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Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans

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ABSTRACT

Alkali-extractable cereal arabinoxylan and oligosaccharides of known structure derived from it by enzymic hydrolysis were treated with endo-(1 → 4)-β-D-xylanases I and III from *Aspergillus awamori* CMI 142717 and the digests subjected to analysis by high performance anion-exchange chromatography. Clear differences in the mode of action of the two endo-(1 → 4)-β-D-xylanases were observed. When counting from the reducing end, at least one unsubstituted xylopyranosyl residue adjacent to singly substituted xylopyranosyl residues or two unsubstituted xylopyranosyl residues adjacent to doubly substituted xylopyranosyl residues cannot be removed by endo-(1 → 4)-β-D-xylanase I. At least two unsubstituted xylopyranosyl residues adjacent to singly or doubly substituted xylopyranosyl residues cannot be removed by endo-(1 → 4)-β-D-xylanase III. β-D-Xylosidase from the same xylanolytic system was able to remove terminal xylopyranosyl residues from the nonreducing end of branched oligosaccharides only when two contiguous unsubstituted xylopyranosyl residues were present adjacent to singly or doubly substituted xylopyranosyl residues.

INTRODUCTION

Endo-(1 → 4)-β-D-xylanases with different physicochemical parameters have been purified from various microbial sources[1]. They hydrolyse glycosidic bonds in the xylan chain in a random fashion yielding a series of linear and branched oligosaccharide fragments.

Endo-(1 → 4)-β-D-xylanases differ in their mode of action in that they are restricted in the hydrolysis of glycosidic linkages in the vicinity of branch points, which is reflected in the many types of heterogeneous oligosaccharides released by these enzymes[2–5]. They also differ in the number of subsites and the location of

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the active site among the subsites, which plays an important role in xylo-oligosaccharide hydrolysis[6,7]. The elucidation of the mode of action of endo-xylanases may also be hampered by the possibility of termolecular hydrolysis and transglycosylation reactions which can occur with increasing concentrations of xylo-oligosaccharides[8].

In this paper, a model is proposed for the mode of action of endo-(1 → 4)-β-D-xylanases I and III and the β-D-xylosidase from *Aspergillus awamori* CMI 142717, based on data for the hydrolysis of polymeric arabinoxylan, a whole range of arabinoxylan-derived oligosaccharides, and some xylo-oligosaccharides.

EXPERIMENTAL

Materials.—Endo-(1 → 4)-β-D-xylanases I and III (Endo I and Endo III, respectively), (1 → 4)-β-xylosidase (β-xylosidase), and (1 → 4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) were purified from *A. awamori* CMI 142717 (refs. 9 and 10). α-L-Arabinofuranosidase A (Arafur A) was purified from *Aspergillus niger*[11]. The preparation and characterisation of alkali-extractable wheat-flour arabinoxylan (BE1-U) is described elsewhere[12].

The isolation of oligosaccharides derived from alkali-extractable wheat-flour arabinoxylan by degradation with Endo I and III, by Bio-Gel P-2 gel permeation chromatography and high performance anion-exchange chromatography, and the elucidation of their structures by ^{1}H NMR spectroscopy were described by Gruppen et al.[13] and Kormelink et al.[14]. The elucidation of the structures obtained from alkali-extractable barley arabinoxylan by degradation with Endo I was described by Viëtor[15].

Xylo-oligosacccharides were derived from steam-exploded birchwood xylan which was a kind gift of Dr. Puls (Institute of Wood Chemistry and Chemical Technology of Wood, BFH, Hamburg, FRG).

Enzyme incubations.—A solution of alkali-extractable wheat arabinoxylan (80 mg) in 50 mM sodium acetate buffer (80 mL, pH 5.0) was incubated with Endo III (0.4 μg/mL) and AXH (0.1 μg/mL) for 24 h at 30°C. After inactivation of the enzyme, the digest was concentrated to 2.0 mL under reduced pressure and fractionated by Bio-Gel P-2 chromatography.

The release of arabinoxylan oligosaccharides from alkali-extractable wheat arabinoxylan by Endo I or III with time (0–48 h) was studied by incubation of 0.2% (w/v) alkali-extractable wheat arabinoxylan with 0.1 μg/mL Endo I or III at 30°C in 50 mM sodium acetate buffer (pH 5.0).

