

# Stereochemical course of hydrolysis catalyzed by arabinofuranosyl hydrolases

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**Abstract** The stereochemical course of hydrolysis catalyzed by various enzymes acting on arabinofuranosyl linkages has been determined.  $^1\text{H}$ -NMR analysis of the action of endo-(1 $\rightarrow$ 5)- $\alpha$ -L-arabinanases from *Aspergillus niger* and *Aspergillus aculeatus* showed that both hydrolyze linear arabinan with inversion of configuration, and may therefore act via a single displacement mechanism. This is consistent with the *A. niger* enzyme's classification in glycosyl hydrolase family 43. The catalytic mechanisms of  $\alpha$ -L-arabinofuranosidases from *A. niger*, *A. aculeatus*, *Aspergillus awamori*, *Humicola insolens*, *Penicillium capsulatum* and *Bacillus subtilis* were investigated using both  $^1\text{H}$ -NMR and high performance anion exchange chromatography to follow glycosyl transfer reactions to methanol. In all cases these enzymes catalyzed the reaction with retention of configuration, and therefore probably operate via double displacement hydrolytic mechanisms. From the results with arabinofuranosidase A and B from *A. niger* we predict that all members of glycosyl hydrolase family 51 and 54 catalyze hydrolysis with net retention of anomeric configuration. Similar studies with (1 $\rightarrow$ 4)- $\beta$ -D-arabinoxylan arabinohydrolases from *A. awamori*, *Trichoderma reesei* and *Bifidobacterium adolescentis* only enabled their tentative classification as inverting enzymes on the basis of their lack of glycosyl transfer to methanol.

**Key words:** Endo-(1 $\rightarrow$ 5)- $\alpha$ -L-Arabinanase;  $\alpha$ -L-Arabinofuranosidase; Arabinoxylan arabinohydrolase; Hydrolysis mechanism; *Aspergillus niger*; Glycosyl hydrolase family

## 1. Introduction

Enzymic hydrolysis of glycosidic linkages occurs via two major mechanisms, which result in either net retention or inversion of anomeric configuration [1]. Both hydrolytic mechanisms involve general acid catalysis and require two critical residues (a proton donor and a nucleophile/base), which in most glycosyl hydrolases are aspartate and/or glutamate residues [2]. Inverting enzymes operate by a single displacement ( $\text{S}_{\text{N}}2$ ) reaction [3], involving protonation of the glycosidic oxygen, followed by nucleophilic attack on the anomeric carbon by water. With retaining enzymes a double displacement ( $\text{S}_{\text{N}}1$ ) mechanism operates [3], with the hydrolysis proceeding through either a covalent glycosyl-enzyme intermediate, or an oxocarbenium ion intermediate stabilized electrostatically by enzyme carboxylate(s).

Early work examining the stereochemical course of enzyme

hydrolysis suggested that glycosidases and endo-glycanases acted with retention of anomeric configuration, while exo-glycanases acted with inversion [4,5]. However, more recent studies have shown that this is not always the case since an increasing number of inverting glycosidases [6,7] and endo-hydrolases [8–12], and retaining exo-hydrolases [12–14] have been reported. Recent work with the classification of enzymes based on amino acid sequence similarities [15–17] has proved more useful in predicting enzymic mechanisms since the same stereoselectivity appears to be exhibited by members of a given enzyme family [18–20].

