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Degradation of arabinans by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*

G. Beldman,* M.J.F. Searle-van Leeuwen, G.A. De Ruiter, H.A. Siliha & A.G.J. Voragen

Department of Food Science, Wageningen Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands

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An endo-arabinanase was purified from an enzyme preparation, derived from *Aspergillus aculeatus*. After SDS-gel electrophoresis a molecular weight of 45 kDa was estimated. The enzyme was reactive with antibodies raised against endo-arabinanase from *Aspergillus niger*, having the same molecular weight. Besides similarities, remarkable differences were observed for endo-arabinanases from *A. niger* and *A. aculeatus*. The enzyme from *A. aculeatus* was optimally active at a higher pH and produced a different spectrum of oligomers after incubation with linear arabinan. This was reflected in a relatively low concentration of oligomers with a degree of polymerization (DP) of 13 and a high concentration of oligomers with a DP of 6–7. Contrary to this, the concentration of oligomers produced by the *A. niger* endo-arabinanase gradually increased, going from DP 20 to DP 3.

Both endo-arabinanases, as well as arabinofuranosidase B from *A. niger*, were studied with respect to the degradation of branched arabinans. Removal of arabinofuranosyl side chains by arabinofuranosidase B was essentially independent of the type of glycosidic linkage but had a tremendous effect on the digestibility by endo-arabinanase.

INTRODUCTION

Arabinans occur in various plant tissues and are composed of α-L-arabinofuranosyl residues which are α-1,5-linked, a varying number of them being substituted with other α-L-arabinofuranosyl residues at the C2 and/or the C3 position. They are structural components of the plant cell wall and in pectic material closely associated with the rhamnogalacturonan part of the pectin molecule (Stephen, 1983). Arabinans and arabinan-degrading enzymes have gained interest in plant biochemistry (Joseleau *et al.*, 1983; Herve du Penhoat *et al.*, 1987), food technology (Villettaz *et al.*, 1981; Ducroo, 1987) and nutritional science (Ukai *et al.*, 1978).

In the food industry arabinans play an important role in processing of fruits and vegetables and occur in by-products of agricultural crops (Voragen *et al.*, 1987). Arabinan degrading enzymes can be used to facilitate processing of fruits and vegetables or to valorize by-products. A particular problem in apple-juice manufacture is the development of haze in a juice from 'pectinase'-treated apple pulp. Haze formation could be ascribed to a linear α-1,5-arabinan, which was formed from the branched arabinan by an arabinofuranosidase present in the pectinase preparation (Voragen *et al.*, 1982; Churms *et al.*, 1983).

Arabinanases from plant, bacterial and fungal sources have been described by several investigators and have been reviewed by Kaji (1984), Whitaker (1984), Voragen *et al.* (1987) and Beldman *et al.* (1992). It is generally accepted that endo-arabinanase is the key enzyme in preventing haze formation in apple juice, by degrading the linear arabinan. Endo-arabinanases have been isolated from several bacteria, such as *Clostridium felsineum* (reported in Kaji, 1984) and *Bacillus subtilis* (Kaji & Saheki, 1975; Weinstein & Albersheim, 1979; Yoshihara & Kaji, 1983; Sakai & Sakamoto, 1990). So far, the only fungal endo-arabinanase, studied in detail, has been isolated from *Aspergillus niger* (Rombouts *et al.*, 1988) and its induction has been studied by Van der Veen *et al.* (1991).

In this study we describe the purification of an endo-arabinanase and partial purification of arabino-

*To whom correspondence should be addressed.

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furanosidases from *A. aculeatus*. Also, the degradation of arabinans by enzymes from *A. aculeatus* as well as from *A. niger* was investigated.

MATERIALS AND METHODS

Substrates

Linear arabinan, isolated from hazy apple juice, was a gift from NOVO-Nordisk (Dittingen, Switzerland). Branched sugar-beet arabinan was from Koch and Light (Colnbrook, Bucks, UK). Apple-juice ultrafiltration retentate arabinan (MHR) was prepared as described by Voragen *et al.* (1986). Wheat arabinoxylan was obtained by an alkaline extraction method (Gruppen *et al.*, 1991). Xylan from oat spelts was from Sigma (St Louis, Missouri, USA). Arabinogalactans from potato and onion were prepared by Van de Vis *et al.* (1991), according to the method of Labavitch *et al.* (1976). Larch-wood arabinogalactan (stractan) was from St Regis Paper Company (Tacoma, Washington, USA).

Avicel cellulose was obtained from Serva (Heidelberg, Germany). H_3PO_4-swollen cellulose was prepared according to Wood (1971). CM-cellulose, type Akucell AF 0305, was from AKZO (Arnhem, The Netherlands). Polygalacturonic acid was purchased from ICN (Cleveland, Ohio, USA) and *p*-nitrophenyl-α-L-arabinofuranoside was from Sigma (St Louis, Missouri, USA).

All other chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany) or Sigma (St Louis, Missouri, USA).

Enzymes

Endo-arabinanase, arabinofuranosidase A and arabinofuranosidase B from *Aspergillus niger* were isolated from the experimental enzyme preparation Pectinase 29 (Gist-Brocades, Delft, The Netherlands) as described by Rombouts *et al.* (1988). Pectinex Ultra SP (NOVO Nordisk, Dittingen, Switzerland) is a product from *Aspergillus aculeatus* and was used for the purification of an endo-arabinanase and arabinofuranosidases B1 and B2.

