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The Arabinanases of *Aspergillus niger* — Purification and Characterisation of Two α-L-Arabinofuranosidases and an *endo*-1,5-α-L-Arabinanase

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ABSTRACT

Three arabinan-degrading enzymes were isolated from a preparation derived from Aspergillus niger. *Steps used in the purification procedure included gel filtration on Bio-Gel, anion exchange on DEAE Bio-Gel A, adsorption on Bio-Gel HTP, treatment with crosslinked alginate and FPLC chromatography on Mono Q. α-L-Arabinofuranosidase A was active on p-nitrophenyl-α-L-arabinofuranoside (specific activity 67 U/mg protein) and on 1,5-α-L-arabinofuranose oligosaccharides, but not on 1,5-α-L-arabinan, beet arabinan, arabinan obtained from apple juice by ultrafiltration, or arabinoxylan.*

α-L-Arabinofuranosidase B was active on all these substrates, but the synthetic sugar derivative was its best substrate (specific activity 147 U/mg protein). Both enzymes released arabinose as the sole product. endo-*1,5-α-L-Arabinanase was active towards UFR arabinan and beet arabinan, but 1,5-α-L-arabinan was its best substrate (specific activity 1·2 U/mg protein). During hydrolysis of this substrate the disaccharide and the trisaccharide accumulated as end-products. All three enzymes were glycoproteins with isoelectric points in the range of pH 4·5 to 6·5. Their optimum pH values were in the range of 3·7 to 5·0 and their optimum temperatures 50 to 60°C. Arabinofuranosidase A (M_r 128000) had a K_m value for p-nitrophenyl-α-L-arabinoside of 6×10^{-4} M, and a V_{max} value of $15{\cdot}7 \times 10^3$ min^{-1}. The corresponding kinetic parameters for arabinofuranosidase B (M_r 60000) were $4{\cdot}8 \times 10^{-4}$ M and $14{\cdot}6 \times 10^3$ min^{-1}. The K_m and V_{max} values for* endo-*arabinanase (M_r 35000) on 1,5-α-L-arabinan were 0·72 g/litre and 73 min^{-1}, respectively.*

INTRODUCTION

Interest in arabinan-degrading enzymes stems primarily from a problem recently encountered in apple juice technology. Juice concentrates derived from apple pulp which had been treated with technical 'pectinase' preparations developed a precipitate ('haze'), a serious quality defect for a juice which is normally consumed as a sparkling clear product. The haze material was isolated and identified as an almost linear (unbranched) 1,5-α-L-arabinan (Voragen *et al.*, 1982; Churms *et al.*, 1983). It was apparently produced from apple arabinan, an α-L-arabinan in which one third of the α-L-arabinose residues are linked (1→3) or (1→2) to the 1,5-α-L-arabinan backbone (Churms *et al.*, 1983). Indeed, the α-L-arabinofuranosidase (EC 3.2.1.55) described from *Asperigillus niger* (from which most technical 'pectinase' preparations are derived) is known to preferentially hydrolyse the α-L-1,3-arabinose substituents from arabinans (Tagawa, 1970), leaving a linear α-L-1,5-arabinan which is much less water soluble (Churms *et al.*, 1983).

Until now the only arabinan-degrading enzyme from *A. niger* described was the α-L-arabinofuranosidase (EC 3.2.1.55) (Kaji & Tagawa, 1970; Tagawa, 1970). Its properties were summarised and discussed by Kaji (1984) and Whitaker (1984). Another type of α-L-arabinofuranosidase, from *Streptomyces purpurascens*, has been described (Komae *et al.*, 1982). This enzyme is also active on such low-molecular weight substrates as *p*-nitrophenyl-α-L-arabinofuranoside and L-arabinose oligosaccharides but, unlike that obtained from *A. niger*, does not act on L-arabinan, L-arabinoxylan, or L-arabinogalactan.

The third type of arabinan-degrading enzyme is the arabinan *endo*-1,5-α-L-arabinosidase, or *endo*-1,5-α-L-arabinanase (EC 3.2.1.99). It has been purified from *Bacillus subtilis* (Kaji & Saheki, 1975; Weinstein & Albersheim, 1979; Yoshihara & Kaji, 1983) and is known to be produced by certain *Clostridium* spp. (Kaji, 1984), but it has so far not been found in fungi. The enzyme is specific for 1,5-α-L-arabinose linkages, its best substrate being 1,5-α-L-arabinan, which it degrades by an *endo* mechanism. Final products are the arabinose disaccharide and the monomer. The enzyme is inactive towards *p*-nitrophenyl-α-L-arabinoside.

This paper describes the separation, purification and general properties of the arabinan-degrading enzymes from a strain of *A. niger*, which appeared to produce all three types of enzymes.

MATERIALS AND METHODS

Substrates

Beet arabinan and xylan from oat spelts were purchased from Koch-Light, Colnbrook, Bucks, UK. 1,5-α-L-Arabinan (haze arabinan) was isolated from turbid apple juice concentrate (Voragen *et al.*, 1982; Churms *et al.*, 1983); 1,5-α-L-arabinofuranose oligosaccharides were obtained by partial hydrolysis of 1,5-α-L-arabinan by *endo*-arabinanase and separation on a Bio-Gel P-2 column (Weinstein & Albersheim, 1979). Apple juice ultrafiltration retentate (UFR) arabinan was obtained by ultrafiltration of clarified apple juice produced from apple pulp using technical pectinase and cellulase enzymes (Voragen *et al.*, 1986). Prior to use the substrates were dialysed against distilled water and lyophilised.

Galactan was isolated from potato fibre obtained from a potato starch factory (Avebe, Veendam, The Netherlands). Prior to extraction of galactan, starch removal was done by gelatinisation for 1 h at 90°C and subsequent treatment with porcine pancreatic α-amylase (EC 3.2.1.1; Merck, Darmstadt, FRG), using five units of enzyme per g of fibre, and incubating for two days at 30°C in 0·1 M sodium phosphate buffer, pH 6·5, with 0·01% sodium azide added as antimicrobial agent. Otherwise the extraction and isolation procedure of the galactan was that used by Labavitch *et al.* (1976) for galactan from citrus pectin.

Citrus galactan was from the laboratory collection. Stractan, a preparation of larch arabinogalactan with an extremely high degree of branching and a significant proportion of arabinopyranose residues, was obtained from St Regis Paper Company, Tacoma, Washington, USA. CM-cellulose (type Akucell AF 0305) was from Akzo, Arnhem, The Netherlands. Polygalacturonic acid was purchased from ICN, Cleveland, Ohio, USA. *p*-Nitrophenyl-α-L-arabinofuranoside and the *p*-nitrophenyl derivatives of *a*-D- and β-D-galactose, glucose and xylopyranose were obtained from Sigma Chemical Company, St Louis, Missouri, USA.

Characterisation of substrates

The neutral sugar composition of substrates was determined as follows. Portions of 5 mg were hydrolysed with 2 M trifluoroacetic acid for 1·5 h at 120°C. The sugars released were then reduced with sodium borohydride, acetylated, and determined by gas liquid chromatography

(Albersheim *et al.*, 1967). Galacturonic acid was determined by the *m*-hydroxybiphenyl method (Thibault, 1979).

