Fig. 2). No significant decrease in the two signals at 5.23 and 5.28 ppm was observed. Incubation with Arafur B showed only a small decrease in the intensity of the two signals at 5.23 and 5.28 ppm (Fig. 3), which could indicate a preference for α-(1 → 3)/α-(1 → 2)-linked arabinofuranosyl substituents of doubly substituted xylopyranosyl residues. A combination of AXH and Arafur B released α-L-arabinofuranosyl groups from singly substituted xylopyranosyl residues as well as from doubly substituted xylopyranosyl residues, as indicated by a decrease in the intensity of all H-1 signals of α-L-arabinofuranosyl groups (Fig. 4).

A difference in the mode of debranching of arabinoxylan by AXH and Arafur B was reflected also in the formation of a haze. By the removal of terminal α-(1 → 3)-linked arabinofuranosyl groups from singly substituted xylopyranosyl residues, a precipitate was formed on prolonged incubation with AXH, which was,

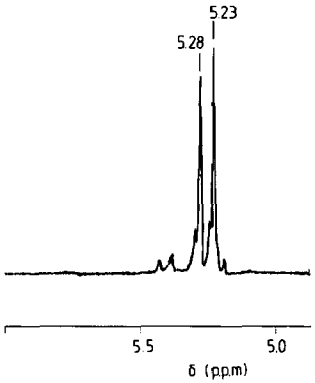



Fig. 2. 600.1-MHz $^1$H NMR spectrum of endo-xylanase-III-pretreated wheat alkali-extractable arabinoxylan after incubation with AXH.

however, not the case for Arafur B. The formation of a precipitate after incubation of arabinoxylan with arabinose-releasing enzymes was reported earlier[18,19] and ascribed to aggregation of partly linearised (arabino)xylan chains.

The results from the methylation analysis are shown in Table II. Incubation with AXH removed most of the arabinofuranosyl groups from singly substituted xylopyranosyl residues, as indicated by a decrease in the mol% of singly substituted xylopyranose which resulted in an increase in the amount of 2,3-$Me_2$-Xyl*p* from 39.4 to 52.8 mol%. No decrease in doubly substituted xylopyranosyl residues was observed.

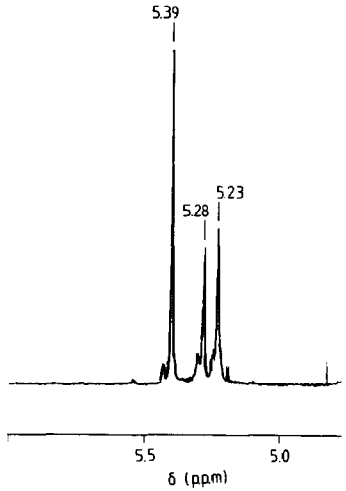



Fig. 3. 600.1-MHz $^1$H NMR spectrum of endo-xylanase-III-pretreated wheat alkali-extractable arabinoxylan after incubation with α-L-arabinofuranosidase B.

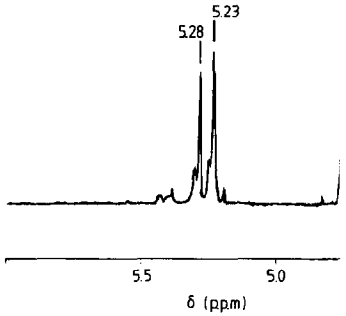



Fig. 4. 600.1-MHz $^1$H NMR spectrum of endo-xylanase-III-pretreated wheat alkali-extractable arabinoxylan after incubation with AXH and α-L-arabinofuranosidase B.

The data presented here also suggest that AXH cannot cleave all the Ara*f*-α-(1 → 2)-Xyl*p* and Ara*f*-α-(1 → 3)-Xyl*p* linkages. Of the 3-*O*-substituted xylopyranose, 84% was converted into unsubstituted xylopyranose. However, by comparing the values for unmethylated Xyl*p* before and after enzymic treatment, an increase could be observed, which was even larger when comparing absolute numbers of methylated xylopyranosyl residues (shown in parentheses). These findings may be partly explained by differences in the extent of methylation.

All these data, however, indicate a preference of AXH for α-(1 → 2)- and α-(1 → 3)-linked arabinofuranosyl groups on singly substituted xylopyranosyl residues.