Enzymes capable of hydrolyzing arabinofuranosidic linkages have been studied in some detail, and much is now known regarding their substrate specificities and action patterns [21–23]. Endo-(1 $\rightarrow$ 5)- $\alpha$ -L-arabinanases ((1 $\rightarrow$ 5)- $\alpha$ -L-arabinan (1 $\rightarrow$ 5)- $\alpha$ -L-arabinanohydrolase, EC 3.2.1.99) hydrolyse the (1 $\rightarrow$ 5)- $\alpha$ -L-arabinofuranosyl linkages in linear arabinan in a random pattern, initially releasing arabinotriose and larger arabino-oligosaccharides as the major hydrolysis products [22]. In contrast,  $\alpha$ -L-arabinofuranosidases ( $\alpha$ -L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55) hydrolyse non-reducing  $\alpha$ -L-arabinofuranosyl residues from arabinofuranose-containing substrates, releasing arabinose as the only hydrolysis product [21]. Two major types of  $\alpha$ -L-arabinofuranosidases can be classified according to substrate specificity, and are typified by the two enzymes isolated from *Aspergillus niger* [21,24]. Arabinofuranosidase A is only active against small substrates, like 4-nitrophenyl  $\alpha$ -L-arabinofuranoside (pNPA) and short-chain arabino-oligosaccharides, while arabinofuranosidase B has similar activity on these substrates, but is also able to hydrolyse polymeric substrates like branched arabinan and arabinoxylan. Recently, other enzymes have been isolated [25,26] that are specific for arabinofuranosyl residues in arabinoxylan, and are termed arabinoxylan arabinohydrolases ((1 $\rightarrow$ 4)- $\beta$ -D-arabinoxylan arabinofuranohydrolase, EC 3.2.1.) (AXH). The AXH from *Aspergillus awamori* has been studied in some detail [23,25,27] and found to only cleave arabinofuranose from single-substituted xylopyranosyl residues in the xylan backbone, and has only low activity against substrates like pNPA or branched arabinan. Similar enzymes from *Bifidobacterium adolescentis* [26] and *Trichoderma reesei* (R. Kavitha, H. Gruppen and A.G.J. Voragen, unpublished) have also been recently isolated that appear to have a slightly different specificity since they are active against arabinofuranosyl groups linked to double-substituted xylopyranosyl residues, and are therefore termed AXH-d.

Apart from studies with the  $\alpha$ -L-arabinofuranosidases from *Monilinia fructigena* [28,29], there has been little work examining the mechanisms by which these enzymes cleave arabinofuranosyl linkages. In this current study the stereochemical course of hydrolysis catalyzed by endo-(1 $\rightarrow$ 5)- $\alpha$ -L-arabina-

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**Abbreviations:** AXH, arabinoxylan arabinohydrolase; pNPA, 4-nitrophenyl  $\alpha$ -L-arabinofuranoside; HPAEC, high performance anion exchange chromatography

nases,  $\alpha$ -L-arabinofuranosidases and AXHs from *Aspergillus* species and several other microbial sources has been investigated.

## 2. Materials and methods

### 2.1. Enzymes

Endo-arabinanase, arabinofuranosidase A and arabinofuranosidase B from *A. niger* were purified from a commercial enzyme preparation, Pectinase 29 (Gist-Brocades, Delft, The Netherlands), according to Rombouts et al. [21]. *Humicola insolens* arabinofuranosidase was isolated from another enzyme preparation, Ultraflo (Novo Nordisk, Bagsvaerd, Denmark) [30], while *A. aculeatus* endo-arabinanase and arabinofuranosidase B were purified from Pectinex Ultra SP (Novo Nordisk, Dittingen, Switzerland) as described by Beldman et al. [22]. Arabinofuranosidase (Ara II) purified from *Penicillium capsulatum* [31] was a gift from the late Professor M.P. Coughlan (University College, Galway, Ireland). *A. awamori* AXH and arabinofuranosidase were purified as previously described [23], while *Bacillus subtilis* arabinofuranosidase (Ara II) was purified according to Weinstein and Albersheim [32]. AXH-d from *B. adolescentis* [26] and *T. reesei* (R. Kavitha, H. Gruppen and A.G.J. Voragen, unpublished) were purified in this laboratory.

### 2.2. Substrates and other chemicals

Wheat arabinoxylan was from Megazyme (Sydney, Australia). Arabinoxylan oligosaccharide 6.1, isolated from arabinoxylan hydrolyzates according to Gruppen et al. [33] and used as a substrate for the *B. adolescentis* AXH-d, was a gift from Ms. Katrien Van Laere of this laboratory. All other substrates used were from sources previously specified [22].