Glycosidic-linkage analysis of arabinose residues was performed according to Talmadge *et al.* (1973). Portions of 5 mg were permethylated by the Hakamori method (Talmadge *et al.*, 1973); the permethylated material was hydrolysed with 2 M trifluoroacetic acid and the aldose derivatives were reduced and acetylated. The partially methylated alditolacetates were identified by GCMS (Talmadge *et al.*, 1973).

Enzyme preparation

Pectinase 29, an enzyme preparation derived from *A. niger*, was kindly provided by Gist-Brocades, Delft, The Netherlands.

Enzyme assays

All enzyme activities were expressed in International Units (*U*). Enzyme protein was measured according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Activity on UFR arabinan was measured in a reaction mixture composed of 0·1 ml 0·5% w/v UFR arabinan solution in distilled water, 0·35 ml 0·05 M sodium acetate buffer, pH 5·0, and 0·05 ml enzyme solution. After incubation at 30°C for 1 h, the increase in reducing end-groups was measured by the method of Nelson–Somogyi (Spiro, 1966), using arabinose as standard.

In the galactanase assay the substrate was 0·1 ml of 0·5% w/v potato galactan, but other conditions were as in the assay on arabinan. However, in the Nelson–Somogyi assay reaction, mixtures were centrifuged prior to absorbance reading at 520 nm, and galactose was used as standard.

The arabinofuranosidase assay was performed by addition of 0·05 ml of enzyme solution and 0·10 ml of 0·1% w/v *p*-nitrophenyl-α-L-arabinofuranoside to 0·35 ml of 0·05 M sodium acetate buffer, pH 5·0. After incubation for 1 h at 30°C the reaction was stopped by the addition of 0·5 ml 0·5 M glycine–sodium hydroxide buffer, pH 9·0, containing 2 mM disodium ethylenediaminetetraacetate. The concentration of *p*-nitrophenol was determined by reading the absorbance at 400 nm and using the extinction coefficient of 13 700 M^{-1} cm^{-1}.

The polygalacturonase and CM-cellulase assays were identical to the arabinanase assay, except for the substrates and standards used, which were 1% polygalacturonic acid neutralised to pH 5·0 and galacturonic

acid respectively in the polygalacturonase assay, and CM-cellulose and glucose respectively for the CM-cellulase assay.

The activity of pure enzymes towards various other substrates was measured as described in the arabinanase assay, with enzyme concentrations of 10 μg/ml and incubation times of 24 h.

Column chromatography

Column chromatography was carried out on Bio-Gel P-10 (100-200 mesh), Bio-Gel P-100 (100-200 mesh), DEAE Bio-Gel A and Bio-Gel HTP (Bio-Rad Laboratories, Richmond, California, USA). Gradient elution was performed with the LKB 11300 Ultrograd Gradient Mixer (LKB-Produkter AB, Stockholm, Sweden), with the level sensor set at an absorbance A_{254} value of 0·01.

Other columns were alginate crosslinked with epichlorohydrin, in a molar ratio of epichlorohydrin to anhydro-uronic acid of 2·34 (Rombouts *et al.*, 1979), the Mono Q column HR 5/5, and the Superose 12 column HR 16/50, the latter two columns from Pharmacia used with the Fast Protein Liquid Chromatography (FPLC) System of Pharmacia (Uppsala, Sweden).

Eluted fractions were analysed for arabinanase activity on UFR arabinan, for arabinofuranosidase activity and for galactanase activity. Chromatography experiments were carried out at 4°C. All buffers contained 0·01% sodium azide as antimicrobial agent. Details on the chromatographic steps are given with the figures.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) gel electrophoresis was performed with the PhastSystem (Pharmacia, Uppsala, Sweden) according to the instructions of the supplier described in Separation Technique Files 110 and 210. Gels coded PhastGel gradient 10–15 (code 17-0540-01) were used with buffer strips (code 17-0516-01).

Samples were twice diluted with sample buffer, consisting of 0·125 M Tris/HCl, pH 6·8, 2·5% (w/v) SDS, 2·5% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol and 0·0025% bromphenol blue and subsequently stored at 4°C for 24 h. Gels were stained for protein with Coomassie brilliant blue 350-R, and for carbohydrate using the periodic acid–Schiff reagent (Seqrest & Jackson, 1972). Bovine serum albumin (M_r 68 000), catalase (M_r 60 000) adolase (M_r 35 000), trypsin inhibitor (M_r 20 000) and cytochrome C (M_r 12 500) were used as standards.

Isoelectric focussing

Analytical thin-layer gel isoelectric focussing was performed in the pH range 3·5–9·5 on the LKB 2117 Multiphor (LKB-Produkter AB, Stockholm, Sweden) using the application note provided by the manufacturer.

Isoelectric focussing and titration curves were also performed with the PhastSystem (Pharmacia) according to the instructions of the supplier. The PhastGel medium used in both techniques was IEF 3–9 (code 17-0543-01). The Coomassie blue staining technique was used for development.

Optimum pH and temperature

The optimum pH of the purified enzymes was determined by measuring the actvity on *p*-nitrophenyl-α-L-arabinoside (arabinofuranosidase A and B) or UFR arabinan (*endo*-arabinanase) at pH values ranging from 3·0 to 8·0, using a series of 0·1 M McIlvaine buffers.

The optimum temperature was determined by measuring the activity of the enzymes at temperatures ranging from 10 to 90°C in reaction mixtures as described under Enzyme assays.

Product analysis by HPLC

In order to identify the reaction products of purified enzymes, the latter were incubated at a concentration of 20 μg/ml with 5 mg substrate in 1 ml 0·05 M sodium acetate buffer pH 5·0 at 30°C for up to 72 h. At certain time intervals the enzymes were inactivated by placing the tubes in a boiling water bath for 5 min. Lead nitrate was added to a final concentration of 0·1 M to precipitate protein and undegraded substrate. Products were analysed by HPLC (Spectra Physics SP 8000) using an Aminex HPX 87P column (Bio-Rad Labs, Richmond, California, USA) and water as eluent (Voragen *et al.*, 1986).

RESULTS

Characterisation of substrates

For the interpretation of the activity of purified enzymes it was necessary to characterise the substrates. The sugar composition is given in Table 1. With the total sugar content set at 100 mol %, the highest arabinose content was found in 1,5-α-L-arabinan from haze (97%), followed by beet

arabinan (88·5%), UFR arabinan (55·1%), potato galactan (24%) and xylan from oat spelts (9%). Most of the substrates were fairly pure heteroglycans with sugar contents ⩾ 90% in the air-dry preparations, but the total sugar content of the potato galactan was low (55%).