From Table II, it can also be seen that Arafur B removed α-(1 → 3)/α-(1 → 2)-linked arabinofuranosyl groups in only small amounts from doubly substituted xylopyranosyl residues. No significant amounts of α-(1 → 2)- or α-(1 → 3)-linked

TABLE II

Glycosidic linkage analysis of alkali-extractable wheat-flour arabinoxylan before and after treatment with AXH, α-L-arabinofuranosidase B, or combinations of both

| Glycosidic linkage | Relative proportions (mol%) [a] | | | |
|---|---|---|---|---|
| | Control | AXH | Arafur B | AXH + Arafur B |
| 2,3,5-$Me_3$-Ara*f* | 33.9 | 25.5 | 27.3 | 21.7 |
| 3,5-$Me_2$-Ara*f* | 0.7 | 1.4 | 1.3 | 1.3 |
| 2,3-$Me_2$-Ara*f* | 3.7 | 2.2 | 5.4 | 1.5 |
| 2,5-$Me_2$-Ara*f* | 0.4 | 0.4 | 5.3 | 0.2 |
| 2,3,4-$Me_3$-Xyl*p* | 2.0 (3.3) [b] | 2.9 (4.1) | 4.4 (7.3) | 4.5 (6.0) |
| 2,3-$Me_2$-Xyl*p* | 39.4 (64.2) | 52.8 (74.9) | 40.2 (66.1) | 57.4 (76.2) |
| 3-Me-Xyl*p* | 1.2 (2.0) | 0.3 (0.4) | 0.7 (1.2) | 0.3 (0.4) |
| 2-Me-Xyl*p* | 9.7 (15.8) | 1.6 (2.3) | 9.8 (16.1) | 1.5 (2.0) |
| Xyl*p* | 9.1 (14.8) | 12.9 (18.3) | 5.7 (9.3) | 11.6 (15.4) |
| Ratio terminal/branching | 1.16 | 0.92 | 1.25 | 0.87 |

[a] Mol% of total arabinofuranose and xylopyranose residues. [b] Data in parentheses represent mol% of total xylopyranosyl residues.

arabinofuranosyl groups were removed from singly substituted xylopyranosyl residues.

AXH in combination with Arafur B resulted in an even larger decrease of $\alpha$-(1 → 3)-linked arabinofuranosyl groups, down to 21.7 mol% in comparison to treatment with AXH alone (25.5 mol%). The amount of unbranched (1 → 4)-linked xylopyranosyl residues increased to 57.4 mol% in comparison to 52.8 mol% for treatment with AXH alone. No significant changes can be observed in the mol% of 3-Me-Xyl*p*.

Despite inaccuracy in the mol% of unmethylated Xyl*p*, trends can be recognised from these data. Arafur B seems to be able to remove $\alpha$-(1 → 3)/$\alpha$-(1 → 2)-linked arabinofuranosyl groups from doubly substituted xylopyranosyl residues when comparing the data after Arafur B treatment with those from the control or by comparing the data after concerted action of AXH and Arafur B with those after AXH treatment.

Nevertheless, AXH seems to be highly active and specific towards (wheat) arabinoxylan. The amount of arabinofuranosyl groups removed from wheat arabinoxylan can be slightly increased by the concerted action of AXH with Arafur B.

The results obtained from oligosaccharide digestion agree with the findings obtained by $^1$H NMR spectroscopy and methylation analysis of AXH-treated arabinoxylan, but do not confirm the results obtained with Arafur B-treated arabinoxylan. Unfortunately, no data are available from other research groups.

## CONCLUSIONS

AXH, an arabinose-releasing enzyme active on arabinoxylans only[3], was able to remove $\alpha$-(1 → 2)- and $\alpha$-(1 → 3)-linked arabinofuranosyl groups from singly substituted xylopyranosyl residues specifically. The low affinity of AXH towards doubly substituted xylopyranosyl residues may be explained by the need of a free hydroxyl group at C-2 or C-3 in the enzyme–substrate complex.

AXH and Arafur A removed arabinofuranosyl groups from terminal as well as nonterminal singly-substituted xylopyranosyl residues in low $M_w$ oligosaccharides, whereas Arafur B could only remove arabinofuranosyl groups from nonterminal singly substituted xylopyranosyl residues. Arafur A only worked on arabinoxylan-derived oligosaccharides, whereas AXH and, although to a lesser extent, Arafur B were active on both high $M_w$ arabinoxylan as well as oligomeric arabinoxylan structures. Both aspects are important for the optimal breakdown of arabinoxylans by endo-(1 → 4)-$\beta$-D-xylanases. The ability of endo-(1 → 4)-$\beta$-D-xylanases to hydrolyse glycosidic linkages in the main chain next to a branchpoint, thus producing oligosaccharides with terminal substituted xylopyranosyl groups, will enhance the action of AXH or $\alpha$-L-arabinofuranosidases when these enzymes act together in the degradation of arabinoxylan.

Because AXH removed arabinofuranosyl groups from arabinoxylans only, and not from other arabinofuranose-containing substrates, the enzyme has require-

ments involving the surrounding types of glycosyl sugar residues. This enzyme can therefore be assigned to the group of enzymes which need two different monosaccharides in the enzyme–substrate complex, for example, α-glucuronidase[20] or rhamnogalacturonase[21].

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