Methyl arabinofuranoside standards were prepared from arabinose by Fischer glycoside synthesis at 35°C according to Mizutani et al. [34]. For high performance anion exchange chromatography (HPAEC) analysis, the synthesis was stopped by addition of pyridine just prior to the disappearance of arabinose (measured reductometrically [35]) to ensure higher concentrations of the methyl arabinofuranosides than the corresponding methyl arabinopyranosides [36]. During preparation of similar standards for  $^1\text{H}$ -NMR analysis, the addition of pyridine was delayed until after the disappearance of arabinose to allow the formation of all four methyl arabinosides [36]. These solutions were then dried at 40°C under reduced pressure to give mixtures containing different proportions of arabinose, methyl  $\alpha$ - and  $\beta$ -arabinofuranosides and methyl  $\alpha$ - and  $\beta$ -arabinopyranosides.

### 2.3. $^1\text{H}$ -NMR analysis

All the enzymes (ca. 1 unit in water) and substrates (5 mg in 500  $\mu\text{l}$  of 0.2 M sodium acetate buffer, pH 5.0) were freeze-dried three times from  $\text{D}_2\text{O}$  (99.96 atom % D, Cambridge Isotope Laboratories, Andover, MA, USA) to exchange labile  $^1\text{H}$  atoms for D. For endo-arabinanase product analysis the substrate was dissolved in 0.7 ml  $\text{D}_2\text{O}$  and the pH adjusted to 5.0 by the addition of 20% DCl in  $\text{D}_2\text{O}$ . The solution was then equilibrated at 30°C in a 5 mm NMR tube and the initial spectrum recorded. The enzymes (20–50  $\mu\text{l}$  in  $\text{D}_2\text{O}$ ) were added and the stereochemical course of hydrolysis followed by recording  $^1\text{H}$ -NMR spectra at intervals during the incubation. Analysis of the arabinofuranosidases and AXHs was carried out in a similar manner except that the reaction mixtures contained 2.5 M deuterated methanol (99.96 atom % D, Cambridge Isotopes).

$^1\text{H}$ -NMR spectra were recorded at 30°C in a Bruker DPX-400 spectrometer operated at 400 MHz and equipped with a 5 mm  $^1\text{H}/^{13}\text{C}$  probe. Spectra were referenced to the residual HDO resonance at 4.74 ppm (measured indirectly with respect to tetramethylsilane at 30°C). Each spectrum was acquired with 56 transients and 2 dummy transients using 32 796 data points over the 4000 Hz spectral width. The plotted spectra were normalized to the peak area of the residual HDO resonance.

### 2.4. HPAEC analysis

For HPAEC examination of glycosyl transfer to methanol the enzyme assay mixtures (500  $\mu\text{l}$ ) contained 2 mM pNPA (1 mg/ml of other substrates), 2.5 M methanol and ca. 30 mU of each enzyme. Samples were taken periodically, the reaction stopped by heating

(100°C for 10 min) and 20  $\mu\text{l}$  aliquots analyzed by HPAEC on a Dionex Bio-LC system (Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (4  $\times$  250 mm) as previously described [22].

Identification of peaks produced upon HPAEC of the methyl arabinoside standard mixture was achieved by  $^1\text{H}$ -NMR spectroscopy of ca. 5 mg of the purified compounds, obtained by preparative HPAEC on a Spectra-Physics system (San Jose, CA, USA) using the same conditions as for analytical HPAEC, but with a Dionex CarboPac PA-100 column (22  $\times$  250 mm) and a flow rate of 20 ml/min. Fractions (5 ml) collected were neutralized with acetic acid, desalted with a mixed-bed anion exchange resin (AG 501-X8, Bio-Rad), and then freeze-dried.

## 3. Results and discussion

### 3.1. Endo-arabinanases

The stereochemical course of hydrolysis catalyzed by *A. niger* and *A. aculeatus* endo-arabinanases was followed by  $^1\text{H}$ -NMR spectroscopy. Reduced short-chain oligosaccharides are often employed as substrates for this type of analysis since they give high resolution  $^1\text{H}$ -NMR spectra and are usually