The types of linkages in which the arabinose residues are involved are listed in Table 2. In agreement with earlier studies (Voragen *et al.*, 1982; Churms *et al.*, 1983) the haze arabinan could be described as a predom-

TABLE 1
Sugar Composition of Substances

Sugar	*UFR arabinan*	*Beet arabinan*	*1,5-α-L arabinan*	*Potato galactan*	*Oat spelts xylan*
Rhamnose/Fucose	5·6	1·5	1·2		
Arabinose	55·1	88·5	90	24	9
Xylose	8·4	1·4	0·3		89
Mannose			0·7		
Galactose	9·9	7·4	1·6	59	1
Glucose		1·5	0·1	13	1
Galacturonic acid	20·9		3·9		
Total sugar content	90	90	98	55	94

The sugar composition is expressed in mole percentages, with the total sugars set at 100 mol%. In addition the total sugar content is given expressed as % w/w.

TABLE 2
Arabinosidic-linkage Composition of Substrates

Arabinose residue	*UFR arabinan*	*UFR (linearised)*	*Beet arabinan*	*Beet (linearised)*	*1,5-α-L arabinan*	*Oat spelts xylan*
1-Ara *f*	25	13	35	11	4	67
1-Ara *p*			2	3		
1,2-Ara *f*	1			1	2	23
1,3-Ara *f*	8	5	7	11		3
1,5-Ara *f*	47	68	28	57	88	7
1,3,5-Ara *f*	11	8	13	7	3	
1,2,5-Ara *f*	3	2	4	5	1	
1,2,3,5-Ara *f*	5	3	10	6		

Data are expressed as mole percentages of arabinose residues with specified linkages: 1-Ara *f* represents an arabinofuranose residue linked through C-1 (a non-reducing terminal residue); 1,2-Ara *f* represents an arabinofuranose residue linked through C-1 and C-2, etc.

inantly linear 1,5-α-L-arabinan, with very few 1,2- and 1,3-α-L-arabinose residues attached. The beet arabinan also has a 1,5-α-L-arabinan backbone, but is highly branched, and the side chains may contain one or more 1,3-linked arabinose residues. The UFR arabinan is less branched and contains important sequences of 1,5-linked arabinose residues, in addition to high numbers of branch-point and terminal arabinose residues, and some 1,3-linked residues. Most of the arabinose residues in xylan are terminally linked. Some of the beet arabinan and UFR arabinan is 'linearised' by partial removal of the side chains with arabinofuranosidase B, according to Yoshihara and Kaji (1983). With respect to the starting material, these substrates contain less terminal, 1,3- and 1,5-linked arabinofuranose residues and concomitantly more 1,5-linked arabinose residues.

Enzyme purification studies

The procedure which was applied for fractionation of Pectinase 29 and isolation and purification of its two arabinofuranosidases and its *endo-*

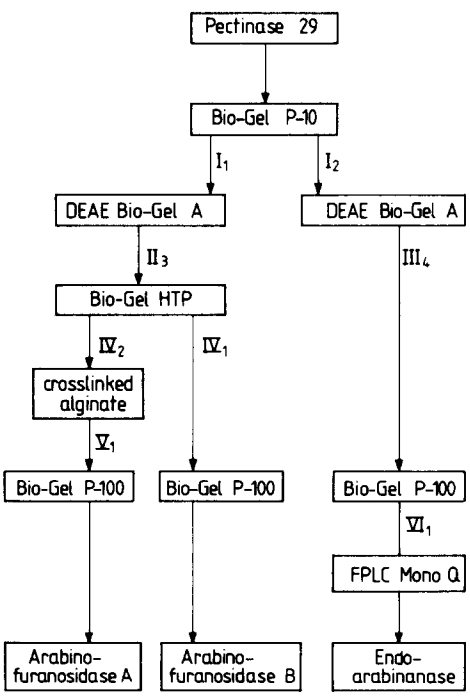

Fig. 1. Flow sheet of the purification of arabinofuranosidases A and B and *endo*-arabinanase from an enzyme preparation of *Aspergillus niger*. The roman numerals with arabic subscripts refer to enzyme pools.

arabinanase is shown in Fig. 1. Pectinase 29 was desalted on a Bio-Gel P-10 column (Fig. 2). An arabinanase peak appeared near the void volume of the column (Pool I_1). A second peak (Pool I_2) which, for unknown reasons, eluted towards the total volume of the column was rich in galactanase activity, but contained arabinanase activity as well.

Samples of both pools were chromatographed by FPLC on Mono Q (not shown). The two arabinofuranosidases A and B, an *endo*-arabinanase and a galactanase could be located in both elution profiles. It could be also be judged that the amounts and specific activities of the two arabinofuranosidases were higher in Pool I_1, whereas the specific activity of *endo*-arabinanase was much higher in Pool I_2.

Pool I_1 was applied to a DEAE Bio-Gel A column (Fig. 3). Elution was performed with the gradient mixer controlled by the level sensor set at $A_{254} = 0{\cdot}01$. This device made it possible to elute protein peaks with a minimum of contamination, by maintaining the concentration of the eluent at a fixed value during elution of peaks (see gradient in Fig. 3). Several peaks (II_2 to II_5) were pooled and analysed by FPLC on Mono Q. Also, the action pattern of the enzymes present in these pools was studied by product analysis on HPLC. It was found that arabinofuranosidases

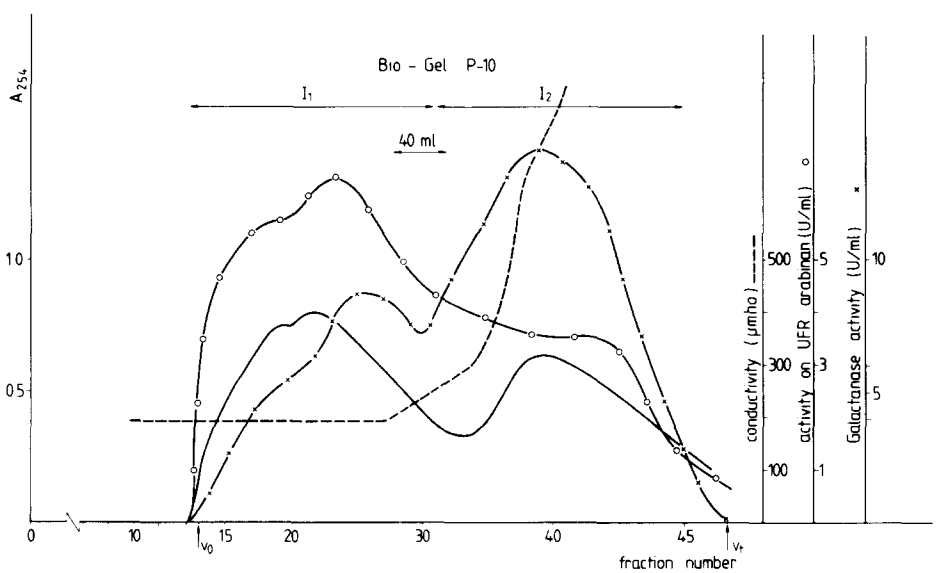

Fig. 2. Bio-Gel P-10 chromatography of crude Pectinase 29. Pectinase 29 (10 g) was dissolved in 30 ml 0·01 M sodium acetate pH 5·0, centrifuged and applied to a Bio-Gel P-10 column (30 × 950 mm) equilibrated and eluted with the same buffer. Flow rate 10 ml/h. Fraction volume: peak fractions 10 ml, otherwise 20 ml. V_0 void volume; V_t total volume.

