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# Action patterns and mapping of the substrate-binding regions of endo-(1 → 5)-α-L-arabinanases from *Aspergillus niger* and *Aspergillus aculeatus*

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**Abstract**

The substrate binding sites of endo-(1 → 5)-α-L-arabinanases (EC 3.2.1.99) from *Aspergillus niger* and *Aspergillus aculeatus* were investigated using reduced and regular (1 → 5)-α-L-arabino-oligosaccharides and high performance anion exchange chromatographic analysis. Calculation of bond cleavage frequencies and $k_{cat}/K_m$ parameters for these substrates enabled the determination of the number of arabinofuranosyl binding subsites and the estimation of the binding affinities of each subsite. The *A. aculeatus* endo-arabinanase has six subsites arranged symmetrically around the catalytic site, while the *A. niger* endo-arabinanase has five subsites; two from the catalytic site towards the non-reducing end of the bound substrate and three toward the reducing end. The two subsites directly adjacent to the catalytic sites in both the *A. niger* and *A. aculeatus* endo-arabinanase have near-zero net free energy of binding. These results are unlike most glycopyranosyl endo-hydrolases studied which have net negative (unfavourable) energies of interaction at these two subsites, and may be related to the greater conformational flexibility of arabinofuranosyl residues than glycopyranosyl residues. The complete subsite maps are also rationalized with regard to the observed action patterns of these enzymes on linear (1 → 5)-α-L-arabinan. © 1997 Elsevier Science Ltd.

*Keywords:* Endo-(1 → 5)-α-L-arabinanase; *Aspergillus niger*; *Aspergillus aculeatus*; Subsite mapping; Substrate binding site

Abbreviations: ABN, endo-(1 → 5)-α-L-arabinanase; $Ara_n$, (1 → 5)-α-L-arabinofurano-oligosaccharide with degree of polymerization *n*; $Ara_n^*$, reduced (1 → 5)-α-L-arabinofurano-oligosaccharide with degree of polymerization *n*; BCF, bond-cleavage frequency; dp, degree of polymerization; HPAEC, high performance anion exchange chromatography; PAD, pulsed-amperometric detection

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## 1. Introduction

The action of polysaccharide hydrolases can be rationalized on the basis of the subsite model, where substrate binding at the active site is considered to involve an array of contiguous subsites that each interact with a single glycosyl residue of the substrate through hydrogen and hydrophobic bonding [1–9]. The catalytic amino acids are positioned between two of these subsites. This substrate–subsite interaction (subsite affinity) can be either favourable or unfavourable for binding of the respective substrate glycosyl residue, with the latter implicated in some cases in lowering the energy barrier of the hydrolysis reaction by introducing strain in the bound substrate [10,11] and stabilizing the glycosyl-cation-like transition state [11–13].

Endo-(1 → 5)-α-L-arabinanases (ABN) [(1 → 5)-α-L-arabinan (1 → 5)-α-L-arabinanohydrolase, EC 3.2.1.99] cleave the (1 → 5)-α-L-arabinofuranosyl backbone of arabinans in a random manner, releasing arabino-oligosaccharides. Enzymes of this type have been found in various bacteria and fungi (see [14]), and also in germinating seeds of higher plants [15]. Fungal ABN's have found commercial applications in fruit and vegetable processing [14,16], particularly in apple juice manufacture where their use prevents haze formation in 'pectinase'-treated apple pulp by depolymerising de-branched arabinan formed by the action of α-L-arabinofuranosidases during this process (see [16]).

The ABN's from *Bacillus subtilis* were the first to be studied in detail [17–22]. More recently the isolation and characterization of similar enzymes from commercial enzyme preparations of *Aspergillus niger* [23–29] and *Aspergillus aculeatus* [25] have been described. Both *Aspergillus* ABN's appear resonably similar in regard to molecular weight, pH optimum, substrate specificity and hydrolytic mechanisms [30], and antibodies raised against the *A. niger* enzyme also show cross-reactivity with the *A. aculeatus* ABN [25]. In contrast to the *B. subtilis* ABN's, which accumulate arabinose and arabinobiose as end-products from linear (1 → 5)-α-L-arabinan [17–20], both *Aspergillus* enzymes release very little arabinose, but instead produce arabinobiose and arabinotriose as the major final hydrolysis products [25–27]. However, some distinct differences in the spectrum of initial oligosaccharide products released from linear arabinan by the *Aspergillus* ABN's have been reported [25], indicating differences in the action patterns of the two enzymes on this substrate.

Like the *B. subtilis* enzymes [17,18], the extent and rate of branched arabinan hydrolysis by the *Aspergillus* ABN's is affected by the degree of substitution on the arabinan backbone by (1 → 3) or (1 → 2)-α-L-arabinofuranosyl residues [23–25,27]. $^{13}C$ NMR studies of the hydrolysis products from *A. niger* ABN action on branched arabinan [31] indicated that the smallest branched oligosaccharide released was a tetrasaccharide with an (1 → 3)-α-L-arabinofuranosyl residue linked to the central residue of (1 → 5)-α-L-arabinotriose (i.e. $3^2$-α-L-arabinofuranosyl (1 → 5)-α-L-arabinotriose). This suggested a requirement for at least one unsubstituted α-L-arabinofuranosyl residue on each side of the substituted residue before hydrolysis by this enzyme could occur. Comparison of the initial hydrolysis products formed by action of each of the *Apergillus* ABN's on branched arabinans gave similar distribution of branched oligomeric products [25], possibly indicating that the two ABN's have a similar tolerance for branched sites.

It is well known that the substrate specificity, action pattern and kinetic parameters of an enzyme is dictated by the number, arrangement and size of the subsite binding affinities, and the position of the catalytic site [32]. Therefore, in this study we report and compare the subsite affinities of the *A. niger* and *A. aculeatus* ABN's in an attempt to understand the reasons behind the differences in their action pattern on linear (1 → 5)-α-L-arabinan. This analysis was performed essentially according to Suganuma et al. [8] by studying the action and kinetics of the enzymes on a series of reduced and regular (unreduced) arabino-oligosaccharides.

## 2. Experimental

*Enzymes and assay conditions.*—Endo-(1 → 5)-α-L-arabinanases from commerical enzyme preparations of *A. niger* (Pectinase 29; Gist-Brocades, Delft, The Netherlands) and *A. aculeatus* (Pectinex Ultra SP; NOVO Nordisk, Dittingen, Switzerland) were purified and assayed according to Rombouts et al. [24] and Beldman et al. [25] respectively. All enzyme assays were performed at 40 °C in 20 mM sodium acetate buffer at pH 5.0 or pH 5.5 for the *A. niger* [24] and *A. aculeatus* [25] ABN's respectively.