The purification of arabinanases from *A. aculeatus* was carried out according to the procedure for rhamnogalacturonase as described by Schols *et al.* (1990), including chromatography on the Bio-Gel HTP column (Bio-Rad, Richmond, CA, USA). After this step, the arabinanase fraction was purified by anion exchange on the Mono-Q HR 5/5 column, using the FPLC system of Pharmacia-LKB (Uppsala, Sweden). For this purpose the enzyme was dialysed against 10 mM piperazine buffer pH 5·5 and applied onto the column, which was equilibrated in the same buffer. Gradient elution was carried out with 0–0·2 M sodium chloride in this buffer. Fractions of 1 ml were collected. The protein content of the arabinanase fractions was determined using the method of Sedmak and Grossberg (1977).

Enzyme assays

Activity towards linear arabinan

Endo-arabinanase activity was measured by mixing 10 µl of enzyme solution, diluted to the appropriate concentration, with 30 µl of a 5 mg/ml solution of linear arabinan (from apple juice) in distilled water and 100 µl of buffer. This buffer was in most cases 50 mM sodium acetate (pH 5·0), except for the measurement of activity in fractions from the Mono-Q column, where a 10 mM piperazine buffer pH 5·5 was used. Incubation was performed at 30°C for 1 h. Subsequently, the concentration of reducing sugars in the reaction mixture was measured with the Nelson-Somogyi method (Somogyi, 1952).

Activity towards p-*nitrophenyl-α-L-arabinofuranoside*

Arabinofuranosidase activity was determined by mixing 10 µl of enzyme solution in the appropriate concentration, with 100 µl of 0·2 mg/ml *p*-nitrophenyl-α-L-arabinofuranoside in 50 mM sodium acetate buffer pH 5·0. Reaction was performed at 30°C for one hour. Subsequently, the *p*-nitrophenol concentration was measured colorimetrically as described by Rombouts *et al.* (1988).

Activity towards various polysaccharides

Side activities of purified arabinanases were measured using an enzyme protein concentration of 1 µg/ml and 1 mg/ml of various polysaccharides in 50 mM sodium acetate pH 5·0. After incubation at 30°C for 20 h, the increase of reducing end groups was measured with the Nelson-Somogyi method.

Influence of pH on activity and stability

The effect of pH on the activity and stability of endo-arabinanase and arabinofuranosidases from *A. aculeatus* was tested in a series of McIlvaine buffers from pH 2·5–8·0.

For the stability measurements of arabinofuranosidases a pre-incubation without substrate was done for 30 min at 40°C, followed by an incubation with substrate (0·1 mg/ml) in 100 mM sodium acetate pH 5·0 for a period of 30 min at 40°C.

Stability of endo-arabinanase was tested by pre-incubating for 45 min at 30°C, after which residual activity was measured in 100 mM sodium acetate pH 5·0 during 30 min at 30°C, using 1 mg/ml linear arabinan (from apple juice) as substrate. Products were analysed as described above.

Degradation of branched sugar-beet arabinan with arabinofuranosidase B

Sugar-beet arabinan (Koch and Light) was dissolved in distilled water and dialysed extensively against distilled water. After freeze-drying, 100 mg samples were re-dissolved in 50 ml of sodium acetate buffer, pH 4·0, and incubated with 200 µl *A. niger* arabinofuranosidase B solution (125 µg/ml) at 55°C for 0·5, 1, 2, 3, 4, 5 and 6 h, respectively. An untreated aliquot of the substrate was used as control. After inactivation of the enzyme by heat treatment (5 min, 100°C) aliquots were taken and analysed for reducing sugars using the method of Nelson-Somogyi. Subsequently, the samples were dialysed against distilled water and freeze-dried. Glycosidic linkages were determined by methylation analysis.

Degradation of arabinans with endo-arabinanases

Arabinans (from apple juice, as well as from sugar-beet pulp), MHR and partially debranched sugar-beet arabinan (see above) were incubated with endo-arabinanases from *A. niger* and *A. aculeatus*. Incubation was performed at 30°C in 50 mM sodium acetate pH 5·0, using an enzyme concentration of 0·1 µg/ml and a substrate concentration of 1 mg/ml. After several incubation times the enzymes were inactivated at 100°C for 5 min. Products were analysed by high-performance anion-exchange chromatography.

Analytical methods

SDS-gel electrophoresis
Purification of the arabinanases from *A. aculeatus* was monitored using SDS-gel electrophoresis on the Pharmacia PhastSystem. The procedure described by Rombouts *et al.* (1988) was used with a slight modification: samples were heated for 5 min at 100°C instead of being stored at 4°C for 24 hours.

For Western blot analysis, SDS-gel electrophoresis was done on the Midget Electrophoresis System (Pharmacia-LKB) as described by Van der Veen *et al.* (1991).