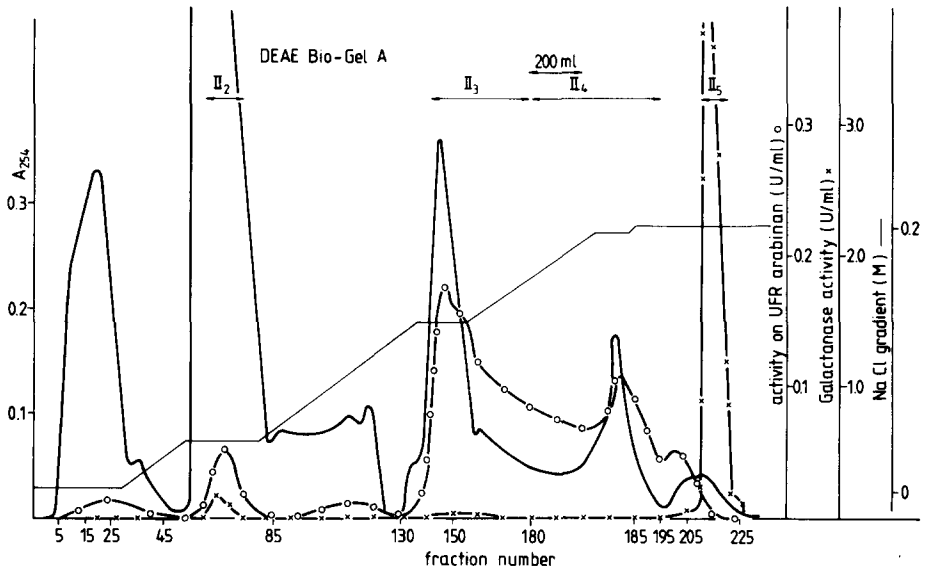

Fig. 3. DEAE Bio-Gel A chromatography of pool I_1. Pool I_1 from the Bio-Gel P-10 column (150 ml) was applied to the DEAE Bio-Gel A column (30 × 200 mm), equilibrated in 0·01 M sodium acetate buffer pH 5·0. Elution was performed using the LKB Ultrograd Gradient Mixer, applying a gradient from the equilibration buffer to a solution of 0·2 M sodium chloride in 0·05 M sodium acetate, pH 5·0. Fraction volume: peak fractions 10 ml, otherwise 20 ml.

A and B were both present in pools II_2 and II_3, but in different proportions, whereas most of the *endo*-arabinanase eluted in pool II_4.

Pool II_3 was applied to a Bio-Gel HTP column (Fig. 4). Much inert protein passed through the column without binding (not shown in Fig. 4). During gradient elution two major peaks were separated. The first of these peaks contained activity towards both UFR arabinan and *p*-nitrophenyl-α-L-arabinofuranoside (arabinofuranosidase B, collected as Pool IV_1) whilst the second was active only towards the synthetic arabinoside (arabinofuranoside A, Pool IV_2).

Pool IV_2 was dialysed against 0·1 M sodium acetate buffer pH 4·2 and passed over a crosslinked alginate column (not shown) to remove polygalacturonase activity, which binds selectively to this column. The eluent and the wash were collected (Pool V_1) and concentrated by freeze-drying, as it was previously observed that concentration by ultrafiltration resulted in aggregation of the enzyme and aberrant elution behaviour on Bio-Gel P-100. Pool IV_1 was also concentrated and for this pool an ultrafiltration cell (Diaflo type 50, Amicon, Danvers, Massachusetts, USA) was used without such problems.

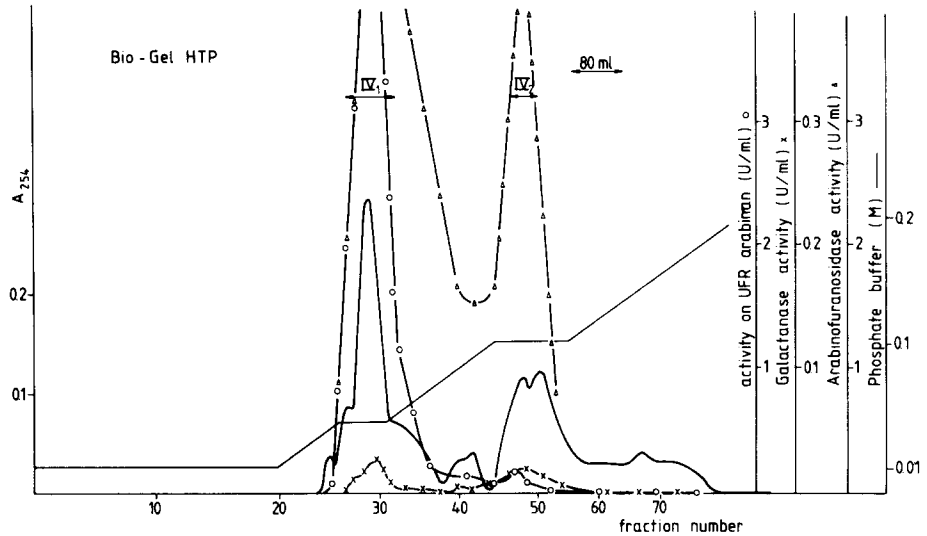

Fig. 4. Bio-Gel HTP chromatography of pool II_3. Pool II_3 from the DEAE Bio-Gel A column (345 ml) was dialysed against 1 mM sodium phosphate–potassium phosphate pH 6·8, and applied to a Bio-Gel HTP column (20 × 140 mm), equilibrated against 10 mM of this buffer. Elution was performed using the LKB Ultrograd Gradient Mixer with the concentration of the phosphate buffer increasing up to 0·2 M. Fractions of 10 ml (protein peaks) or 20 ml were collected at a flow rate of 20 ml/h. V_0 void volume; V_t total volume.

The enzyme pool V_1 redissolved in 5 ml distilled water, and enzyme pool IV_1, concentrated to a volume of about 5 ml were applied separately, onto Bio-Gel P-100 (Figs 5 and 6, respectively). Fractions 15 to 19 of V_1 (Fig. 5) were collected as arabinosidase A and 15 to 20 of IV_1 (Fig. 6) as arabinosidase B. Gel filtration chromatography allowed for the separation of some contaminating arabinofuranosidase B from A in V_1 (Fig. 5) and some residual *endo*-arabinanase from arabinofuranosidase B in IV_1 (Fig. 6).

Purification of the *endo*-arabinanase was started from Pool I_2 of the Bio-Gel P-10 column (Figs 1, 2). This pool was chromatographed on the DEAE Bio-Gel A column (Fig. 7). Pools III_2 and III_3 with relatively small amounts of activity on UFR arabinan contained probably the same enzymes as Pools II_2 and II_3 in Fig. 3 and were discarded. Pool III_5 was a galactanase (identical to II_5) which was not further studied. Pool III_4 was applied to the Bio-Gel P-100 column (Fig. 8). A reasonable separation of the *endo*-arabinanase from the galactanase was achieved. Fractions 22 to 25 were collected (Pool VI_1, 37 ml) so as to eliminate the galactanase activity.