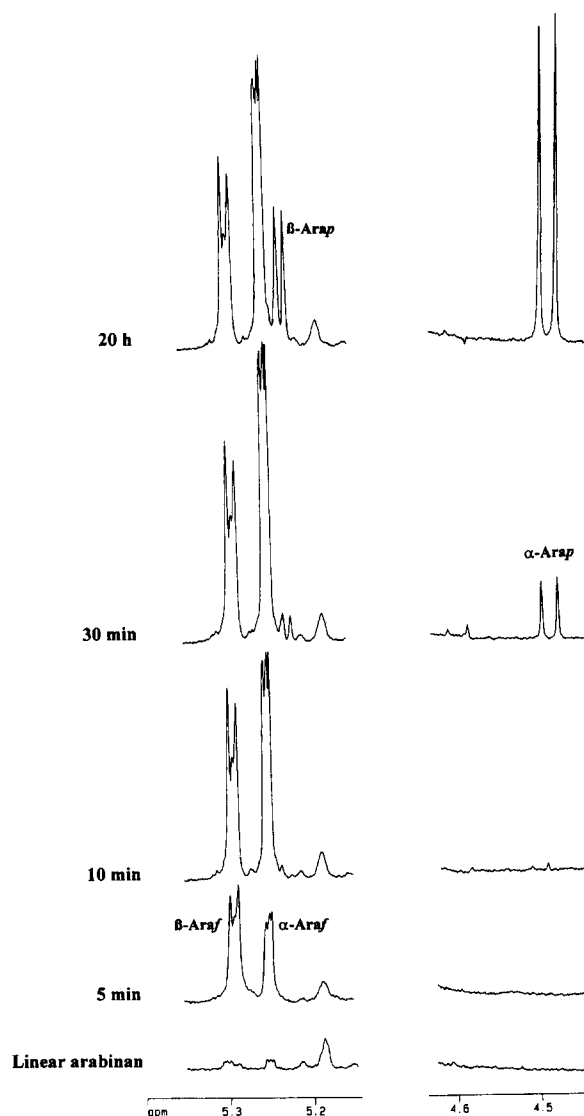


Fig. 1. Partial  $^1\text{H}$ -NMR spectra showing the stereochemical course of hydrolysis of linear arabinan by *A. niger* endo-arabinanase. H-1 resonances of the reducing-end residues of the arabinofurano-oligosaccharide products are indicated ( $\alpha$ -Araf and  $\beta$ -Araf), as are those of the free arabinopyranose also released ( $\alpha$ -Arap and  $\beta$ -Arap).

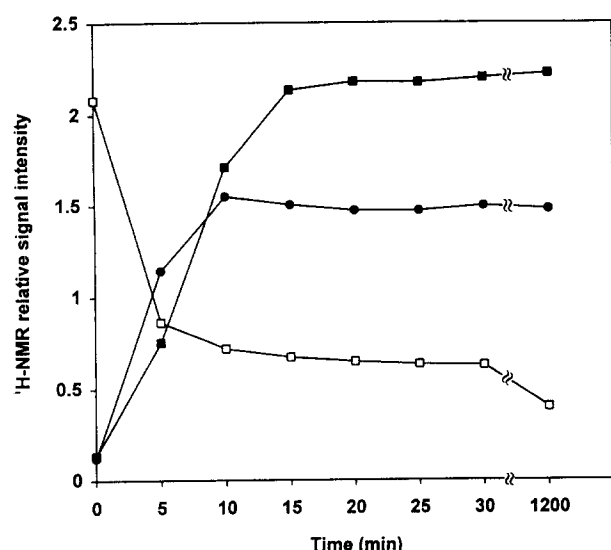


Fig. 2. Relative intensity of the arabinofuranosyl anomeric proton resonances detected by  $^1\text{H}$ -NMR during linear arabinan hydrolysis by *A. niger* endo-arabinanase. H-1 signal of linear arabinan (□) ( $<10^{-1}$ ); H-1 $\alpha$  (■) and H-1 $\beta$  (●) of arabinofuranosyl oligosaccharide hydrolysis products. All values are the result of peak integration and are expressed as a percentage of the residual HDO resonance.

only hydrolyzed to a single reducing hydrolysis product. However, they have the disadvantage that they are only slowly cleaved by most endo-hydrolases. Therefore, since the rate of hydrolysis must be greater than that of mutarotation, these substrates are not particularly suitable for stereochemical analysis of endo-arabinanases where very rapid mutarotation of the hydrolysis products (arabinofuranosyl oligosaccharides) was observed to occur. Since several recent reports have described the successful use of natural polymers as substrates for stereochemical analysis with  $^1\text{H}$ -NMR [37–39], we have employed linear (apple) (1 $\rightarrow$ 5)- $\alpha$ -L-arabinan for this purpose in the current study.