Antibodies and Western blotting
Antibodies against endo-arabinanase and arabino-furanosidases A and B from *A. niger* (Rombouts *et al.*, 1988) were raised in mice as described by Van der Veen *et al.* (1991) and obtained from the Department of Genetics of the Agricultural University (Wageningen, The Netherlands). Cross-reactivity between these antibodies and the arabinanases from *A. aculeatus* was tested by Western blotting. Nitrocellulose blots were incubated with antisera and subsequently visualized using anti-mouse goat IgG, conjugated with alkaline phosphatase, as described by the supplier (Bio-Rad).

Methylation analysis
Methylation of arabinans was carried out using sodium dimethyl sulphinyl anion, according to the Hakomori method (Hakomori, 1964), as modified by Sandford and Conrad (1966). The methylated material was converted to partially methylated alditol acetates and subsequently analysed by gas chromatography as described by Gruppen *et al.* (1992).

HPAEC analysis
High-performance anion-exchange chromatography (HPAEC) of the samples was performed on a Dionex Bio-LC system (Sunnyvale, CA, USA) coupled to an SP 8880 autosampler (Spectra-Physics, San Jose, CA, USA). The system was equipped with a CarboPac PA1 column (4 × 250 mm) in combination with a CarboPac guard column and run at 20°C. Separation was performed with a flow rate of 1 ml/min using a combined gradient of two eluants. Eluant A: 0·1 M NaOH prepared from a 50% NaOH solution (Baker, 7067, Deventer, The Netherlands) in distilled water. Eluant B: 1 M sodium acetate (Merck, Darmstadt, Germany) in 0·1 M NaOH. The eluants were degassed by flushing helium, and pressurized continuously with the Dionex eluant degas module.

Eluants A and B were mixed, resulting in the following linear gradient of sodium acetate in 0·1 M NaOH: 0–5 min, 0 mM; 5–40 min, 0–500 mM; 40–45 min, 500–1000 mM; 45–50 min, 1000 mM; 50–50·1 min, 1000–0 mM; 50·1–60 min, 0 mM. Samples (20 µl) were injected, using a Spectra Physics SP8880 autosampler. The effluent was monitored using a pulsed electrochemical detector in the pulsed-amperometric mode (PAD) with a Dionex gold working electrode and an Ag/AgCl reference electrode, to which potentials of E1 = 0·1 V, E2 = 0·6 V and E3 = −0·6 V were applied for duration times T1 = 0·5 s, T2 = 0·1 s and T3 = 0·1 s. Chromatograms were recorded and integrated using a Spectra Physics Winner system.

RESULTS

Purification of arabinanases from *A. aculeatus*

The purification of arabinanases from *A. aculeatus* was based on the fractionation scheme for the isolation of rhamnogalacturonase (Schols *et al.*, 1990). The first four steps of this procedure were: desalting on Bio-Gel P10, anion-exchange on DEAE Bio-Gel A, affinity chromatography on cross-linked alginate and adsorption chromatography on Bio-Gel HTP. The latter step resulted in two relevant fractions: an arabinanase fraction, which was not bound to the column and a rhamnogalacturonase fraction which was eluted from the column by a phosphate gradient. In this study, the further fractionation of the arabinanase pool was

investigated. Analytical fractionation of the arabinanase pool on an anion-exchange column (Mono-Q) at pH 5·5 showed one major peak with activity on linear arabinan and two minor peaks with activity on *p*-nitrophenyl-α-L-arabinofuranoside (not shown). The separation of the endo-arabinanase (active towards linear arabinan) and the two arabinofuranosidases was not complete. For instance the peak-top fraction, containing 58 U/ml endo-arabinanase, was still contaminated with 0·02 U/ml arabinofuranosidase. Focusing on the endo-arabinanase, the anion-exchange fractionation on Mono-Q was carried out three times, after which the fractions containing most of the endo-arabinanase were collected. For a final purification, this pool was applied onto the same column again and eluted with a sodium chloride gradient (Fig. 1). One major protein peak, containing the endo-arabinanase (Endo-ara A) was found. The contaminating arabinofuranosidases (Arafur B1 and B2) eluted just before and also about 10 fractions after this peak.

Endo-arabinanase in fraction 18 showed as one band upon SDS-gel electrophoresis and coomassie staining. The molecular weight was 45 kDa. Silver staining of the same gel revealed some additional minor protein bands.

The purity was also checked by incubation with other substrates (Table 1). Endo-ara A was highly active towards linear arabinan and showed also minor activity towards branched arabinans. No side activity towards the other polysaccharides was observed, except for potato arabinogalactan and CM-cellulose. The ability of endo-ara A to release reducing groups from potato arabinogalactan is probably due to the presence of linear arabinan-type side chains in this substrate (Voragen *et al.*, 1987). The positive reaction after 20 h of incubation with CM-cellulose equals 0·5% of the main activity towards linear arabinan.

Upon SDS-gel electrophoresis, arabinofuranosidases B1 and B2 appeared as major bands with a molecular weight of 37 kDa. Some contamination with other proteins was observed. Although arabinofuranosidase was the major activity (Table 1), Arafur B1 was clearly contaminated with endo-arabinanase, because of its high activity towards linear arabinan. Both enzyme preparations showed minor side-activity towards arabinose-containing polysaccharides.