*Substrates.*—Linear (1 → 5)-α-L-arabinan, isolated from hazy apple juice [33], was a gift from NOVO Nordisk (Dittingen, Switzerland). (1 → 5)-α-L-Arabinofurano-oligosaccharides from arabinobiose to

arabinooctaose ($Ara_2$ to $Ara_8$) were obtained from Megazyme (Sydney, Australia). Since $Ara_8$ contained substantial proportions of $Ara_7$ and $Ara_9$, 10 mg of this preparation was further purified by preparative high performance anion exchange chromatography (HPAEC) on a Spectra-Physics system (San Jose, CA, USA) equipped with a CarboPac PA-100 column (22 × 250 mm; Dionex, Sunnyvale, CA, USA). Elution was performed with flow rate of 20 mL $min^{-1}$ and a linear sodium acetate gradient of 0 to 0.5 M in 0.1 M NaOH. Collected fractions (5 mL) were neutralized with acetic acid, desalted with a mixed bed ion exchange resin (AG 50-1X8, Bio-Rad) at 4 °C and concentrated by freeze-drying.

Arabitol ($Ara^*$) and reduced arabino-oligosaccharides ($Ara_2^*$ to $Ara_8^*$) were prepared by treating ca. 5 mg of the pure mono- and oligosaccharides with 2 mL of 250 mM $NaBH_4$ in 1 M $NH_4OH$ for 2 h at 30 °C. Reaction mixtures were then acidified by dropwise addition of glacial acetic acid, desalted using a mixed bed ion exchange resin (AG 50-1X8) at 4 °C, and freeze-dryed. All reduced and regular arabino-oligosaccharides were redissolved in distilled water to give stock solutions of 2 mM, with the concentrations determined colorimetrically with an automated orcinol/$H_2SO_4$ assay [34] using arabinose as standard. The purity of these preparations was reassessed by HPAEC (see below), and in all cases the level of contamination from other arabino-oligosaccharide homologues was less than 1%.

*Kinetics of arabino-oligosaccharide hydrolysis.*— The kinetic parameter, $k_{cat}/K_m$, was determined for reduced and regular arabino-oligosaccharides by incubation of 4 mL of 25 μM substrate in 20 mM acetate buffer, pH 5.0 and 5.5 for the *A. niger* and *A. aculeatus* ABN's respectively. All incubation mixtures also contained 15 μM xylose as internal standard. Samples (400 μL) were taken at intervals, heated for 10 min at 100 °C to inactivate the enzymes, and evaporated to dryness under a stream of air. The dried samples were then redissolved in 100 μL of distilled water (resulting in a 4-fold concentration over the original sample) and the degree of substrate hydrolysis quantified by integration of peaks obtained from HPAEC (see below). Under the experimental conditions used (i.e. $[E]_o \ll [S]_o \ll K_m$, where $[E]_o$ and $[S]_o$ represent the initial enzyme and substrate concentrations respectively) the hydrolysis can be considered a first-order reaction with a first-order rate constant ($k$) which is equal to $(k_{cat}/K_m)[E]_o$ [8,35]. Therefore, using the integrated first-order rate equation,

$$kt = \ln([S]_o/[S]) \quad (1)$$

where $[S]_o$ and $[S]$ represent the substrate concentrations at zero time and after a reaction time $t$, respectively, $k_{cat}/K_m$ can be determined from the gradient of a plot of $\ln([S]_o/[S])$ versus time [8,35]. To ensure the accuracy of the linear regressions all analyses were performed in duplicate, and incubation times and enzyme concentrations (Table 1) were such that the initial reaction rates (linear portion of the plots) were estimated from at least six (duplicate) data points.

$K_m$ determinations for $Ara_8$ were performed over the substrate concentration range 0.1 to 2.0 mM, with hydrolysis monitored by the increase in reducing sugars, determined by the Somogyi–Nelson method [36,37]. The resultant data were analysed by Michaelis–Menten kinetics using a non-linear regression program [38].

Table 1
Kinetic parameters, $k_{cat}/K_m$ for the *A. niger* and *A. aculeatus* ABN's during hydrolysis of regular arabino-oligosaccharides of dp 2-8

| Substrate (dp) [a] | *A. niger* ABN | | *A. aculeatus* ABN | |
|---|---|---|---|---|
| | $[E]_o$ (nM) [b] | $k_{cat}/K_m$ ($s^{-1} \cdot M^{-1}$) | $[E]_o$ (nM) [b] | $k_{cat}/K_m$ ($s^{-1} \cdot M^{-1}$) |
| 2 | 571 | $3.50 \times 10^{-1}$ | 1111 | 2.40 |
| 3 | 28.6 | $3.92 \times 10^{2}$ | 111 | $2.44 \times 10^{2}$ |
| 4 | 4.29 | $5.24 \times 10^{3}$ | 6.67 | $6.26 \times 10^{3}$ |
| 5 | 2.86 | $2.30 \times 10^{4}$ | 2.22 | $4.93 \times 10^{4}$ |
| 6 | 1.43 | $3.37 \times 10^{4}$ | 1.11 | $2.13 \times 10^{5}$ |
| 7 | 1.43 | $3.45 \times 10^{4}$ | 0.44 | $2.54 \times 10^{5}$ |
| 8 | 1.43 | $3.55 \times 10^{4}$ | 0.44 | $2.68 \times 10^{5}$ |

[a] Substrates were all supplied at 25 μM.
[b] Concentrations based on molecular weights of 43 and 45 kDa for the *A. niger* [29] and *A. aculeatus* [25] ABN's respectively.

*Bond-cleavage frequencies.*—Bond-cleavage frequencies (BCFs) for reduced and regular arabino-oligosaccharides were determined in the same manner as for the kinetic parameters. The distribution of the individual hydrolysis products during the reaction were quantified by HPAEC (peak areas), taking into account the different pulsed-amperometric detection (PAD) response factors for oligosaccharides of different dp [39–41]. Incubation times and enzyme concentrations (Table 1) were such that the formation of second generation products (i.e. more than one cleavage of the original substrate) were minimized. No apparent differences in the product distributions for a particular enzyme/substrate combination were observed over time, at least in the initial stages of the hydrolysis. Therefore, to increase accuracy, BCF values were obtained from averaging the relative product distributions at various times during the initial stages of the hydrolysis. Again, all analyses were performed in duplicate.