Fig. 1 shows the partial  $^1\text{H}$ -NMR spectrum of the anomeric region of linear arabinan, and several spectra recorded at intervals after the addition of the *A. niger* endo-arabinanase, indicating the anomeric form of the products released. Reso-

nances at 5.30 ppm and 5.26 ppm appeared from the earliest stages of the reaction and were assigned to H-1 $\beta$  and H-1 $\alpha$  respectively of the reducing-end arabinofuranosyl residues of the oligomeric products [40]. The complex appearance of these signals probably arose from the presence of a wide range of oligomeric hydrolysis products of different chain length [22], with the resonances at 5.26 ppm appearing to consist of three separate doublets ( $J \sim 1$  Hz). Consistent with earlier studies [22], free arabinose was only observed later in the incubation, identified by resonances at 5.24 ppm ( $J$  3.5 Hz) and 4.52 ppm ( $J$  7.7 Hz) arising from H-1 $\beta$  and H-1 $\alpha$  of the pyranose residues respectively [41]. From these results it can be seen that the rate of mutarotation between the  $\alpha$ - and  $\beta$ -anomers of the reducing-end arabinofuranosyl residues was very rapid, since both anomers were observed from the start of the reaction. However, a comparison of the  $\alpha/\beta$  ratio during the reaction (Fig. 2) unambiguously shows that the initial arabinofuranoligosaccharides formed during the hydrolysis are in the  $\beta$ -configuration. After 5 min the relative intensities of these  $\alpha$ - and  $\beta$ -anomer resonances were 2:3, while the mutarotational equilibrium ratio of ca. 3:2 was observed after the reaction was left for 20 h.

Similar results were obtained for the *A. aculeatus* enzyme (see Table 1), and therefore it appears that for both endo-arabinanases examined, hydrolysis proceeds with inversion of anomeric configuration, probably through a single displacement mechanism.

### 3.2. Arabinofuranosidases

Standard  $^1\text{H}$ -NMR analysis of the stereochemistry of hydrolysis of *p*NPA by the arabinofuranosidases proved impossible due to the instability of the released arabinofuranose residues [43], and the resultant rapid mutarotation between the  $\alpha$ - and  $\beta$ -anomeric forms and tautomerization of these to both arabinopyranose anomers. Unlike the reducing-end arabinofuranosyl residues of (1 $\rightarrow$ 5)- $\alpha$ -L-arabino-oligosaccharides described earlier, free arabinofuranose is not stabilized in the furanose form by substitution at 0-5 [44]. In fact, when  $^1\text{H}$ -NMR analysis was attempted, even with very high hydrolysis rates, the four main arabinose tautomers ( $\alpha$ - and  $\beta$ -L-arabinopyranose and  $\alpha$ - and  $\beta$ -L-arabinofuranose) were ob-

Table 1  
Stereochemical course of hydrolysis catalyzed by arabinofuranosyl hydrolases

Enzyme type	Source	Mechanism
Endo-arabinanase	<i>A. niger</i>	Inverting
	<i>A. aculeatus</i>	Inverting
Arabinofuranosidase A	<i>A. niger</i>	Retaining
Arabinofuranosidase B	<i>A. niger</i>	Retaining
	<i>A. aculeatus</i>	Retaining
	<i>P. capsulatum</i> (Ara II)	Retaining
	<i>H. insolens</i>	Retaining
	<i>B. subtilis</i> (Ara II)	Retaining
	<i>A. awamori</i>	Retaining
	<i>M. fructigena</i> (AF I) [42]	Retaining [28]
	<i>M. fructigena</i> (AF III) [42]	Retaining [28]
AXH	<i>A. awamori</i>	Inverting <sup>a</sup>
AXH-d	<i>B. adolescentis</i>	Inverting <sup>a</sup>
	<i>T. reesei</i>	Inverting <sup>a</sup>

<sup>a</sup>Only tentatively classified as inverting enzymes on the basis of their lack of glycosyl transfer to methanol.