Some characteristics of arabinanases from *A. aculeatus*

Influence of pH on activity and stability

The influence of pH on the activity and stability of the arabinanases from *A. aculeatus* was tested in McIlvaine buffers with pH values between 2·5 and 8. Arabinofuranosidase B1 was optimally active at pH 3·0–3·5. The enzyme is rapidly inactivated below pH 3·0 and above pH 5·5. The pH optimum for Arafur B2 was found to be between pH 4·0 and 4·5. Pre-incubation of Arafur B2 below pH 4·0 or above 6·0 resulted in considerable loss of activity.

Endo-arabinanase showed a pH optimum of 5·5 and was only stable in the narrow pH range from 5·5 to 6·3.

Cross-reactivity with antibodies against A. niger *arabinanases*

Antibodies, raised in mice against endo-arabinanase and arabinofuranosidases A and B from *A. niger*, were

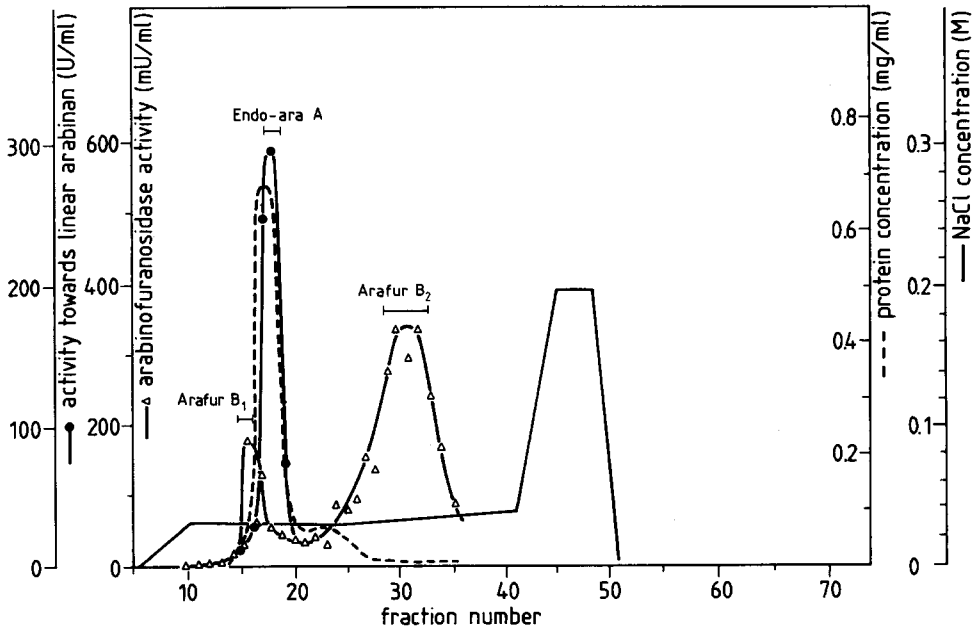

Fig. 1. Anion-exchange chromatography on the Mono-Q column of the *A. aculeatus* endo-arabinanase fractions obtained after pre-fractionation on the same column.

Table 1. Activity of arabinanases from *A. aculeatus* on various substrates

Substrate	Endo-arabinanase	Arabinofuranosidase	
	A	B1	B2
p-Nitrophenylarabinofuranoside	−	++	++
Sugar-beet arabinan (branched)	+	+	+
Apple arabinan (linear)	++	++	+
MHR arabinan	+	+	+
Potato arabinogalactan	+	+/−	+/−
Onion arabinogalactan	−	−	−
Stractan	−	−	−
Wheat arabinoxylan	−	+/−	−
Oat spelts arabinoxylan	−	+/−	−
Avicel	−	−	−
CM-cellulose	+/−	+/−	−
Polygalacturonic acid	−	−	−

Activity towards polysaccharides was measured with the Nelson-Somogyi method, using 1 μg/ml of enzyme. The reaction was strongly positive after 1 h (++), positive after 1 hour (+), positive after 20 h (+/−) or negative after 20 h (−).

used for Western blot analysis (Fig. 2). Antibodies raised against Arafur B (*A. niger;* mol. wt. 67 kDa) were reactive with Arafur B1 and B2 from *A. aculeatus* (mol. wt. 37 kDa), also in the crude *A. aculeatus* preparation. Essentially no Arafur B type of enzyme, with a molecular weight of 67 kDa, could be detected in the latter sample.

Arabinofuranosidases B1 and B2 did not react with antibodies against arabinofuranosidase A from *A. niger.* The presence of this type of enzyme could be demonstrated in the commercial preparation derived from *A. niger,* but not in the *A. aculeatus* preparation.

Anti-endo-arabinanase from *A. niger* was cross-reactive with endo-ara A from *A. aculeatus.* Both enzymes appeared to have the same molecular weight and were also detected in the crude preparations. Apparently, no other endo-arabinanases with the same immunochemical reactivity and different molecular weights were present in these commercial preparations.