*Subsite affinity determinations.*—The number of substrate binding subsites and transition-state subsite affinities were determined according to the method of Suganuma et al. [8]. This procedure requires the input of $k_{cat}/K_m$ values and BCFs (calculated from first generation degradation products) for an oligosaccharide series, and the $K_m$ value of a substrate longer than the total number of subsites. $k_{cat}/K_m$ parameters must be obtained under conditions of pseudo-first order kinetics ($[S]_o \ll K_m$) to simplify the Michaelis–Menten equation, and to prevent bi-substrate processes such as transglycosylation and condensation [8].

Subsite affinities ($A_i$) for the *A. niger* and *A. aculeatus* ABN's were calculated from the difference in the molecular binding affinities (free energy of binding) of oligosaccharides of dp $n$ and $n-1$, where the two substrates complex with the enzyme in the same binding mode. The subsites are labelled from the catalytic site, with negative numbers for subsites to the left (non-reducing end side) and positive numbers to the right (reducing end side) according to the recently proposed nomenclature of Davies et al. [42]. Therefore, affinities for subsites $-5$ to $-2$ (i.e. $i \leq -2$) can be calculated from Eq. (2), while affinities for subsites $+2$ to $+5$ (i.e. $i \geq +2$) can be determined according to Eq. (3) [3,5,8,9,43]:

$$\text{for } i \leq -2, \quad A_i = RT \ln\left[(k_{cat}/K_m)_n \cdot \mathrm{BCF}_{n,i+n}/(k_{cat}/K_m)_{n-1} \cdot \mathrm{BCF}_{n-1,i+n}\right], \tag{2}$$

$$\text{for } i \geq +2, \quad A_i = RT \ln\left[(k_{cat}/K_m)_n \cdot \mathrm{BCF}_{n,i}/(k_{cat}/K_m)_{n-1} \cdot \mathrm{BCF}_{n-1,i-1}\right], \tag{3}$$

where $i$ is the subsite number and $\mathrm{BCF}_{n,i}$ is the bond-cleavage frequency of the $i$-th glycosidic linkage (counting from the reducing end) of an oligosaccharide of dp $n$. Mean subsite affinities and standard deviations were determined from pairs of positional isomers of enzyme–substrate complexes where BCFs were $\geq 0.020$.

Unfortunately, there is no direct method known to determine the individual subsite affinites for the two subsites adjacent to the catalytic site ($-1$ and $+1$) [9]. However, the sum of the binding affinities of these two subsites can be approximated using the $K_m$ of a substrate with dp greater than the total number of subsites to determine the total affinity of all subsites ($\Sigma A_i$) according to Eq. (4) [5,8,44].

$$1/[K_m \cdot (n-m+1)] = 0.018 e^{\Sigma A_i/RT} \tag{4}$$

where $n$ is the dp of the oligosaccharide, $m$ is the number of subsites in the enzyme, and the factor 0.018 is the correction for the change in the unitary free energy of non-specific enzyme–substrate interaction in water [5,8]. The overall binding affinity of $-1$ and $+1$ can then be determined by the energy difference between $\Sigma A_i$ and the sum of the known subsite affinities.

*High performance anion exchange chromatography.*—Quantitative analysis of reduced and regular arabino-oligosaccharide hydrolysis were performed by HPAEC on a Dionex Bio-LC system (Sunnyvale, CA, USA) equipped with a CarboPac PA-100 column ($4 \times 250$ mm), essentially as described previously [25]. Samples (20 $\mu$L) applied were eluted at a flow rate of 1 mL min$^{-1}$ by the following linear sodium acetate gradient in 150 mM NaOH: 0–3 min, 0 mM; 3–5 min, 0–100 mM; 5–25 min, 100–500 mM. The column was re-equilibrated with 8 mL of 150 mM NaOH before application of the next sample. After every 10 samples the column was washed with 15 mL of 1 M sodium acetate and re-equilibrated with 15 mL of 150 mM NaOH. As mentioned previously, an internal standard of xylose was included in all samples to account for any fluctuation in response of the pulsed-amperometric detector over time. Chromatograms were recorded and integrated using PC1000 System Software (Thermo Separation Products, San Jose, CA, USA). A typical HPAEC elution

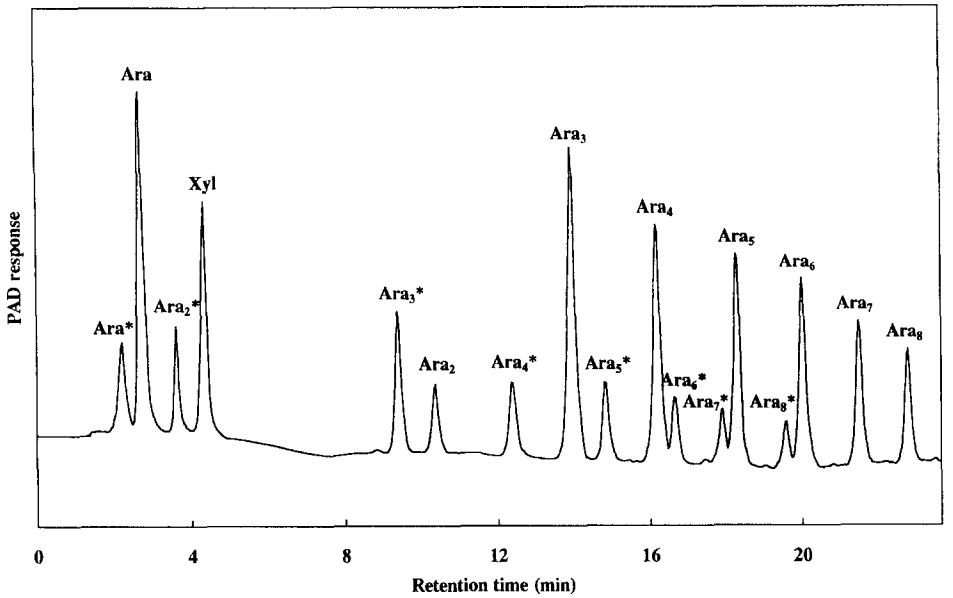


Fig. 1. Separation of regular and reduced arabino-oligosaccharides by HPAEC. Elution of regular (Ara to $Ara_8$) and reduced (1 → 5)-α-L-arabino-oligosaccharides (Ara * to $Ara_8$ *) and the internal standard xylose (Xyl) are shown.

pattern for a standard mixture of reduced and non-reduced arabino-oligosaccharides is shown in Fig. 1.