Arabinoxylan-derived oligosaccharides (30 μg/mL) were incubated with 1.0 μg/mL Endo I, Endo III, β-xylosidase, or a combination of Endo III and Arafur A for 24 h at 30°C in 50 mM sodium acetate buffer (pH 5.0).

Xylo-oligosaccharides, i.e., X_2, X_3, X_4, X_5, and X_6 (100 μg/mL), were incubated with 1.0 μg/mL Endo I or III for 24 h at 30°C in 50 mM sodium acetate buffer (pH 5.0).

After incubation and enzyme inactivation (100°C, 10 min), the mixtures were analysed by HPAEC using well-characterised oligosaccharides[13,14] as standards.

Bio-Gel P-2 gel permeation chromatography.—Arabinoxylan-digests and steam-exploded birchwood xylan were fractionated by Bio-Gel P-2 gel permeation chromatography (100 × 2.6 cm, 200–400 mesh; Bio-Rad; thermostated at 60°C). Water was used as eluent (20 mL/min). Thiomersal (0.01% w/v) was added as preserving agent. Fractions (2.4 mL) were collected and monitored for total neutral sugar content[16].

High performance anion-exchange chromatography (HPAEC).—Digests containing arabinoxylan oligosaccharides were analysed by HPAEC as described previously[13]. Samples (20 μL) were injected on the CarboPac PA-1 column. Elution (1 mL/min) involved linear gradients of sodium acetate in 0.1 M NaOH of 0–100 mM during 5 min, then 100–400 mM during 35 min at 20°C.

RESULTS

Alkali-extractable wheat-flour arabinoxylan, which has an Ara–Xyl ratio of 0.52, was treated with Endo I or III for 24 h and the digest fractionated by Bio-Gel P-2 gel permeation chromatography[13,14]. Endo I converted ca. 72% of the arabinoxylan into oligosaccharides with a degree of polymerisation (dp) lower than 10. Endo III, however, converted only 50% of the arabinoxylan into oligosaccharides with a dp lower than 10. No significant amounts of monomers to tetramers could be observed in the latter case. These observations indicate a difference in the mode of action of Endo I and III towards arabinofuranosyl-substituted xylans and apparently reflect the number of subsites available to form an effective enzyme–substrate complex.

Figs. 1 and 2 schematically summarise the structures of oligosaccharides released from wheat arabinoxylan by Endo I and Endo III, respectively. As can be seen from Fig. 1, xylose, xylobiose, and xylotriose were only released by Endo I. Three groups of arabinoxylan-oligosaccharides could be identified: (*i*) oligosaccharides containing singly substituted xylopyranosyl residues only (Endo I: structures **3.2**, **4.1**, **5.2**, and **6.2**; Endo III: structures **5.4**, **6.4**, **7.3**, **8.3^{I}**, **8.3^{II}**, **9.3^{I}**, and **9.3^{II}**); (*ii*) oligosaccharides containing singly and doubly substituted xylopyranosyl residues (Endo I: structures **6.3**, **7.1**, and **8.1**; Endo III: structures **8.1**, **9.4^{I}**, **9.4^{II}**, and **8.4**); and (*iii*) oligosaccharides containing doubly substituted xylopyranosyl residues only (Endo I: structures **5.1**, **6.1**, **8.2**, **9.1**, and **10.1**; Endo III: structures **6.1**, **7.2**, **9.1**, and **10.1**).

Close observation of the structures suggest the need for Endo I of at least one unsubstituted xylopyranosyl residue adjacent to singly substituted xylopyranosyl residues and at least two unsubstituted xylopyranosyl residues adjacent to doubly substituted xylopyranosyl residues. In the case of Endo III, at least three unsubstituted xylopyranosyl residues adjacent to singly or doubly substituted xylopyranosyl residues are needed.