Degradation of linear arabinan

The degradation of linear apple arabinan by endo-arabinanases from *A. aculeatus* and *A. niger* was followed for a reaction period of 10 h. By high-performance anion-exchange chromatography, linear oligomeric products with a degree of polymerization (DP) of 1–22 could be identified (Fig. 3). Because both endo-arabinanases do not have the same specific activity, different incubation times were used in order to obtain the same degree of degradation. The presence of branched oligomers, eluting as small peaks in between the larger peaks from linear oligomers, could also be demonstrated. The peak preceding the arabinotriose (A3) peak in the chromatogram of the

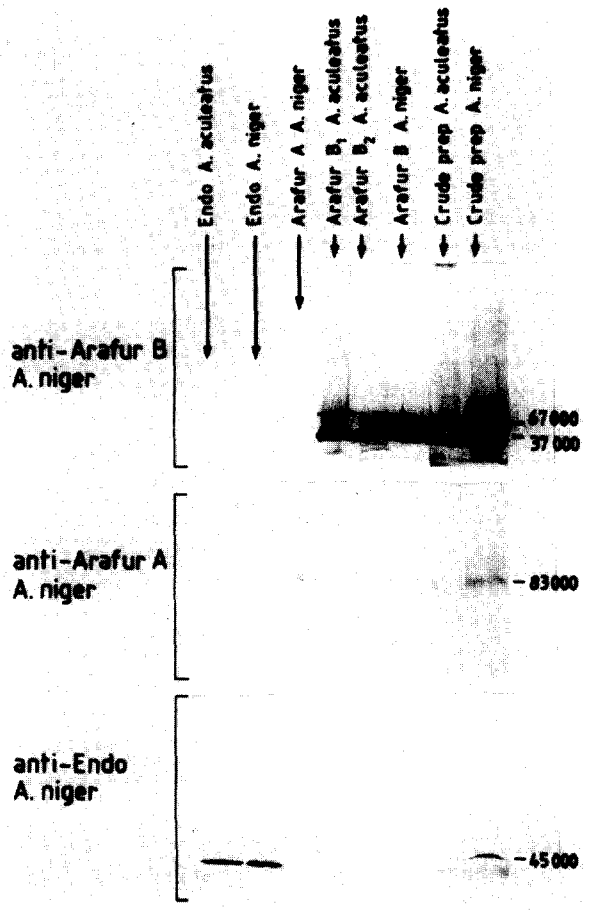

Fig. 2. Western blot analysis of endo-arabinanases and arabinofuranosidase from *A. niger* and *A. aculeatus.* After SDS-gel electrophoresis of the enzymes on different gels, incubation was performed with antibodies against arabinofuranosidase B (upper part), arabinofuranosidase A (middle part) and endo-arabinanase (lower part), all from *A. niger.*

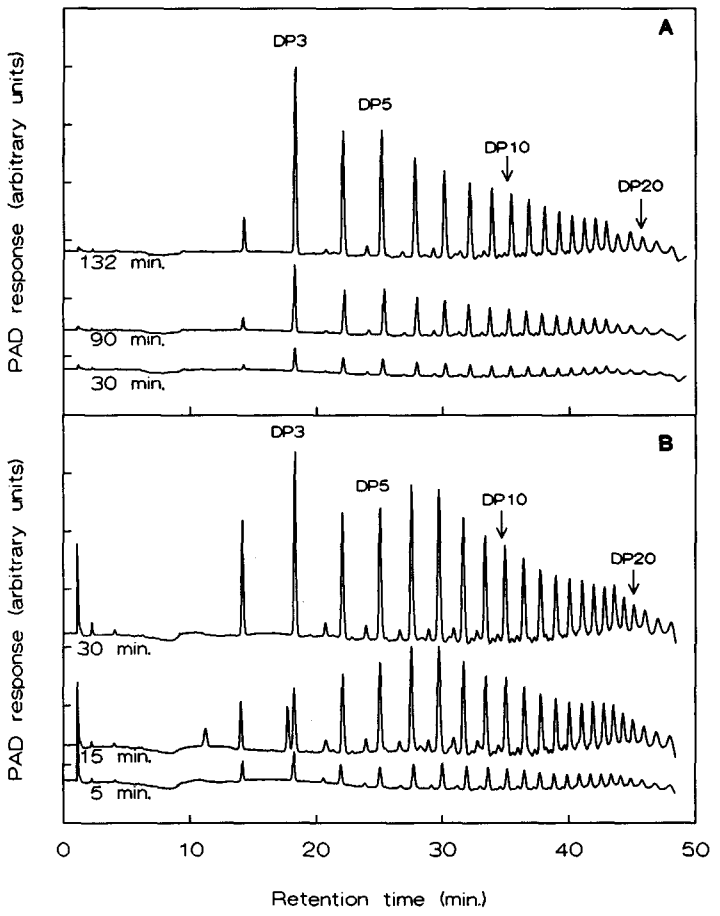

Fig. 3. High-performance anion-exchange chromatography of reaction products from linear arabinan, after incubation with endo-arabinanases from *A. niger* (A) and *A. aculeatus* (B) for varying times. DP3, arabinotriose; DP5, arabinopentaose; etc.

sample after 15-min incubation with endo-arabinanase from *A. aculeatus* (Fig. 3(B)) could not be identified. As it was not found in any of the other chromatograms, this peak may be an artefact.

Both enzymes were not able to release much arabinose; this product appeared in small amounts only after a long incubation time (not shown). The distribution of oligomers in the products from both endo-arabinanases showed some remarkable differences. Compared with endo-arabinanase from *A. niger* (Fig. 3(A)), the enzyme from *A. aculeatus* produced relatively much more dimer of arabinose (A2) (Fig. 3(B)). After a prolonged incubation (10 h) this difference diminished and the ratio of (A2) to (A3) gradually reached the values 1·0 (*A. aculeatus*) and 0·7 (*A. niger*) (not shown).