## 3. Results

*Action patterns on linear arabinan.*—HPAEC analysis of the initial hydrolysis products released from linear (1 → 5)-α-L-arabinan by the *A. niger* and *A. aculeatus* ABN's are shown in Fig. 2. Although both ABN's produced very little Ara during the initial stages of the hydrolysis, the distribution of oligomeric products varied markedly in the hydrolysates of the two enzymes. For example, the *A. aculeatus* ABN produced a series of oligomers of which the concentration decreased progressively from dp 3 to dp 25, and only a small amount of $Ara_2$. However, the *A. niger* ABN initially released more larger oligomers (particularly $Ara_6$ and $Ara_7$) and a higher proportion of $Ara_2$ than the *A. aculeatus* enzyme. These results are a correction to our previous study [25] where the hydrolysis patterns of the *A. niger* and *A. aculeatus* ABN's were erroneously shown in reverse.

*Action patterns on reduced and regular arabino-oligosaccharides.*—The action patterns of the *A. niger* and *A. aculeatus* ABN's were determined against a series of reduced and regular arabino-oligosaccharides (dp 2–8) by HPAEC analysis of the hydrolysis products. Under the conditions used (Table 1), no bi-substrate reactions such as condensation or transglycosylation were observed, with the latter not surprising since both enzymes are known to act with inversion of configuration [30]. Comparison of the product ratios also indicate the absence of multiple attack mechanisms with the substrates used. Thus, the prerequisites of Suganuma et al. [8], required for the validity of subsite mapping, appear to be met by the assay conditions employed.

In our previous reports, $Ara_2$ was not considered a substrate for either the *A. niger* or *A. aculeatus* ABN's [24,25]. However, in the current study, using relatively high enzyme concentrations and the sensitive HPAEC-PAD detection method, hydrolysis of this substrate by both enzymes has been observed, albeit at very low rates. Similar results have also been reported in a recent study with the *A. niger* ABN [27]. In contrast, arabinobiitol ($Ara_2^*$) was not hydrolysed by either ABN, even when very high enzyme concentrations were employed.

*Bond - cleavage frequencies.*—BCF's for both ABN's with regular arabino-oligosaccharides are shown in Fig. 3a. The values obtained for the *A. niger* ABN correlate reasonably well with those reported in a similar study by Dunkel and Amadò [27] considering that in this previous study no correction for the often subtantial differences in PAD response factors for arabino-oligosaccharides of different dp was performed. While BCF's stated in Fig. 3a for symmetrical cleavages are genuine, there is no way of determining the actual frequencies of non-symmetrical cleavages with these unlabelled substrates. In-

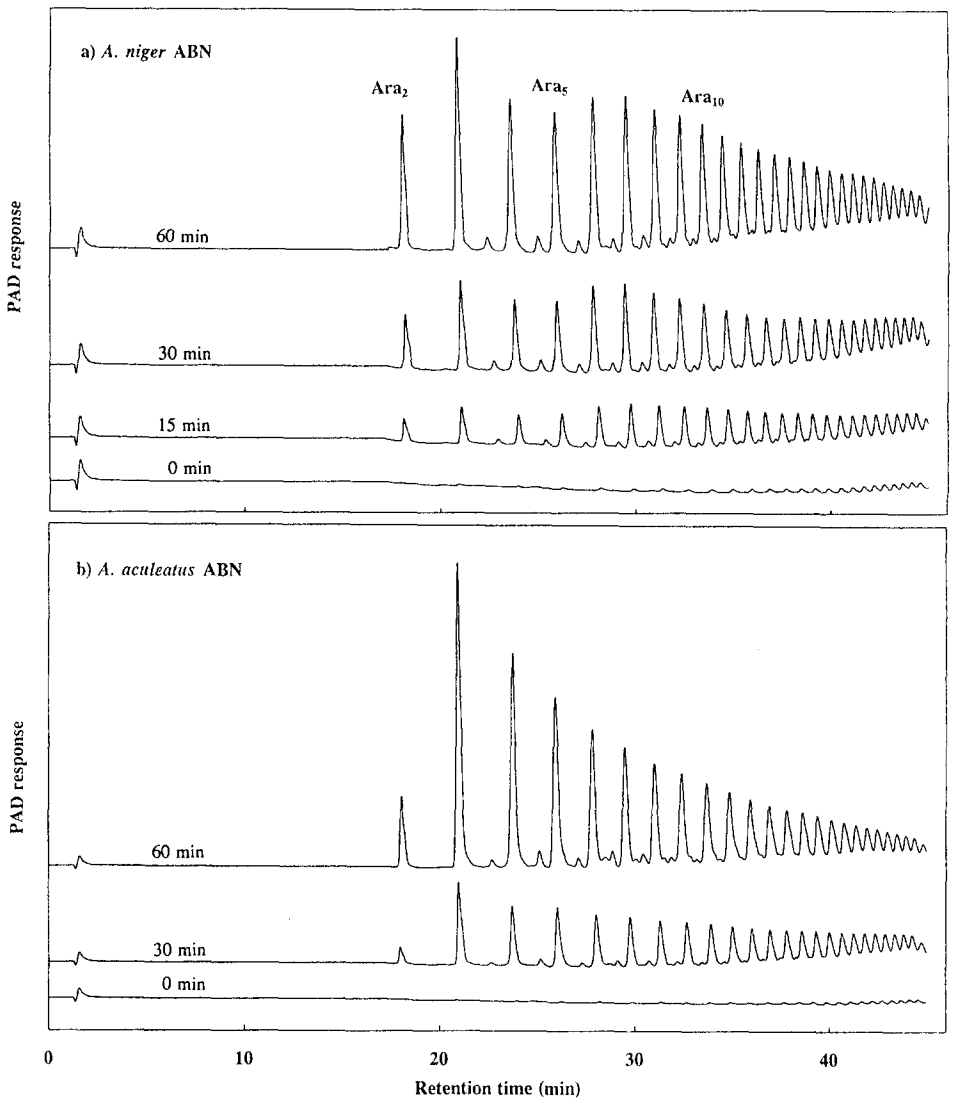


Fig. 2. HPAEC of initial hydrolysis products released from linear arabinan by (a) *A. niger* ABN, and (b) *A. aculeatus* ABN after various times.

stead, only the total frequency distributed over two linkages can be determined, as shown in Fig. 3a. For example, approximately 46% of the enzyme–substrate encounters with *A. niger* ABN and $Ara_7$ result in the formation of $Ara_2$ and $Ara_5$, through cleavage at either linkage *2* or *5*. To calculate these BCF's, the reducing end of the substrates were labelled by reduction with borohydride prior to hydrolysis since the cleavage preferences can be easily determined from HPAEC analysis of the proportion and size of reduced and unreduced products (see Fig. 1).