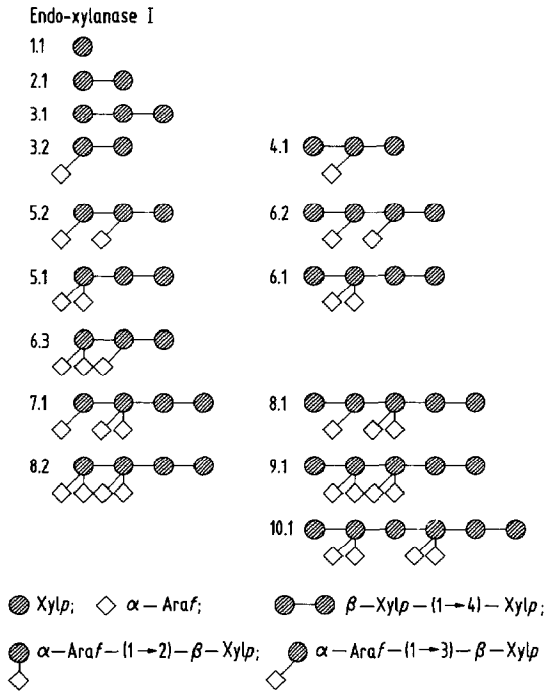


Fig. 1. Arabinoxylan-derived oligosaccharides released by Endo I from alkali-extractable wheat-flour arabinoxylan.

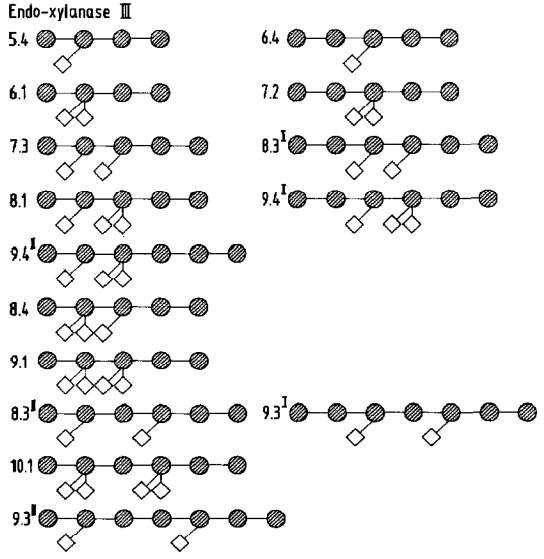


Fig. 2. Arabinoxylan-derived oligosaccharides released by Endo III from alkali-extractable wheat-flour arabinoxylan.

Structures similar to those in Fig. 1 were also released from barley arabinoxylan by Endo I[15]. The isolation of a tetrasaccharide 2^3-α-L-Ara*f*-Xyl_3 from barley arabinoxylan treated with Endo I[15] could suggest that the hypothesis is only valid for Endo I when arabinofuranosyl groups are α-(1 → 3)-linked to xylopyranosyl residues. Endo I is thus more inhibited in its action by α-(1 → 2)-linked arabinofuranosyl groups than by α-(1 → 3)-linked arabinofuranosyl groups.

The extent of hydrolysis by Endo III could be strongly increased by addition of AXH (Fig. 3), an enzyme able to remove arabinofuranosyl groups from singly substituted xylopyranosyl residues[17]. This results in the formation of new linear sites in the xylopyranosyl backbone and thus in the creation of new subsites for Endo III. After AXH treatment, xylose, but especially xylobiose, and xylotriose could be released in significant amounts (Fig. 3b), which was not the case by treatment with Endo III alone (Fig. 3a).

The availability of purified arabinoxylan-derived oligosaccharides as reference compounds made it possible to study more closely the action of Endo I and Endo III by monitoring the release of oligosaccharides from alkali-extractable wheat-flour arabinoxylan (Figs. 4 and 5, respectively). A slower release of oligosaccharides by Endo I can be observed in the first hour; however, much larger amounts (particularly for xylose to xylotetraose) are released in the longer term.

Higher oligosaccharides (dp > 4) released by Endo III, and not present in the Endo I digests, were incubated with Endo I (Table I). By HPAEC analysis, it was demonstrated that Endo I was able to release xylose from oligosaccharides contain-

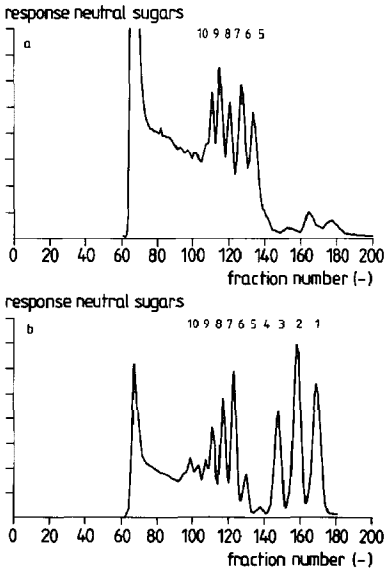


Fig. 3. Bio-Gel P-2 elution patterns of arabinoxylan digests obtained with Endo III (from Kormelink et al.[14]) (a), and a combination of Endo III and AXH (b). The peak number corresponds to the degree of polymerisation of the oligosaccharide.