There was also a difference in the distribution pattern of the larger oligomers in the products. After an intermediate incubation time the enzyme from *A. niger* produced a series of oligomers of which the concentration progressively increased, going from DP 20 to DP 3 (Fig. 4). For both incubation times, there are small 'valley points' at DP 13 and DP 4. The distribution pattern of the oligomers released by the endo-arabinanase from *A. aculeatus* was clearly different. The distribution of the oligomer mixture formed by endo-arabinanase from *A. aculeatus* showed a maximum at DP 6–7 and a minimum at DP 13, after incubation times where about the same degree of degradation of the arabinan was reached for both types of enzyme.

Degradation of branched arabinans

Incubation with arabinofuranosidase B. The mode of action of arabinofuranosidase B from *A. niger* on branched sugar-beet arabinan was studied by isolating the intermediate polymeric products and subsequent methylation analysis. The relative proportions of the various glycosidic linkages, present in these partially debranched arabinans, are presented in Table 2. The original beet arabinan was highly branched and contained about 30% terminal arabinofuranosyl groups. Also, some terminal galactopyranosyl groups were detected. As can be expected, the ratio of the amount of terminal sugar residues to the total amount of branch points was close to 1. Due to the action of arabinofuranosidase B, the amount of terminal arabinofuranosyl units decreased to about 50% of the original level, after incubation for 6 h. This was reflected by an increase of reducing sugars in the

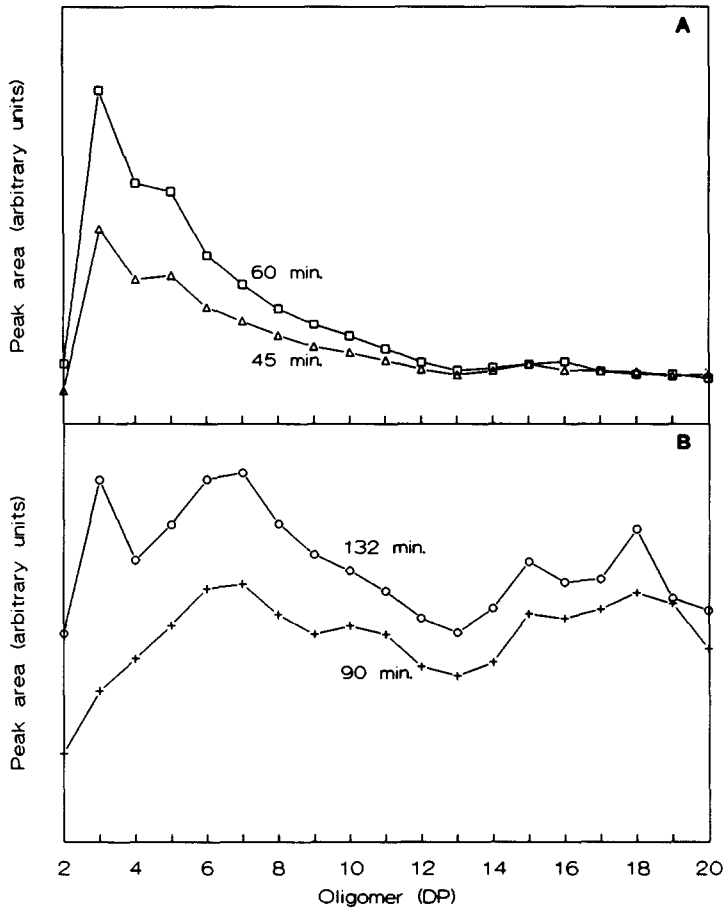

Fig. 4. Distribution patterns of oligomers from linear arabinan, after incubation with endo-arabinanases from *A. niger* (A) and *A. aculeatus* (B) for varying times.

Table 2. Relative amounts of the various glycosidic linkages, present in arabinans after debranching with arabinofuranosidase B from *A. niger* during various incubation times[a]

Binding type	Incubation time (h)						
	0	0.5	1	2	4	5	6
t-Ara*p*[b]	1·2	1·2	1·5	1·4	2·1	1·7	1·5
t-Ara*f*[c]	30·3	28·3	28·5	27·0	20·4	19·9	16·3
1,2-Ara*f*[d]	0·3	0·0	0·3	0·0	0·0	0·2	0·3
1,5-Ara*f*	27·4	30·9	32·7	35·2	42·5	45·7	49·5
1,3-Ara*f*	7·9	7·2	7·7	7·6	8·5	8·6	8·8
1,3,5-Ara*f*	12·6	14·3	13·1	12·6	12·0	10·1	9·6
1,2,5-Ara*f*	4·8	3·8	3·8	4·1	3·7	3·6	3·7
1,2,3,5-Ara*f*	10·5	9·5	8·2	7·7	6·2	5·5	5·1
t-Gal*p*[e]	4·5	4·4	3·8	4·0	4·4	4·7	5·0
1,4-Gal*p*[f]	0·5	0·5	0·4	0·4	0·4	0·1	0·3
Terminal/total branches	0·94	0·91	1·02	1·01	0·96	1·07	0·97