BCF's obtained with reduced arabino-oligosaccharides are shown in Fig. 3b. From these results it can be clearly seen that reduction changes the pattern of ABN action on these substrates. However, unlike some other studies [45,46], the effect of reduction seems predictable since, it appears to effectively shorten the arabino-oligosaccharide chain by one residue (from the reducing end) as far as ABN action is concerned. That is, the BCF's for a regular arabino-oligosaccharide of dp $n$ are quite similar for a reduced oligomer of dp $n + 1$ (Fig. 3), particularly with the larger oligosaccharides. This is most easily seen by comparison on the BCF's of 'symmetrical' linkages and the sum of BCF's for equivalent linkages on either side of the 'symmetrical' linkages for regular oligomers of dp $n$ and reduced oligomers of dp $n + 1$. For example, comparison of the BCF's for *A. niger* ABN with $Ara_6$ and $Ara_7^*$ (Fig. 3) show that the enzyme has similar preferences for the 'symmetrical' linkage *3* in both substrates (0.370 compared to 0.353). Also, the combined BCF's for linkages 2 and

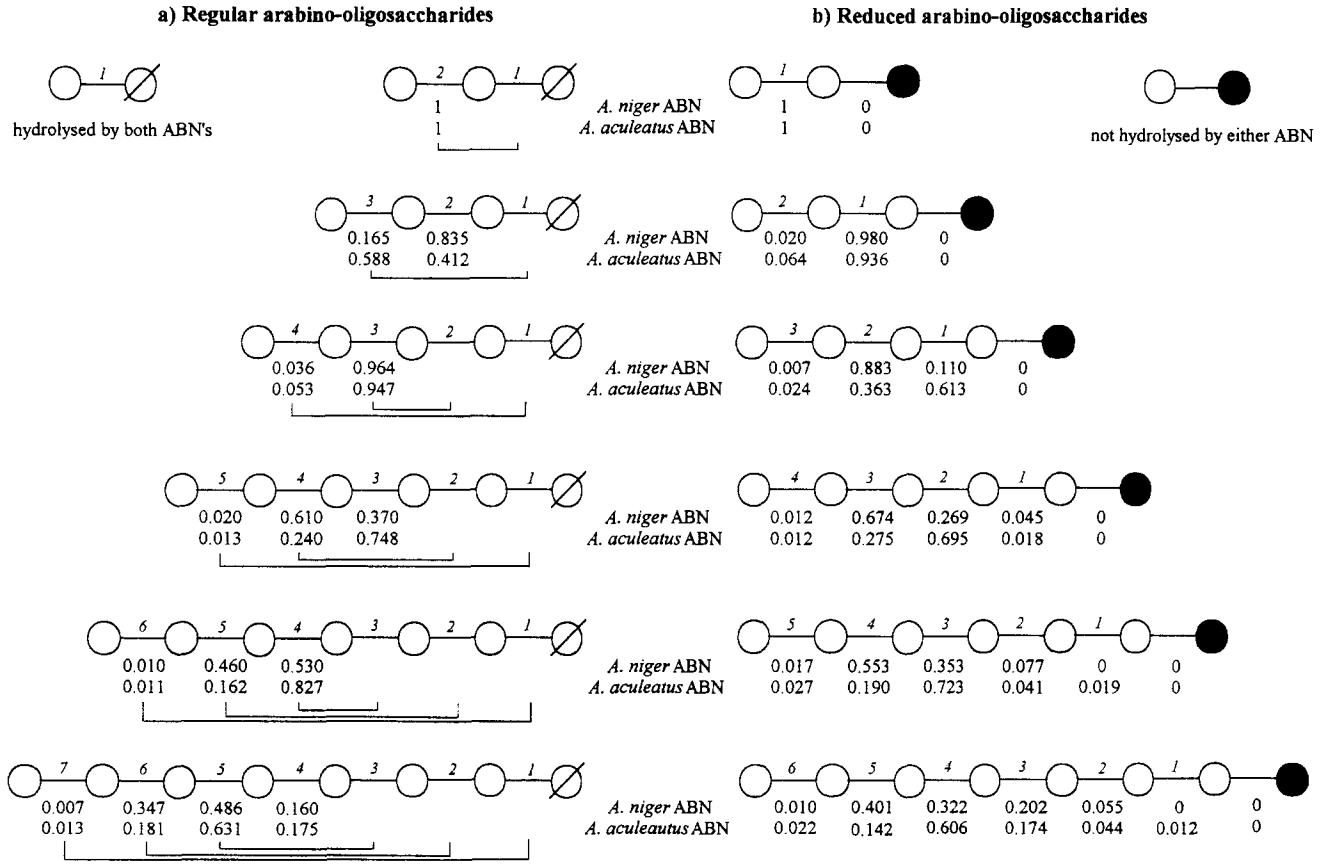


Fig. 3. BCF's of *A. niger* and *A. aculeatus* ABN hydrolysis of (a) regular ($Ara_2$ to $Ara_8$), and (b) reduced (1 → 5)-α-L-arabino-oligosaccharides ($Ara_2^*$ to $Ara_8^*$). For regular arabino-oligosaccharides the BCF's for symmetrical linkages are real, but those given for non-symmetrical linkages are the sum of the corresponding linkages on either side of the symmetrical linkage (see text). ○, arabinofuranosyl residue; ∅, reducing-end arabinofuranosyl residue; ●, reduced arabinofuranosyl residue; ————, (1 → 5)-α-L-arabinofuranosidic linkage. *1* to *7* identify the substrate linkages, numbering from the (former) reducing end. This numbering scheme does not include the reduced residues since these linkages no longer appear to be available for hydrolysis by the ABN's (see text).

*4* give similar values with each substrate (0.610 compared to 0.630).