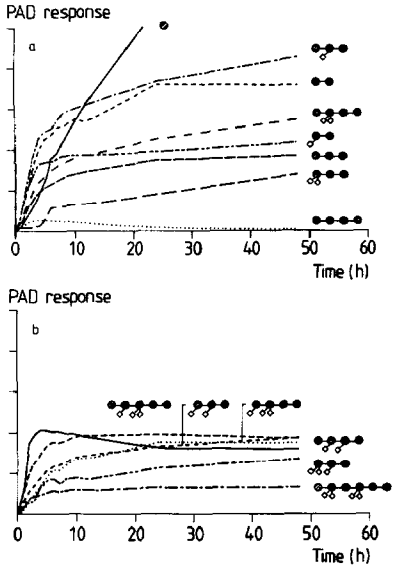


Fig. 4. Release of arabinoxylan oligosaccharides by incubation of alkali-extractable wheat-flour arabinoxylan with Endo I as a function of incubation time: a, small oligosaccharides; and b, higher oligosaccharides.

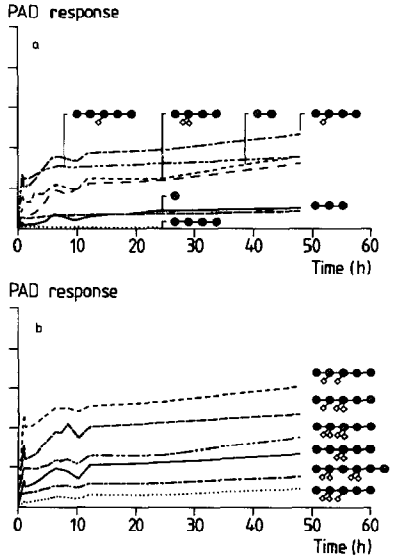
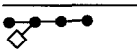
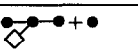
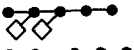
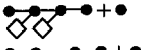
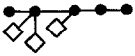
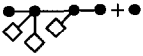
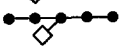
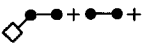
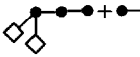
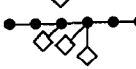


Fig. 5. Release of arabinoxylan oligosaccharides by incubation of alkali-extractable wheat-flour arabinoxylan with Endo III as a function of incubation time (a and b, see Fig. 4).

TABLE I

Hydrolysis of oligosaccharides by Endo I as determined by HPAEC using pulsed amperometric detection

Oligosaccharide	R_t [a] (min)	Hydrolysis products	R_t [a] (min)
	12.35		12.01
	17.04		17.09
	20.21		20.49
	12.59		12.41
	14.15		14.70
	18.93		20.07

[a] R_t = Retention time.

ing two unsubstituted xylopyranosyl residues adjacent to a singly substituted xylopyranosyl residue (structures **5.4**, **6.4**, **7.3**, and **8.4**). Xylose could not be released when the xylopyranosyl residue was doubly substituted (structures **7.2** and **9.4**[I]). At the sites towards the nonreducing end, xylobiose was released by Endo I from oligosaccharides containing two unsubstituted xylopyranosyl residues at the nonreducing end adjacent to a singly or doubly substituted xylopyranosyl residue (structures **6.4**, **7.2**, and **9.4**[I]).

Even after prolonged incubation, Endo III was not able to release xylose or xylobiose from structures **5.4** and **6.4** containing one or two unsubstituted xylopyranosyl residues at the nonreducing and reducing end. Concerted action of Arafur A (1 μg/mL) and Endo III (1 μg/mL) on oligosaccharide **8.4** (30 μg/mL) for 24 h showed the formation of structure **6.1** (results not shown). The removal of the arabinofuranosyl group from the singly substituted xylopyranosyl residue by Arafur A (ref 17) enables Endo III to attack the three unsubstituted xylopyranosyl residues towards the reducing end in order to release xylose from this modified oligosaccharide. Thus, Endo III is able to release xylose from the reducing end when three unsubstituted xylopyranosyl residues are present next to a substituted xylopyranosyl residue.