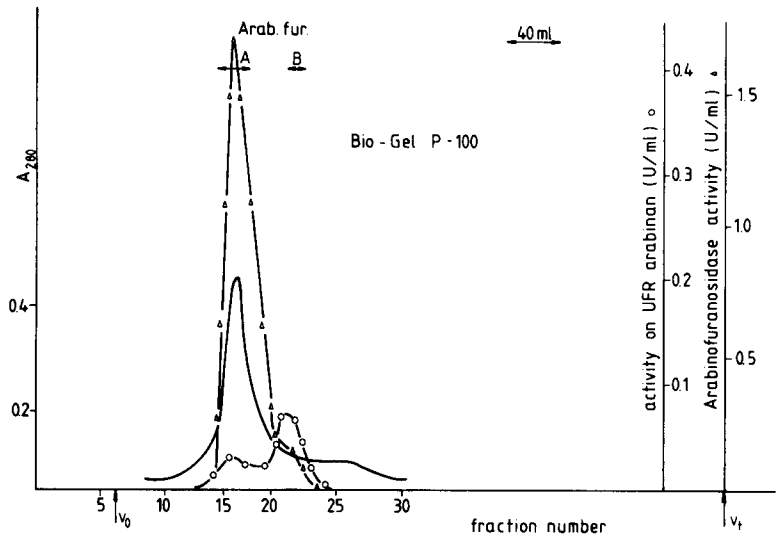

Fig. 5. Bio-Gel P-100 chromatography of pool V_1. Pool V_1 from the crosslinked alginate column was freeze-dried and redissolved in 3 ml 0·01 M sodium acetate buffer pH 5·0, and applied to the Bio-Gel P-100 column (20 × 900 mm). The column was eluted at a rate of 10 ml/h with the sodium acetate buffer, and fractions of 5 ml (protein peaks) or 10 ml were collected. V_0 void volume; V_t total volume.

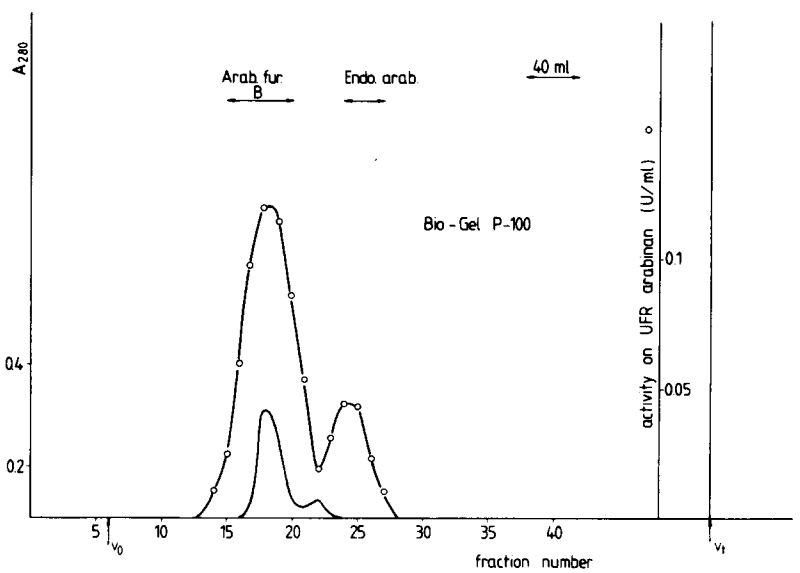

Fig. 6. Bio-Gel P-100 chromatography of pool IV_1. The preparation was concentrated by ultrafiltration to about 3 ml and applied to the column (20 × 900 mm). Elution was performed as in Fig. 7.

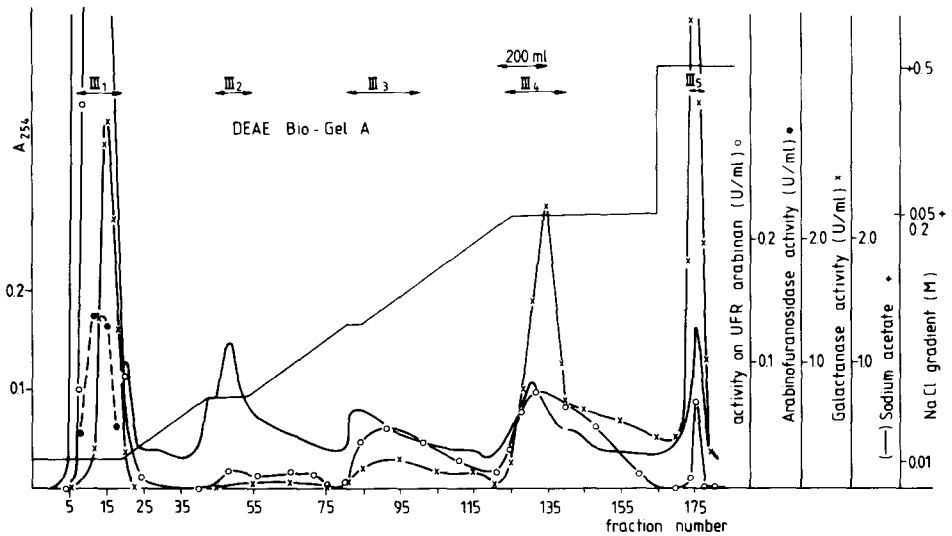

Fig. 7. DEAE Bio-Gel A chromatography of pool I_2. Pool I_2 from the Bio-Gel P-10 column (152 ml) was dialysed against 0·01 M sodium acetate buffer pH 5·0, and applied to the DEAE Bio-Gel A column (30 × 200 mm) equilibrated against the same buffer. Elution was performed at a flow rate of 20 ml/h with a gradient as described in Fig. 4, and then with 0·5 M sodium chloride in 0·05 M sodium acetate, pH 5·0.

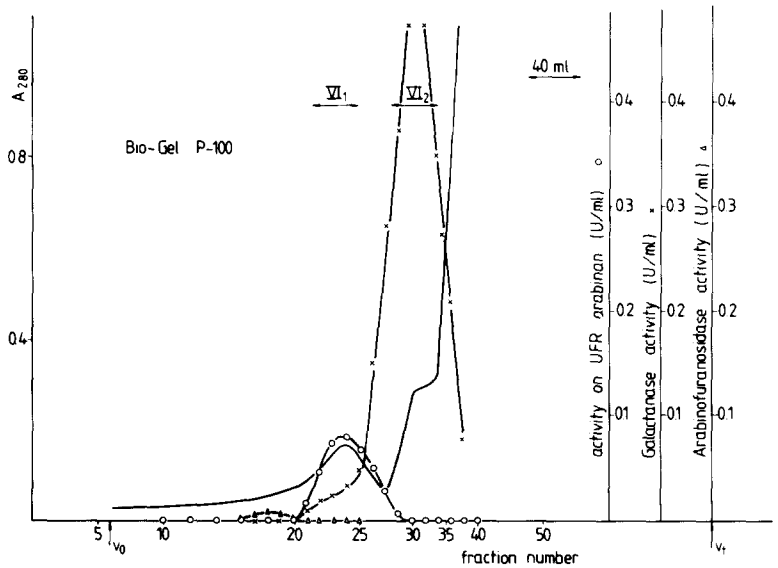

Fig. 8. Bio-Gel P-100 chromatography of pool III_4. Pool III_4 (252 ml) from the column described in Fig. 7 was concentrated by ultrafiltration to about 3 ml and applied to the Bio-Gel P-100 column (20 × 900 mm). Elution was performed as in Fig. 5.