[a]Values are expressed as relative mole percentage of the total amount of glycosidic linkages present in the polymeric material.
[b]t-Ara*p* = terminal arabinopyranose.
[c]t-Ara*f* = terminal arabinofuranose.
[d]1, 2-Ara*f* = 1,2-linked arabinofuranose, etc.
[e]t-Gal*p* = terminal galactopyranose.
[f]1,4-Gal*p* = 1,4-linked galactopyranose.

reaction mixture (not shown) and also by an increase of the number of α-(1,5)-linked arabinofuranosyl residues. Double-branched arabinosyl residues [α-(1,2,3,5)-linked] continuously disappeared during the reaction, resulting in the intermediate products with a single branch [α-(1,2,5)- and α-(1,3,5)-linked] or the unbranched product [α-(1,5)-linked]. After a short reaction time (30 min), the relative amount of α-(1,3,5)-linked arabinofuranosyl residues slightly increased, followed by a gradual decrease after prolonged incubation. The amount of α-(1,2,5)-linked residues continuously decreased. Table 2 also shows that the amount of α-(1,3)-linkages, presumably present in longer side chains of the α-(1,5)-linked main chain, remained almost constant.

Incubation with endo-arabinanase. In order to investigate the effect of branch points on the action of endo-arabinanase, partially debranched beet arabinan, made with arabinofuranosidase B (see above), was incubated with endo-arabinanase from *A. niger*. The degrees of branching of the substrates used (expressed as terminal arabinofuranosyl residues) varied from 16·3 to 28·3%. In Fig. 5 it is demonstrated that the release of oligomers by endo-arabinanase increased with decreasing degrees of branching. After the incubation time used (60 min), the product contained predominantly small oligomers (A2, A3 and A4). Lowering the degree of branching from 28·3% to 27·0% resulted in almost doubled peak areas of these oligomers during the HPAEC analysis. A more pronounced debranching of the substrate to 20·4 and 16·3% had relatively less effect on the increase of oligomeric products.

Product formation by endo-arabinanases from *A. niger* and *A. aculeatus* was compared using a moderately branched apple MHR arabinan, which contained 25% branch points (Rombouts *et al.*, 1988). After 45 min of

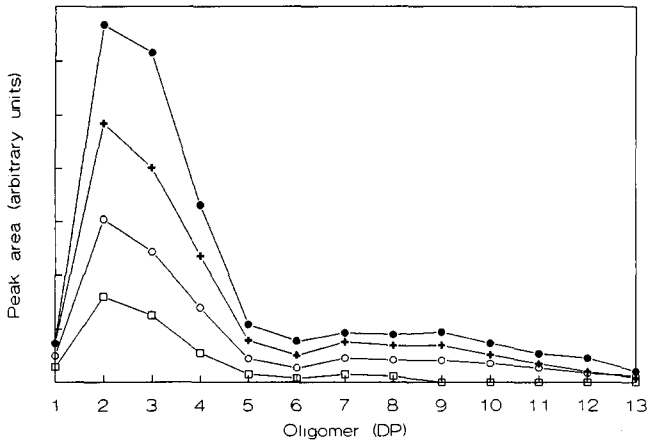

Fig. 5. Distribution patterns of oligomers from arabinans with different degrees of branching, after incubation with endo-arabinanase from *A. niger*. The amounts of oligomers were determined with HPAEC. Degrees of branching: ●, 16·3%; +, 20·4%; ○, 27·0%; □, 28·3%.

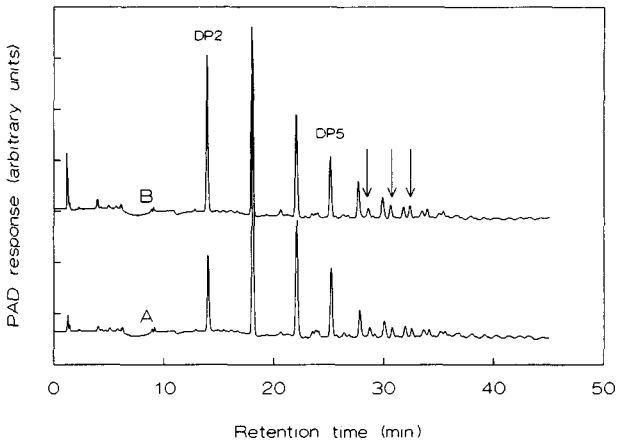

Fig. 6. High-performance anion-exchange chromatography of products from MHR arabinan, after incubation with endo-arabinanases from *A. niger* (line A) and *A. aculeatus* (line B). Arrows indicate branched oligosaccharides.

incubation, low molecular weight products were obtained predominantly with both enzymes (Fig. 6). The A2 to A3 ratio in the product of the *A. niger* endo-arabinanase was lower than that of the *A. aculeatus* enzyme, as was also found with the linear substrate (Fig. 3). However, in contrast to the products from this linear arabinan, the distribution of higher oligomers in the MHR digest was almost the same for both enzymes. The use of this branched substrate also resulted in the appearance of relatively higher peaks of branched oligomers.