Endo I and III have also been compared with respect to their mode of action towards linear xylo-oligosaccharides (Table II). Dimers up to hexamers of xylose were incubated with Endo I and III, respectively, for 24 h and the ratios of the main end-products were determined, i.e., X_3, X_2, and X.

As can be seen from Table II, X_6 is split by Endo I to X_3, X_2, and X in ratios of 0.6 : 1.5 : 1.0. Endo I also splits X_5 to X_3, X_2, and X, but with a somewhat higher value for X_2 (2.0). X_4 is split by Endo I to X_2 and X mainly, with smaller amounts of X_3; X_3 is degraded only slowly to give X_2 and X.

TABLE II

Ratios of X_3, X_2, and X, released from linear β-(1 → 4)-linked D-xylose oligosaccharides by Endo I or III from *A. awamori*

Substrate	Enzyme	X_3:X_2:X
X_6	Endo I	0.6:1.5:1.0
	Endo III	4.9:5.1:1.0
X_5	Endo I	0.7:2.0:1.0
	Endo III	6.0:6.5:1.0
X_4	Endo I	0.3:1.5:1.0
	Endo III	1.6:7.2:1.0
X_3	Endo I	0.8:0.7:1.0
	Endo III	4.8:1.2:1.0
X_2	Endo I	n.d. [a]
	Endo III	n.d.

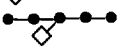
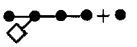
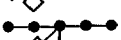
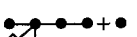
[a] n.d., Not detected.

By observation of the data for Endo III, it can be seen that Endo III releases X_3 and X_2 mainly, with smaller amounts of X from X_6. A similar observation can be made for the degradation of X_5, with even smaller amounts of xylose. A remarkable fact is the degradation of X_4 into mainly X_2. There is little degradation of X_3 into X_2 and X. Both Endo I and III cannot degrade X_2.

As β-xylosidase is important in the optimal breakdown of arabinoxylans, digests of the arabinoxylan oligosaccharides with β-xylosidase were analysed for any breakdown by HPAEC (Table III). Data presented in Table III indicate that β-xylosidase is only able to release xylose from the nonreducing end of arabinoxylan oligosaccharides, when at least two unsubstituted xylopyranosyl residues are present adjacent to a substituted xylopyranosyl residue.

TABLE III

Oligosaccharide hydrolysis by β-xylosidase as determined by HPAEC using pulsed amperometric detection

Oligosaccharide	R_t [a] (min)	Hydrolysis products	R_t [a] (min)
	12.35	n.d. [b]	
	12.59		12.34
	13.98	n.d.	
	14.15		13.95
	19.09	n.d.	
	18.93		18.93

[a] R_t = Retention time. [b] n.d., Not detected.

DISCUSSION

The endo-(1 → 4)-β-D-xylanases I and III, purified from *A. awamori* CMI 142717, not only differ in their physicochemical parameters[10], but were also shown to differ greatly in their mode of action towards arabinoxylan, arabinoxylan oligosaccharides, and xylo-oligosaccharides.

Monomers to tetramers were clearly present in the wheat arabinoxylan digest obtained with Endo I, but only in trace amounts in the digest obtained with Endo III (Figs. 1, 2, and 3). In the case of oat spelts arabinoxylan, also, larger amounts of xylotriose, xylobiose, and xylose were released by Endo I[10].

Endo III is thus more sensitive to arabinofuranosyl substitution than Endo I, which explains the lower degree of hydrolysis of the arabinoxylan by Endo III. This is also reflected in the large void fraction in the Bio-Gel P-2 column fractionation[14].

Our results show that the arabinofuranosyl groups on the xylan backbone clearly inhibit the action of endo-xylanases on glycosidic linkages in the vicinity of the site of substitution.

Endo I is able to split the glycosidic linkage towards the nonreducing end of a singly or doubly substituted xylopyranosyl residue. This fact corresponds with observations made by others[18–20]. Glycosidic linkages towards the reducing end of a xylopyranosyl residue can only be split when the xylopyranosyl residue is unsubstituted and adjacent to an unsubstituted or singly substituted xylopyranosyl residue.

Endo III on the other hand is unable to split the glycosidic linkage towards the nonreducing end of a singly or doubly substituted xylopyranosyl residue. It can split the glycosidic linkage of the adjacent unsubstituted xylopyranosyl residue or the successive unsubstituted xylopyranosyl residues towards the nonreducing end. Neither the glycosidic linkage towards the reducing end of a singly or doubly substituted xylopyranosyl residue nor the next glycosidic linkage of the neighbouring unsubstituted xylopyranosyl residue towards the reducing end can be split. Only the glycosidic linkage at the next neighbouring xylopyranosyl residue can be split when the residue is not substituted.