While the BCF's can be determined with reduced substrates, for subsite analysis it is preferable to use substrates without substituents like alditol groups which may have unspecific interactions with the enzyme binding site [45]. Therefore, actual BCF's for the regular arabino-oligosaccharides, shown in Fig. 4, were determined by combining the results obtained from both the reduced and regular substrates. This was done by retaining all of the BCF's for the regular substrates, but determining the distribution of the cleavage preferences for non-symmetrical linkages from the data obtained from the reduced arabino-oligosaccharide BCF's. Unfortunately, BCFs for non-symmetrical linkages of $Ara_8$ cannot be determined using this procedure because $Ara_9^*$ was not available.

*Kinetics of arabino-oligosaccharide hydrolysis.*— The kinetic parameters $k_{cat}/K_m$ obtained for the *A. niger* and *A. aculeatus* ABN during hydrolysis of a series of arabino-oligosaccharides are shown in Table 1. For both ABN's, rates of hydrolysis increased sharply as the dp of the substrates increased, reaching an apparent maximum at dp 5 for the *A. niger* enzyme and ca. dp 6 for the *A. aculeatus* enzyme (Fig. 5). These maximum $k_{cat}/K_m$ values for the ABN's are reasonably consistent with the values obtained during $K_m$ determinations of $Ara_8$ ($k_{cat}/K_m$ values of $3.51 \times 10^4$ and $2.84 \times 10^5$ $s^{-1} \cdot M^{-1}$ for the *A. niger* and *A. aculeatus* ABN's respectively).

Kinetic parameters $k_{cat}/K_m$ for the hydrolysis of the reduced arabino-oligosaccharides by both ABN's (Fig. 5) are consistent with the results obtained during BCF determinations, since $k_{cat}/K_m$ values for reduced arabino-oligosaccharides of dp $n+1$ are

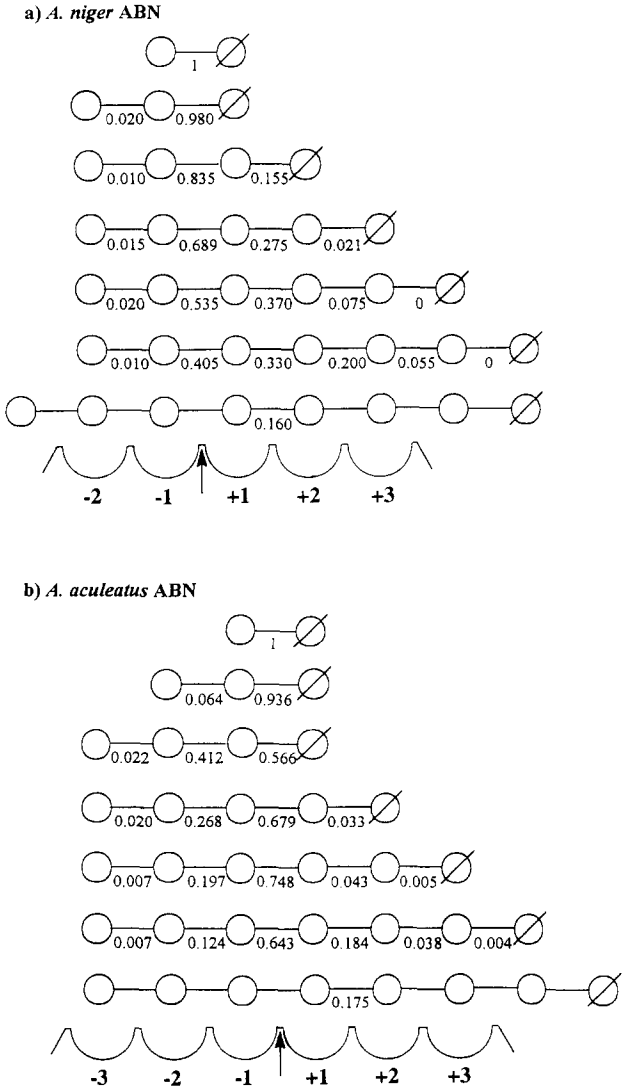


Fig. 4. Enzyme-substrate complexes and BCF's of *A. niger* and *A. aculeatus* ABN's during hydrolysis of regular arabino-oligosaccharides ($Ara_2$ to $Ara_8$). BCF's were obtained from combination of the hydrolysis data for regular and reduced arabino-oligosaccharides (see text). The arrow indicates the relative position of the catalytic amino acids. See Fig. 3 for key to other symbols.

quite similar to values obtained for regular arabino-oligosaccharides of dp *n*. This would appear to further confirm that the alditol group produced during reduction is not involved in substrate binding in either ABN.

*Subsite mapping*.—BCF and $k_{cat}/K_m$ values were used to calculate affinities of ten hypothetical subsites of the *A. niger* and *A. aculeatus* ABN's, including the two subsites adjacent to the catalytic site (−1 and +1). The positional isomers of enzyme–substrate complexes used to calculate the subsite binding affinities of the *A. niger* ABN, and the binding affinities themselves, are given in Table 2.

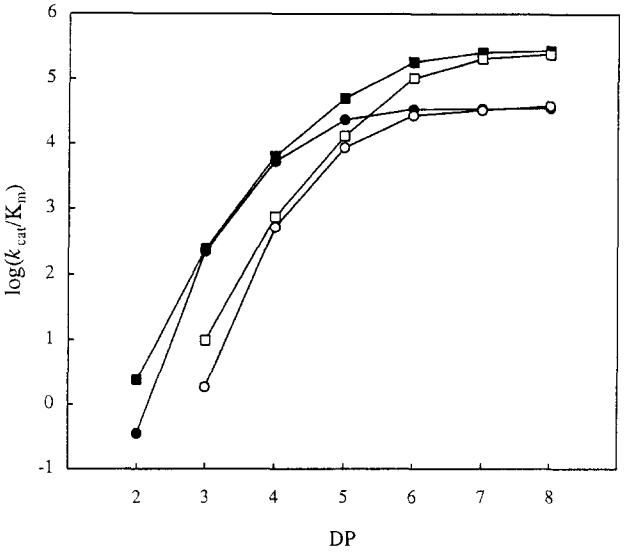


Fig. 5. Dependance of rate parameters $k_{cat}/K_m$ for the *A. niger* and *A. aculeatus* ABN's on regular and reduced arabino-oligosaccharide dp. ● and ○ represent $k_{cat}/K_m$ values for the *A. niger* ABN for normal and reduced arabino-oligosaccharides respectively. Similarly, ■ and □ represent $k_{cat}/K_m$ values for the *A. aculeatus* ABN for regular and reduced arabino-oligosaccharides respectively.