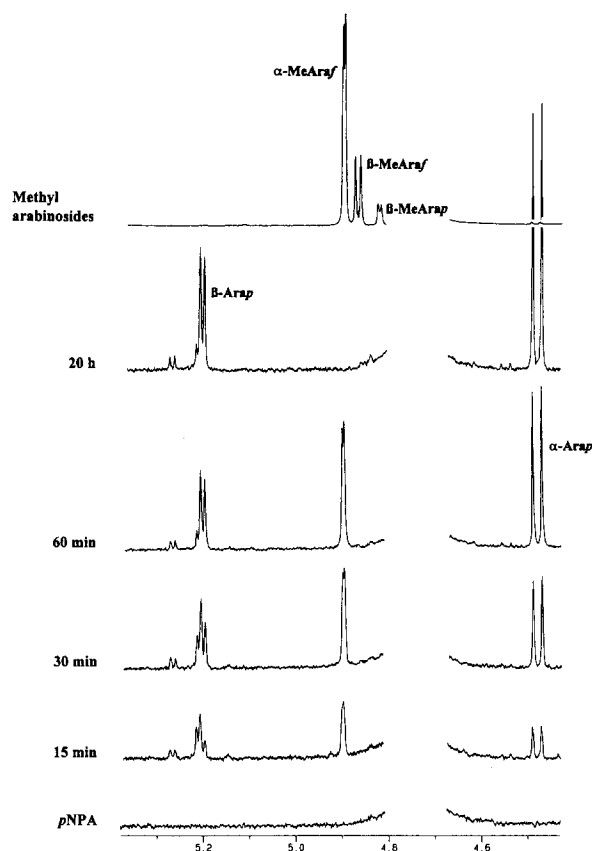


Fig. 3. Partial  $^1\text{H}$ -NMR spectra of the anomeric region during the hydrolysis of *p*NPA in methanol by *A. niger* arabinofuranosidase B. H-1 resonances of the methyl  $\alpha$ - and  $\beta$ -L-arabinofuranosides ( $\alpha$ -MeAraf and  $\beta$ -MeAraf) and methyl  $\beta$ -L-arabinopyranoside ( $\beta$ -MeArap) standards are indicated.

served at mutarotational equilibrium proportions (i.e. 58:34:5:3.5 [40,45]) from the very start of the reaction.

Encouraged by similar stereochemical studies with the arabinofuranosidases from *M. fructigena* [28], *p*NPA hydrolysis was carried out in the presence of 2.5 M methanol and followed by  $^1\text{H}$ -NMR spectroscopy. The resultant spectra for the hydrolysis catalyzed by the *A. niger* arabinofuranosidase B (Fig. 3) shows, in addition to the  $\beta$ - and  $\alpha$ -arabinopyranose anomeric signals at 5.20 ppm ( $J$  3.5 Hz) and 4.48 ppm ( $J$  7.7 Hz) respectively, the production of a doublet at 4.90 ppm ( $J$  1.7 Hz), assigned to the anomeric proton of methyl  $\alpha$ -L-arabinofuranoside [46]. This product, with retained anomeric configuration, was assumed to be due to the enzymic transfer of  $\alpha$ -L-arabinofuranosyl residues from the *p*-nitrophenyl glycoside to methanol since no condensation products were observed during incubation of the enzyme and arabinose in methanol, or with incubation of *p*NPA and methanol alone. After extended incubations (20 h) this product disappeared (Fig. 3), presumably due to its hydrolysis by the arabinofuranosidase, to eventually yield arabinose and methanol.

The hydrolysis in methanol was also followed by HPAEC analysis, using *p*NPA, branched (sugar-beet) arabinan (Fig. 4) and arabinoxylan as substrates, and the production of methyl  $\alpha$ -L-arabinofuranoside was clearly seen in each case. Similar results were also obtained for all the other arabinofuranosidases examined (see Table 1), indicating that all these enzymes act with net retention of anomeric configuration, initially releasing  $\alpha$ -L-arabinofuranose from their substrates. Therefore,

it appears that, despite their diverse microbial origins and quite different substrate specificities, all seven arabinofuranosidases (and those from *M. fructigena* [28]) catalyze hydrolysis with a similar double displacement mechanism.