In the case of doubly substituted xylopyranosyl residues, the next two (1 → 4)-glycosidic linkages towards the reducing end seemed to be resistant to Endo I or III degradation. The same observation was made for α-(1 → 2)-arabinofuranosyl-substituted xylopyranosyl residues in the case of Endo I degradation. Comtat and Joseleau[21] made the same observation with α-(1 → 2)-linked glucopyranosyluronic acid substituents, and even suggested that only the position and not the type of substituent is determinative.

It is unknown whether the presence of arabinofuranosyl groups interferes with the fit of the (1 → 4)-β-D-xylan backbone into the binding site. It has been suggested[18,22] that the presence of arabinofuranosyl groups may not impede enzyme binding to the arabinoxylan in the vicinity of the substituent groups, because of the rapid hydrolysis of α-L-arabinofuranosyl-xylopentaose and α-L-

arabinofuranosyl-xylotetraose by hemicellulase I and II from *Ceratocystis paradoxa*, respectively. However, Brillouet[23] observed that the degree of polymerisation of the lowest oligosaccharide undergoing hydrolysis was increased by 1 when an arabinofuranosyl group is present at the nonreducing terminal xylopyranosyl residue, and therefore suggested that the presence of these substituent groups prevents a good fit between the substrate and the enzyme binding site.

The degradation patterns of xylo-oligosaccharides indicate a difference in the location of the active site among the subsites of Endo I and III. The degradation of xylotetraose shows the most important difference. The main end-products of xylotetraose degradation with Endo I and III were X, X_2, and X_3 in ratios of 1.0 : 1.5 : 0.3 and 1.0 : 7.2 : 1.6, respectively. In the latter case, the enzyme thus shows a very high affinity towards the glycosidic linkage between the second and the third xylopyranosyl residue counting from the reducing end. In the first case, the affinity towards this linkage has to be much lower, resulting in higher concentrations of other products.

The model proposed by Comtat and Joseleau[21] for the mode of action of endo-xylanase II from *Sporotrichum dimorphosporum* appears also to apply for Endo I (Fig. 6A). In our model, the binding of Endo I also requires a region of the xylan chain consisting of three D-xylosyl residues. For cleavage of glycosidic linkages, the O-2 of residue III, and O-2 and O-3 of residue II have to be unsubstituted. Residue I may be unsubstituted, singly substituted, or doubly substituted. Cleavage takes place between residues I and II. If residue III (Fig. 6A) is singly substituted at O-2, or doubly substituted at O-2 and O-3, two out of three

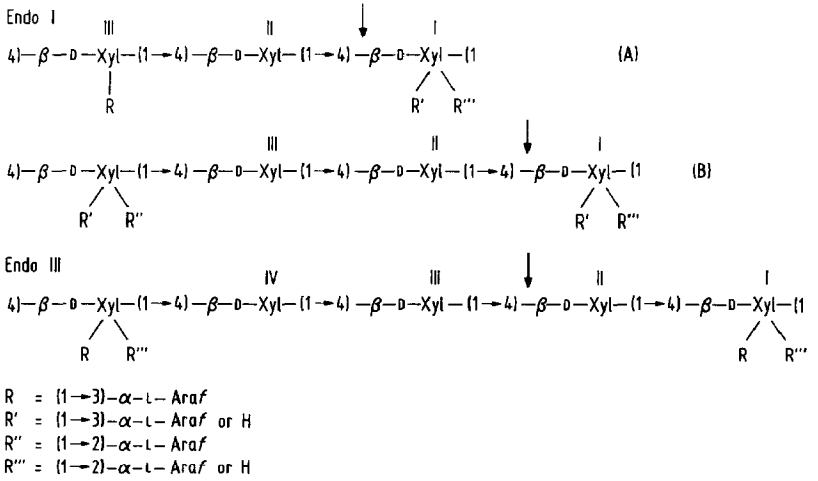


Fig. 6. Model for the mode of action of Endo I (A and B) and III from *A. awamori* towards arabinoxylan.

unsubstituted xylopyranosyl residues are needed for cleavage of the glycosidic linkage (Fig. 6B).