Pool VI_1 was checked for activity on various substrates. It appeared to be contaminated with CM-cellulase activity. This contaminant was efficiently removed by FPLC chromatography on Mono Q (Fig. 9).

The pure enzymes in 0·01 M sodium acetate or piperazine buffer at pH 5·0 were stored dispensed in small tubes and frozen at −20°C.

Chemical properties

Some of the properties of the purified enzymes are listed in Table 3. In view of the different substrate specificities of the enzymes, specific activities were measured under standard conditions on two different substrates. Values of 67 and 147 U/mg were found for arabinosidases A and B, respectively, on *p*-nitrophenyl-α-L-arabinofuranoside. In contrast, the specific acitivity of *endo*-arabinanase was as low as 0·36 U/mg on the UFR arabinan. In spite of the pH optima of the enzymes, which range from 3·7 to 5·0 (Table 3), a pH value of 5 was selected in the assay for all three enzymes in order to escape from chemical hydrolysis of some of

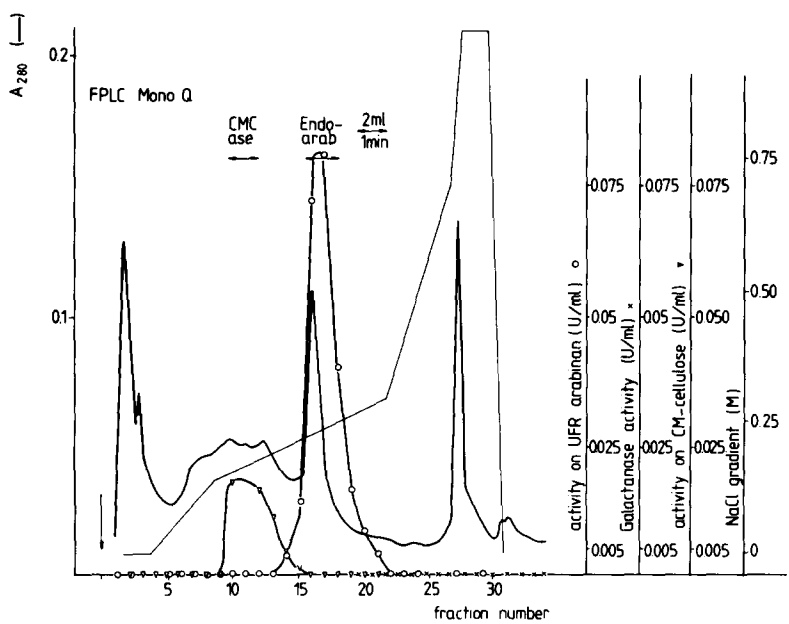

Fig. 9. FPLC chromatography of pool VI_1 on Mono Q. Part of the pool was dialysed against 0·02 M piperazine buffer, pH 6·0 and 0·5 ml portions were applied to the column. After washing with the same buffer, elution was performed with a gradient of 0 to 1 M sodium chloride in the buffer. Fractions of 1 ml were collected at a flow rate of 2 ml/min.

TABLE 3
Some Properties of Arabinan-degrading Enzymes of *A. niger*

	Arabinofuranosidase		*endo-Arabinanase*
	A	*B*	
Specific activities (at pH 5)			
on *p*-nitrophenylarabinoside	67	147	nd
on UFR arabinan	nd	1·6	0·36
on 1,5-α-L-arabinan	nd	nd	1·2
M_r (SDS electrophoresis)	128000	60000	35000
Glycoprotein	Yes	Yes	Yes
Isoelectric point (pH)	6–6·5	5·5–6	4·5–5·5
Optimum pH	4·1	3·7	5·0
Optimum temperature (°C)	50	60	50

nd Not determined.

the substrates, which becomes apparent upon prolonged incubation at pH 4. Since the activities of arabinofuranosidase A and B at pH 5 are 75% and 70%, respectively, of those at their pH optimum, it is apparent that the specific activity values of these two enzymes, as recorded in Table 3, are underestimated.

The molecular weights could be estimated from results of the SDS–polyacrylamide gel electrophoresis (PhastSystem), in which the enzymes moved as single bands. Values of 128000, 60000 and 35000 were found for arabinofuranosidase A, B and *endo*-arabinanase, respectively. Staining gels with periodic acid–Schiff reagent revealed that all three enzymes were glycoproteins.

Attempts to determine the isoelectric points of the enzymes with the conventional thin-layer gel electrofocussing technique gave unsatisfactory results, even when the most appropriate ampholines with a pH range of 2·5 to 5·0 were used. Problems were also encountered with the PhastSystem. When this technique was used for making titration curves, it was observed that the enzymes at the low-pH side of the gel would not move towards the cathode, but remained at the site of application, or moved somewhat to the anode. No explanation was found for the aberrant behaviour of these proteins in isoelectric focussing. Reliable values for the isoelectric points can therefore not be given, but they may be at pH 6 to 6·5 for arabinofuranosidase A, 5·5 to 6 for arabinofuranosidase B and 4·5 to 5·5 for *endo*-arabinanase.

Optimum temperatures were 60°C for arabinofuranosidase B, and 50°C for the other two enzymes. In addition, stability studies in which the

enzymes were held in 0·1 M sodium acetate buffers at pH 5·0 for 90 min at different temperatures, indicated full retention of activity of all three enzymes up to 50°C, and a sharp drop in activity beyond that temperature.

Substrate specificity and action pattern

Table 4 summarises the activity of the three enzymes on different substrates. Arabinofuranosidase A was characterised as an enzyme which preferentially splits *p*-nitrophenyl-α-L-arabinoside and small 1,5-α-L-arabinose oligomers, whereas its activity on pentamer in comparison to that on *p*-nitrophenyl-α-L-arabinoside, was already much reduced. Arabinose was the only product detected by HPLC (see Fig. 10). This enzyme showed a slight activity upon prolonged incubation with some of the high molecular weight substrates, namely those with sequences of 1,5-α-L-arabinose residues (see Table 2). As revealed by the Nelson–Somogyi test, these substrates show slight chemical hydrolysis under such conditions. That is why the activity observed may well arise from enzymic hydrolysis of oligomers, formed by chemical hydrolysis.