Table 2
Subsite binding affinities of *A. niger* ABN

| Subsite affinity | Calculated values [a] | | Average (±S.D.) |
|---|---|---|---|
| $A_{-5}$ | −0.72 | ($Ara_{7,2}$–$Ara_{6,2}$) [b] | −0.72 |
| $A_{-4}$ | −1.75 | ($Ara_{8,4}$–$Ara_{7,4}$) | |
| | −1.49 | ($Ara_{7,3}$–$Ara_{6,3}$) | −1.85±0.42 |
| | −2.31 | ($Ara_{6,2}$–$Ara_{5,2}$) | |
| $A_{-3}$ | −1.16 | ($Ara_{7,4}$–$Ara_{6,4}$) | |
| | −0.61 | ($Ara_{6,3}$–$Ara_{5,3}$) | 0.26±1.40 |
| | 0.93 | ($Ara_{5,2}$–$Ara_{4,2}$) | |
| | 1.89 | ($Ara_{4,1}$–$Ara_{3,1}$) | |
| $A_{-2}$ | 15.94 | ($Ara_{4,2}$–$Ara_{3,2}$) | 16.78±1.20 |
| | 17.63 | ($Ara_{3,1}$–$Ara_{2,1}$) | |
| $A_{-1}+A_{+1}$ | | | 1.49 |
| $A_{+2}$ | 5.17 | ($Ara_{5,2}$–$Ara_{4,1}$) | 5.65±0.68 |
| | 6.13 | ($Ara_{4,2}$–$Ara_{3,1}$) | |
| $A_{+3}$ | 2.53 | ($Ara_{7,3}$–$Ara_{6,2}$) | |
| | 1.71 | ($Ara_{6,3}$–$Ara_{5,2}$) | 2.50±0.77 |
| | 3.25 | ($Ara_{5,3}$–$Ara_{4,2}$) | |
| $A_{+4}$ | −0.49 | ($Ara_{8,4}$–$Ara_{7,3}$) | |
| | −0.23 | ($Ara_{7,4}$–$Ara_{6,3}$) | −0.13±0.41 |
| | 0.32 | ($Ara_{6,4}$–$Ara_{5,3}$) | |
| $A_{+5}$ | −0.64 | ($Ara_{7,5}$–$Ara_{6,4}$) | −0.64 |

[a] Calculated from the difference in molecular binding affinities of the two positional isomers of substrate–enzyme complexes shown in parentheses (BCFs ≥ 0.020) according to Suganuma et al. [8].

[b] Positional isomers of substrate–enzyme complexes. The first subscript indicates the dp of the arabino-oligomer and the second is the number of the linkage cleaved (counting from the reducing end).

Almost identical positional isomer combinations were used for subsite affinity calculations for the *A. aculeatus* enzyme (not shown). The sum of the binding affinities of subsites −1 and +1 were calculated using the $K_m$ values determined for $Ara_8$ (0.388 and 0.592 mM for the *A. niger* and *A. aculeatus* ABN's respectively), resulting in near-zero overall affinities for these two subsites in each enzyme. It should be noted that these estimations of the net binding affinities of subsites −1 and +1 must be made with caution since calculation of these values relies on the validity of intrinsic subsite binding affinity additivity, which may not always hold true (see [9]).

The binding affinities obtained for the *A. niger* and *A. aculeatus* ABN's are shown in Fig. 6, where positive values indicate binding between the enzyme and aligned arabinofuranosyl residues, while negative values indicate repulsion. Although the sum of the affinities of the two subsites −1 and +1 are often diplayed in the literature as a single histogram spanning the catalytic site [8,35,47], we have adopted the approach recently employed by Hrmova et al. [48] and averaged the sum of the binding affinities over these two sites and displayed them as two separate binding affinities. We have done this to emphasise that there are actually two arabinofuranosyl binding subsites in this region of the active site, and accept that major differences are likely to exist in the affinities of these two subsites. While binding affinities for ten putative subsites have been calculated from the experimental data, the possible existence of subsites more remote from the catalytic site cannot be discounted.

Comparison of the subsite maps of the *A. niger*

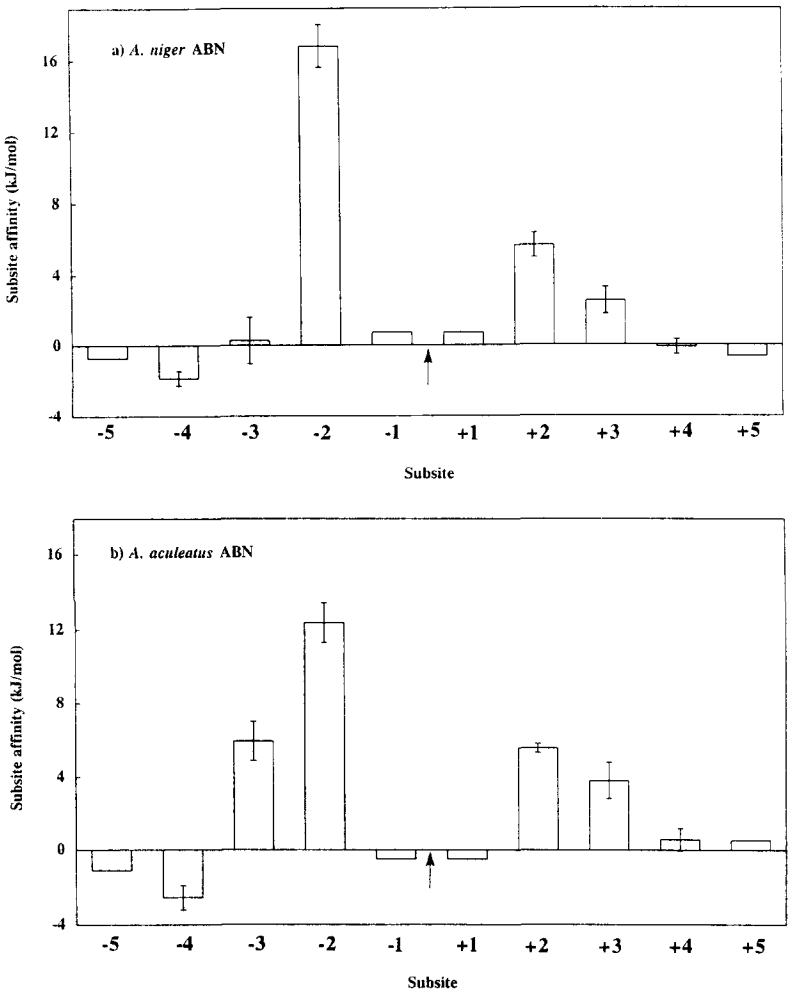


Fig. 6. Subsite maps of *A. niger* and *A. aculeatus* ABN's. The arrow indicates the relative position of the catalytic amino acids. Positive values indicate binding between the enzyme and aligned arabinofuranosyl residues, while negative values indicate repulsion. The sum of the affinities of subsites −1 and +1 were averaged and displayed as two separate binding affinities to emphasise that there are actually two arabinofuranosyl binding subsites in this region of the active site. Error bars indicate standard deviations.