DISCUSSION

This study shows that an endo-arabinanase, with a similar molecular weight as the *A. niger* enzyme, can also be isolated from *A. aculeatus*. For final purification of the enzyme, rechromatography on the anion-exchange (Mono-Q) column was necessary. Although the endo-arabinanase did react with antibodies against the *A. niger* endo-arabinanase, indicating antigenic similarities, it also showed some remarkable differences. The pH optimum of the *A. aculeatus* endo-arabinanase (pH 5·5) was about one pH unit higher than that of the *A. niger* enzyme (pH 4·5–5·5).

The most striking difference was found with respect to product formation. The amount of arabinobiose at the initial stage of hydrolysis of linear apple arabinan with the *A. niger* endo-arabinanase was much higher compared to the *A. aculeatus* enzyme.

High-performance anion-exchange chromatography on the CarboPac PA1 column made it possible to identify oligomeric products with a degree of polymerization up to 22. The distribution profile of the higher oligomers showed that both enzymes act according to different mechanisms. The results indicate that the affinity of *A. niger* endo-arabinanase for the various

oligomers progressively decreases, going from DP 20 to DP 3. The arabinotriose is not, or only very slowly, hydrolysed by this enzyme and accumulates in the reaction mixture. The appearance of a valley point at DP 13 in the distribution profile of oligomers in the reaction mixture of the *A. aculeatus* enzyme may be due to a relatively high affinity for oligomers of that length, or to the fact that these oligomers are only slowly formed. Both phenomena must be related to structural features of the enzyme and not of the substrate, because with the *A. niger* endo-arabinanase there was almost no formation of a valley point at DP 13.

A possible explanation for the relatively high amounts of oligomers with DP 6–7, can be the relatively low affinity of the enzyme for these oligomers (as is the case for DP 3 with the *A. niger* enzyme). High affinity for oligomers smaller than DP 6–7 can be excluded, because there is no accumulation of monomer, dimer, trimer or tetramer. More insight into the affinity and the number of sub-sites, can be obtained with kinetic experiments, using purified oligomers as substrates.

During purification of the endo-arabinanase from *A. aculeatus*, there was always co-elution of arabinofuranosidase activity, until the Mono-Q column. After chromatography on this column, two arabinofuranosidase fractions could be distinguished, eluting just before and after the endo-arabinanase peak. Besides different chromatographic behaviour, the pH-optimum and pH-stability curves for both enzyme pools were also different. Arafur B1 showed its optimal activity at a lower pH than Arafur B2. Also, the latter enzyme was less stable at a lower pH value. On the other hand, both enzymes showed some similarities. Their molecular weights were similar and they were both reactive with antibodies against arabinofuranosidase B from *A. niger* and did not react with antibodies against arabinofuranosidase A from the same source. Possibly, the contamination of the Arafur B1 and B2 pools with other proteins may have influenced the chromatographic and pH properties of these enzymes.

Arabinofuranosidase B from *A. niger* splits off arabinofuranosyl residues present as side chains, as well as terminal α-(1,5)-linked residues in the main chain. Because of the relatively higher concentration of terminal α-(1,3)- and α-(1,2)-linked arabinosyl residues in branched beet arabinan, the rate of release of these residues is much higher than for the terminal α-(1,5)-linked arabinofuranosyl units. Kinetic studies have indicated that the affinity of arabinofuranosidase B for terminal residues in branch points is of the same order of magnitude as for terminal residues in a linearized substrate (Rombouts *et al.*, 1988; Beldman *et al.*, 1992). In this study, the methylation analysis of intermediate polymeric products, obtained from the branched beet arabinan by incubation with arabinofuranosidase B, revealed that the enzyme is not very specific. Arabinose was released from single- as well as from double-substituted arabinofuranosyl residues in the backbone. There was no clear difference in the rate of hydrolysis of α-(1,2)- or α-(1,3)-linkages. The results indicate that in double-branched residues of the backbone (α-1,2,3,5-arabinofuranoside), the α-(1,2)-linkage is slightly more susceptible to enzymatic degradation than the α-(1,3)-bond, which leads to a temporary increase of the amount of α-(1,3,5)-linked residues. This mode of action is completely different from the way an arabinofuranosidase from *A. awamori*, specific for arabinoxylans, acts on its substrate (Kormelink *et al.*, 1991). This arabinoxylan arabinofuranohydrolase only splits off arabinose residues from single-substituted xylose units leaving the double substituted residues fully intact.

Degradation of branched arabinans with endo-arabinanase gave rise to predominantly small oligomeric products. Due to the relatively long incubation time, intermediate oligomeric products must have been degraded further. Therefore these results represent an end-point situation and are a measure for the digestibility of the substrate by endo-arabinanases. It was clear that the action of the endo-arabinanase was strongly inhibited by the presence of arabinofuranosyl branches. Debranching of the beet arabinan had a tremendous effect on the digestibility by endo-arabinanase. The removal of a small amount of branches by Arafur B at an early stage of the reaction improved the digestibility by endo-arabinanase much more than the removal of a larger amount of branches at a later stage of the reaction. This can be explained by assuming a non-random distribution of arabinofuranosyl side chains over the backbone, leading to clusters which are relatively highly branched and other clusters with less branch points. Removal of a few branches from the latter type of cluster will more effectively lead to longer stretches of α-1,5-linked arabinofuranosyl residues, improving the digestibility by endo-arabinanase. Further research on the structure and abundance of the intermediate oligomeric products is needed to gain more evidence for this hypothesis.

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