TABLE 4
Activity of Arabinan-degrading Enzymes of *A. niger* on Various Substrates

Substrate	*Arabinofuranosidase*		*endo-Arabinanase*
	A	*B*	
p-Nitrophenylarabinoside	+ + +	+ + +	−
Arabinose disaccharide	+ +	+ +	−
Arabinose trisaccharide	+ +	+ +	+
Arabinose tetrasaccharide	+ +	+ +	+
Arabinose pentasaccharide	+	+	+ +
1,5-α-L-Arabinan	−	+	+ + +
UFR arabinan	±	+ +	+ +
Beet arabinan	+	+ + +	+ +
Potato galactan	−	±	+
Citrus galactan	−	+ +	±
Stractan	−	−	−
Xylan from oat spelts	−	+	−

The activity towards the substrates was measured by HPLC (Fig. 11) or by the Nelson–Somogyi method and found to be positive after 1 h (+ + +), 5 h (+ +), 72 h (+), weakly positive after 72 h (±), or negative (−).

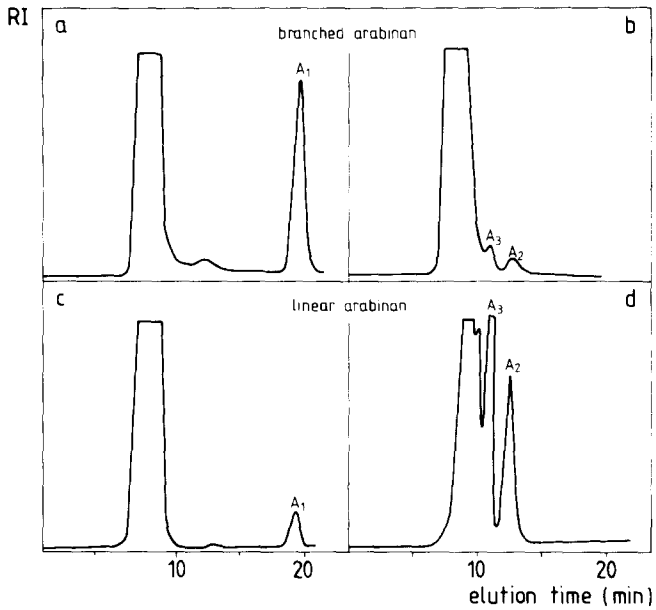

Fig. 10. HPLC analysis of products released from beet arabinan and 1,5-α-L-arabinan. Arabinofuranosidase B (a, c) and *endo*-arabinanase (b, d) were incubated at a concentration of 20 μg/ml with beet arabinan (a, b) and 1,5-α-L-arabinan (c, d) at a concentration of 4 mg/ml, in 0·1 M sodium acetate buffer, pH 5·0 for 1 h at 30°C. a_1, arabinose; a_2, α-L-arabinosyl (1 → 5) arabinoside (dimer); a_3, trimer.

The enzyme is apparently not capable of splitting 1,3-α-L- or 1, 2-α-L-linked arabinose substituents from the main chain of polysaccharides, or arabinose from longer sequences of 1,5-α-L-linked arabinose residues.

In contrast, arabinofuranosidase B appeared to be capable of splitting these types of bonds, in addition to those split by enzyme A. This is clearly demonstrated by its activity on the highly-branched beet arabinan and UFR arabinan, the xylan with its arabinofuranose substituents and the almost unbranched 1,5-α-L-arabinan. Arabinose was again the only product detected (see Figs 10 and 11).

The best substrate for the *endo*-arabinanase was the 1,5-α-L-linked arabinan, and its activity was progressively reduced as the 1,5-α-L-sequences became shorter or more highly substituted (Table 4). Its action pattern is shown in Figs 10 and 11. In the course of the reaction on 1,5-α-L-arabinan or beet arabinan there was a depolymerisation of the substrate, as indicated by the shift in the elution time of the bulk of the material in HPLC (Fig. 10). There was concomitant appearance and increase of peaks of the 1,5-α-L-linked dimers and trimers. At that stage

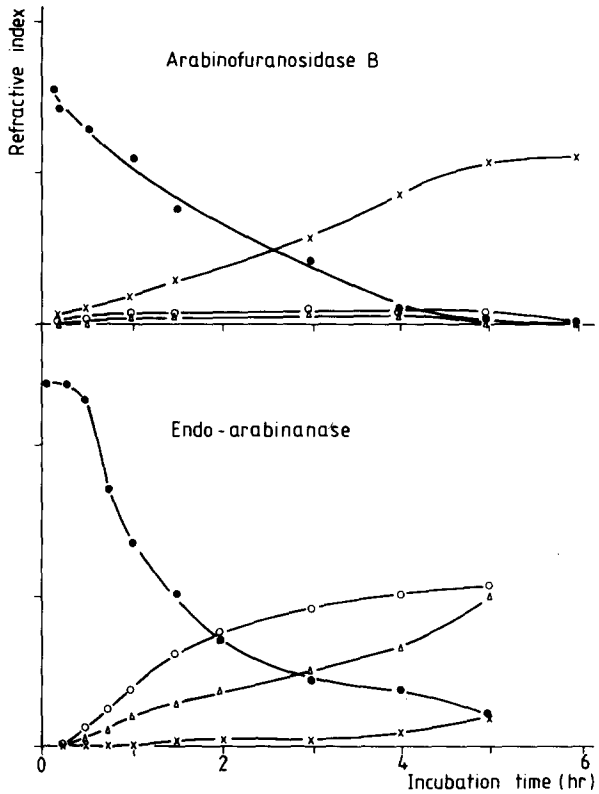

Fig. 11. Time-course studies of the degradation of 1,5-α-L-arabinan by arabinofuranosidase B and *endo*-arabinanase. Arabinofuranosidase B (45 μg/ml) and *endo*-arabinanase (9·6 μg/ml) were incubated at 30°C with 0·8 mg/ml of substrate in 0·04 M sodium acetate buffer, pH 5·0. At time intervals samples were analysed by HPLC. (●) 1,5-α-L-Arabinan; (×) arabinose; (○) α-L-arabinosyl (1→5) arabinoside (dimer); (△) trimer.

no monomers were formed. Upon exhaustive degradation of the substrate some monomers were eventually produced (Fig. 11).

Kinetic parameters on some substrates

Table 5 lists K_m and V_{max} values for the three enzymes on their best substrates. These data are a further substantiation of the results in Table 4. The activity measurements on which the data in Table 5 are based were systematically corrected for chemical degradation of the substrates during incubation. *p*-Nitrophenyl-α-L-arabinofuranoside was the best substrate for the two arabinofuranosidases, but it was possible to determine K_m and V_{max} values of arabinofuranosidase B on the other sub-

TABLE 5
Kinetic Parameters of Arabinan-degrading Enzymes from *A. niger* on Some Substrates

Substrate	*Arabinofuranosidase A*				*Arabinofuranosidase B*				*endo-Arabinanase*			
	Enzyme concentration (μg/ml)	K_m *(g/litre)*	K_m *(mol/litre)*	V_{max} *(min^{-1})*	*Enzyme concentration (μg/ml)*	K_m *(g/litre)*	K_m *(mol/litre)*	V_{max} *(min^{-1})*	*Enzyme concentration (μg/ml)*	K_m *(g/litre)*	K_m *(mol/litre)*	V_{max} *(min^{-1})*
p-Nitrophenyl-α-L-arabinoside	0·1	0·16	6×10^{-4}	15700	0·1	0·13	$4{\cdot}8 \times 10^{-4}$	14600				
UFR arabinan					4	3·9	$3{\cdot}7 \times 10^{-3}$	470	16	15	26×10^{-3}	200
UFR (linearised)					4	7·0	$2{\cdot}9 \times 10^{-3}$	380	16	4·2	$9{\cdot}3 \times 10^{-3}$	106
1,5-α-L-Arabinan					4	17	$4{\cdot}5 \times 10^{-3}$	360	16	0·72	$4{\cdot}2 \times 10^{-3}$	73