and *A. aculeatus* ABN's (Fig. 6) reveals the existence in the *A. aculeatus* enzyme of an additional arabinofuranosyl-binding subsite (−3) not present in the enzyme from *A. niger*. However, apart from this difference, there appear many similarities in the distribution of the binding affinities over the other subsites of these two ABN's. These include, near-zero net affinities at across subsites −1 and +1, large binding affinities at subsite −2, stronger binding at subsite +2 than +3, and replusion at subsite −4.

## 4. Discussion

A number of different approaches have been utilized to identify cleavage preferences during subsite mapping of glycosyl endo-hydrolases. Most methods employed rely on labeling the reducing end residue of the substrate and determining the proportion and size of labelled and unlabelled products released during hydrolysis. Tritium is the most common label used, with the radioactive hydrolysis products quantified with a scintillation counter after separation by TLC [43,47–50]. This method is often preferred because the substrate is not altered significantly, but the inability to determine unlabelled products represents a major disadvantage since the absence of second generation degradation products cannot be verified, nor can multiple attack mechanisms be easily identified. Other labeling methods used include reduction with borohydride [45,46,51–54] or addition of chromophoric groups like 4-nitrophenyl [55–58] or 4-methylumbelliferyl [45,57,59]. These methods are advantageous since labelled and unlabelled products can be separated and quantified chromatographically, although difficulties in developing a subsite map can arise if the attached label influences subsite binding [9,45,46,60]. In the present study, we have followed the hydrolysis of reduced and regular arabino-oligosaccharides by HPAEC to determine BCF values for ABN subsite mapping by procedures similar to that employed recently by Bray and Clarke [53] and Vincken et al. [46]. The effects of the reduced residue on the action of both ABN's did not appear substantial since the BCF's and kinetic data for regular ($n$) and reduced ($n+1$) oligosaccharides were comparable and would suggest the validity of our approach to subsite affinity determinations.

The present work represents the first characterization of the substrate binding sites of ABN's, although McCleary [31] previously proposed that the *A. niger* enzyme possessed at least 5 or 6 subsites based on limited kinetic data. The substrate binding sites of the *A. niger* and *A. aculeatus* ABN's appear to be composed of 5 and 6 subsites respectively, with the catalytic site in both enzymes appears located between the third and fourth subsite from the reducing-end side of the bound substrate. Interestingly, the *A. niger* and *A. aculeatus* ABN's have near-zero net free energies of binding across the two subsites adjacent to the catalytic site. While this would account for the low, but detectable activity the enzymes show for $Ara_2$, these results differ from those obtained for a wide range of glycopyranosyl endo-hydrolases, including lysozyme [10], amylases [4,8,35], (1 → 3)-β-glucanases [48] and xylanases [47,49], which often have quite large net negative (repulsive) affinities across subsites −1 and +1.

Although there is no direct method currently known to determine the individual affinities of subsites −1 and +1 [9], computer simulation of amylase action [2,4,6,7] suggested that subsite −1, holding the glycosyl residue that undergoes cleavage, has a strong negative affinity, while subsite +1 has a weak positive affinity. By analogy to lysozyme action [10,11], the negative interaction at subsite −1 may increase catalytic efficiency by distortion of the glycopyranosyl residue into a sofa or half chair conformation more closely resembling the planar oxocarbonium ion-like transition state [61], as well as stereoelectronically stabilising this transition state [11–13]. This putative mechanism may also be applicable for ABN action even though the arabinose residues in (1 → 5)-α-L-arabinan are in the furanose form. However, unlike the pyranose sugars, where the different conformations are separated by substantial energy barriers, there is little difference in free energy between the various furanose envelope and twist conformations [62]. As a result, rapid interconversion can easily occur between these arabinofuranosyl conformers [63–65]. Therefore, it would be expected that, compared to glycopyranosyl endo-hydrolases, only a small negative interaction at subsite −1 of the ABN's would be required to stereoelectronically stabilize the arabinofuranosyl-cation-like transition-state in an envelope conformation like $^3E$ or $E_3$ (i.e., a conformation with C-1, C-2, C-4 and O-4 coplanar [61]). This would be consistent with the observed less negative (i.e., near-zero) net affinities across subsites −1 and +1 of the ABN's compared to glycopyranosyl endo-hydrolases.

The differences in hydrolysis products released from linear (1 → 5)-α-L-arabinan by the *A. niger* and *A. aculeatus* ABN's [25] can be rationalized with

regard to the acquired subsite maps. As mentioned previously, the major difference between the action of these enzymes on the polymeric substrate is the much lower proportion of $Ara_2$ released by the *A. aculeatus* ABN than with the *A. niger* enzyme. Clearly, the occurrence of three substrate binding sites to the left of the catalytic site in the *A. aculeatus* enzyme (particularly with strong affinities at subsites $-2$ and $-3$), combined with the negative energy of subsite $-4$, would lead to preference in binding oligosaccharides with the non-reducing end residue adjacent to subsite $-3$. Thus, this would result in a preference in the formation of $Ara_3$ from these substrates. In contrast, the *A. niger* ABN has only two substrate binding sites to the left of the catalytic site, and a near-zero binding energy at imaginary subsite $-3$. This would be expected to result in approximately equal binding preferences for the non-reducing end residue of oligosaccharides adjacent to subsites $-2$ and $-3$, leading to the observed similar proportions of $Ara_2$ and $Ara_3$ formation. Other differences in the distribution of larger oligosaccharide products formed from linear arabinan by the two ABN's [25] are more difficult to interpret with reference to the subsite maps developed here, and may result from other enzyme–substrate interactions further from the catalytic site than could be examined by the methods employed in this study, or from interactions with the helical three-dimensional structure of the linear polysaccharide [65].

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