Because of the degradation of xylotetraose into xylobiose mainly and the need of at least three contiguous unsubstituted xylopyranosyl residues for cleavage, it is supposed that the binding of Endo III needs a region of the xylan chain consisting of four D-xylosyl residues, in which the O-2 and O-3 of residues II, III, and IV are unsubstituted. Residue I does not have to be unsubstituted. Cleavage takes place between residues II and III (Fig. 6).

Biely et al.[7] found that the substrate binding site of an *A. niger* endo-xylanase consisted of seven subsites, whereas Meagher et al.[24] found five subsites. Conclusions concerning the exact number of subsites cannot be made in the case of Endo I and Endo III, because kinetic parameters have not been determined on oligosaccharide hydrolysis. However, Endo III must have a higher turnover number on alkali-extractable wheat-flour arabinoxylan than Endo I, because of the higher initial rate for release of oligosaccharides.

Fig. 7 shows an example for the sites of attack of Endo I and III towards a fictitious arabinoxylan, based on all these observations. As can be seen from Fig. 7, Endo III has many fewer cleavage-sites than Endo I, which explains the absence of small end-products.

No arabinofuranosyl groups were linked to the reducing xylopyranosyl residue of oligosaccharides obtained by Endo I or Endo III. Only in the case of hydrolysis of rice straw arabinoxylan with an *A. niger* xylanase[25] were α-L-arabinofuranosyl-(1 → 4)-β-xylobiose and α-L-arabinofuranosyl-(1 → 4)-β-xylotriose produced with the arabinofuranosyl group on the reducing residue. The enzyme could thus hydrolyse the (1 → 4)-glycosidic linkage towards the reducing end of a singly substituted xylopyranosyl residue and must therefore have a mode of action which differs from those of Endo I and Endo III.

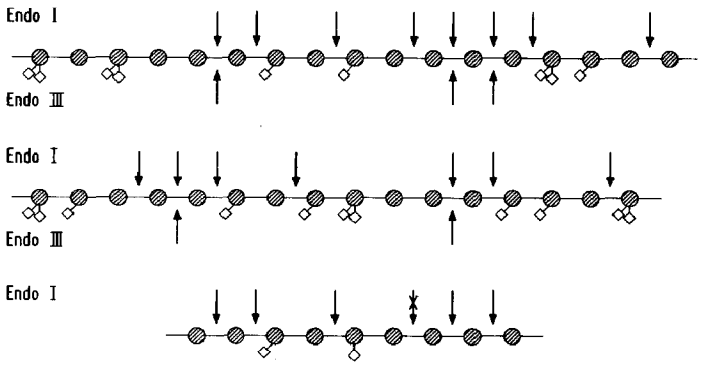


Fig. 7. Sites of attack of Endo I and III from *A. awamori* towards a fictitious arabinoxylan (see Fig. 1 for legend).

Structure **3.2** (3^2-α-L-Ara*f*-Xyl_2)[21,23,26–30] and structure **4.1** (3^2-α-L-Ara*f*-Xyl_3)[30,31] have been described before in arabinoxylan digests. Because only 1 or 2 oligosaccharides were purified in these cases, no model could be presented as complete as the model proposed here. A similar range of oligosaccharides as obtained by Endo III was found by enzymic treatment of wheat arabinoxylan by Hoffmann et al.[32,33]. However, in the latter case, the enzyme was not pure, as indicated by the presence of free arabinose in the arabinoxylan digest. Also, the degradation was not complete, as shown by the presence of structures with three unsubstituted xylopyranosyl residues adjacent to a substituted xylopyranosyl residue towards the reducing end.

The structures of the arabinoxylan oligosaccharides obtained by Endo I, Endo III, and others[21,23,25–33] indicate the existence of at least three groups of endo-xylanases which differ in their mode of action towards arabinoxylan.

β-Xylosidase was shown to split off xylopyranosyl residues from the terminal nonreducing end of oligosaccharides, but is limited by arabinofuranosyl groups. Different observations were made by Takenishi and Tsujisaka[25], who incubated 3^1-α-L-Ara*f*-Xyl_2 with an *A. niger* β-xylosidase which resulted in the release of 3-α-L-Ara*f*-Xyl and xylose. Differences can thus be expected also in the mode of action of β-xylosidases towards substituted xylo-oligosaccharides.

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