Incubation was done in 0·1 M sodium acetate buffer, pH 5·0, at 30°C for 1 h. K_m values (mol/litre) for arabinofuranosidase B activity on the arabinans are expressed as mol/litre of terminally linked arabinose residues and for *endo*-arabinanase activity on the same substrates as mol/litre of unbranched 1,5-α-L-linked arabinose residues, using data from Tables 1 and 2. V_{max} (min^{-1}) values represent moles of arabinose or reducing groups released per mole of enzyme per min, using the M_r values for the enzymes from Table 3.

strates. V_{max}/K_m may be taken as a measure of the catalytic efficiency and specificity. If K_m is expressed in g/litre, the values are 121, 54 and 21 for UFR arabinan, UFR (linearised) and 1,5-α-L-arabinan, respectively. However, if K_m is expressed as mol/litre of terminal arabinose residues, the values for these three substrates ($12{\cdot}7 \times 10^4$, $13{\cdot}1 \times 10^4$ and $8{\cdot}0 \times 10^4$, respectively) approach each other rather closely.

For the *endo*-arabinanase the lowest K_m value was found on 1,5-α-L-arabinan and the highest value on UFR arabinan. For K_m in g/litre the values of V_{max}/K_m are 13, 25 and 101, for UFR arabinan, UFR (linearised) and 1,5-α-L-arabinan, respectively. Expressing K_m values in mol/litre of unbranched 1,5-α-L-linked arabinose residues yields values of $7{\cdot}7 \times 10^3$, $11{\cdot}4 \times 10^3$ and $17{\cdot}4 \times 10^3$, respectively. These latter values indicate that the 1,5-α-L-arabinan is the best substrate for *endo*-arabinanase.

DISCUSSION

Purification

The scheme adopted for the purification of the arabinanases of the *A. niger* preparation was primarily based on that used for the purification of the cellulase enzymes from a preparation of *Trichoderma viride* (Beldman *et al.*, 1985). In view of their widely different molecular weights the enzymes should be easily separated. However, the isolation of the three enzymes was very difficult and the use of the level sensor controlled gradient mixer was of critical value in the purification procedure. The problems encountered in purification are similar to those in isoelectric focussing experiments. Part of these problems may be explained by a tendency of the enzymes to associate, which was clearly demonstrated (e.g. see Figs 1, 3 and 7). It is possible that the addition of reducing agents, such as cysteine or dithiothreitol, to the chromatography buffers would have facilitated enzyme purification, but it has not been tried.

A summary of the purification, including yields and all the data on the specific activity of the enzymes in subsequent steps is not included in this paper. These are meaningless, as the enzymes exhibit considerable synergistic effects. Because of the synergistic effects separation always goes at the expense of the apparent activity, even without real loss of enzyme activity. However, the specific activities of the purified enzymes on two different substrates are listed in Table 3.

Properties of the enzymes

Until now only a single type of arabinan-degrading enzyme has been isolated from *A. niger* (Kaji & Tagawa, 1970; Tagawa, 1970). With regard to substrate specificity this so-called *A. niger* type of α-L-arabinofuranosidase is similar to α-L-arabinofuranosidase B described in this paper. It was described as being active towards simple synthetic α-L-arabinoside derivatives and to release the side-chain L-arabinose residues from α-L-arabinan, L-arabinoxylan and L-arabinogalactan. It also hydrolysed the 1,5-α-L-arabinan but at a lower rate (Tagawa, 1970). This type of enzyme has been isolated from a number of sources including other fungi (Kaji & Yoshihara, 1971; Brillouet *et al.*, 1985) and bacteria (Weinstein & Albersheim, 1979; Greve *et al.*, 1984).

α-L-Arabinofuranosidase B differs in a number of properties from the enzyme isolated from *A. niger* by Tagawa (Kaji & Tagawa, 1970; Tagawa, 1970). The molecular weight of this latter enzyme was 53000, its isoelectric point 3·6, its optimum pH 3·9, its specific activity on synthetic α-L-arabinoside 397 U/mg, its K_m for branched arabinan 0·26 g/litre and its V_{max} for that substrate $2{\cdot}0 \times 10^3$ min^{-1}. In relation to this it may be mentioned that Waibel *et al.*, (1980) produced evidence for the existence of two forms of α-L-arabinofuranosidase B in *A. niger*, but they did not fully purify them and, apart from their substrate specificities, no further properties were reported.

In addition to type B we found another α-L-arabinofuranosidase (type A) which, in its substrate specificity, resembled very much the so-called *Streptomyces purpurascens* type (Komae *et al.*, 1982), as both enzymes are active only towards synthetic α-L-arabinosides and the lower oligosaccharides derived from 1,5-α-L-arabinan. This type of enzyme has also been demonstrated in *Bacillus subtilis* (Weinstein & Albersheim, 1979). Molecular and kinetic properties of the type A enzyme differ from those of the *S. purpurascens* enzyme (Komae *et al.*, 1982), while those of the *Bacillus* enzyme have not been reported.

The *endo*-arabinanase described in this paper is similar in its substrate specificity and action pattern to the *endo*-arabinanase from *B. subtilis*, (described by Kaji & Saheki, 1975; Weinstein & Albersheim, 1979; Yoshihara & Kaji, 1983), as both enzymes are inactive on synthetic arabinosides and arabinogalactan, and the best substrate is 1,5-α-L-arabinan for both enzymes. However, the *endo*-arabinanase described here degrades 1,5-α-L-arabinan with accumulation of the disaccharide and the trisaccharide as the end-products, whilst arabinose monomers and dimers were the end-products of the *B subtilis* enzyme.

Other differences between the two enzymes are molecular weight (35000 versus 32000–33000 for that of *B. subtilis*), isoelectric points (4·5–5·5 versus 9·3) and optimum pH (5·0 versus 6·0). Detailed kinetic properties of *B. subtilis* enzymes are not available in literature, but Yoshihara and Kaji (1983) reported the initial rate of reaction of *Bacillus endo*-arabinanase on 1,5-α-L-arabinan (15 μmol min^{-1} mg^{-1} protein) to be slightly higher than that on beet arabinan (12·5 μmol min^{-1} mg^{-1}); which is true also for the *A. niger endo*-arabinanase.

A fourth type of arabinan-degrading enzyme has recently been described from *Erwinia carotovora* (Kaji & Shimokawa, 1984). It is a true *exo*-arabinanase as it releases arabinose trisaccharides from the non-reducing end of 1,5-α-L-arabinan, and does not show activity on synthetic α-L-arabinosides. No evidence was found for the existence of this enzyme in the *A. niger* preparation.

During this study it was noticed that the three enzymes exert considerable synergistic effects. These are now being studied in detail and will be the subject of a future communication.

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