For all the arabinofuranosidases examined an arabinose/methyl arabinofuranoside product ratio of ca. 2:1 was observed in the early stages of the reaction (see Figs. 3 and 4). This indicates methanol to be ca. 10 times more reactive than water on a molar basis towards the arabinofuranosyl-enzyme intermediate, which is comparable to the values obtained for the *M. fructigena*  $\alpha$ -L-arabinofuranosidases [28].

### 3.3. Arabinoxylan arabinohydrolases

In light of the success achieved in determining the stereochemical course of hydrolysis catalyzed by the arabinofuranosidases, particularly with the use of arabinoxylan as substrate, similar studies with a range of AXHs were also attempted. Arabinoxylan was used as the substrate, and since, like the arabinofuranosidases, these enzymes release arabinofuranose as the only hydrolysis product [24], the reaction was carried out in 2.5 M methanol. However, under these conditions arabinose was the only hydrolysis product observed using  $^1\text{H}$ -NMR or HPAEC analysis. No methyl arabinofuranoside transfer products were seen with any of the enzymes at any stage of the incubation. During further studies with the *A. awamori* AXH no transfer products could be detected even when the incubations were performed at up to 60°C or 10 M methanol, or with *p*NPA as substrate, even though some arabinose formation was seen under all these conditions.

Since glycosyl hydrolases that act with overall inversion of anomeric configuration cannot carry out glycosyl transfer to methanol [1], it would be tempting to speculate that all AXHs examined in this study act with inversion of anomeric configuration, releasing  $\beta$ -arabinofuranose. However, since it is possible that the absence of glycosyl transferase activity in the AXHs is an artifact of the experimental conditions employed, no firm conclusions can be drawn from these results.

### 3.4. Conclusions

Mechanistic data for glycosyl hydrolases can best be interpreted in terms of membership in glycosyl hydrolase families

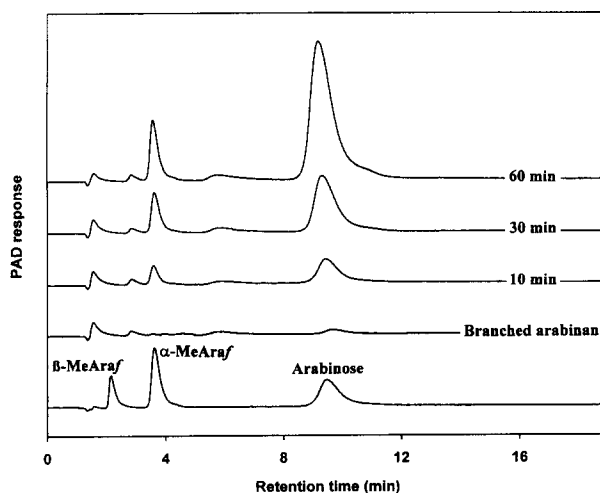


Fig. 4. High performance anion exchange chromatography of reaction products from the hydrolysis of branched arabinan in methanol by *A. niger* arabinofuranosidase B.

based on amino acid sequence similarity [15–17]. This better reflects structural features of the enzymes than parameters like substrate specificity, and since the stereochemistry of hydrolysis is governed by the three dimensional structure of the active site [47] it is not surprising that members of a given family exhibit the same stereoselectivity [18–20]. According to this classification scheme, the *A. niger* endo-arabinanase belongs to glycosyl hydrolase family 43. Therefore, the results of this current study are in good agreement with findings from the only other enzyme in this family that has been examined in detail, a  $\beta$ -xylosidase from *Bacillus pumilus*, which also acts with inversion of anomeric configuration [6]. Similarly, since *A. niger* arabinofuranosidase A and B belong to glycosyl hydrolase families 51 and 54 respectively [17], we predict that all enzymes belonging to these families catalyze hydrolysis with net retention of anomeric configuration.

Unfortunately, amino acid sequences are yet not available for any of the other enzymes examined in this study, and therefore their classification into glycosyl hydrolase families is not currently possible.

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