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Purification and physico-chemical properties of an *endo*-1,5-α-L-arabinanase (EC 3.2.1.99) isolated from an *Aspergillus niger* pectinase preparation

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Using low and medium (FPLC) pressure column chromatographic separation methods, an *endo*-1,5-α-L-arabinanase (EC 3.2.1.99) was purified from an *Aspergillus niger* pectinase preparation. The purification procedure combined different anion exchange and hydrophobic interaction chromatographies followed by gel filtration, yielding a highly pure and specific enzyme. The isolated *endo*-1,5-α-L-arabinanase was most active on linear 1,5-α-L-arabinan (90·2 U/mg), whereas branched arabinans from sugar beets were degraded to a lesser extent (~ 14 U/mg). No other significant carbohydrate degrading activities could be detected. The enzyme was shown to be electrophoretically pure after silver staining and its identity was confirmed through specific binding to an antiserum directed against *endo*-1,5-α-L-arabinanase. The major physico-chemical characteristics of the enzyme were the following: M_r 42 500 (SDS–PAGE), M_r 33 900 (MALDI-TOF-MS), pI ≤ 3·0, pH optimum 4·8, temperature optimum 55°C, pH stability 3·5–8·0, temperature stability ≤ 45°C, K_m = 0·205 mg/ml, $V_{max} = 1{\cdot}77 \times 10^{-4}$ μmol/min, E_A = 12·20 kcal/mol; Zn^{2+} and Hg^{2+} showed potent inhibitory effects.

INTRODUCTION

Cell walls have important functions in plant tissue and affect the processing of plant material to a large extent. Primary plant cell walls are thought to consist of complicated cross-linked macromolecular networks, in which cellulose fibres are embedded in a matrix of pectic and hemicellulosic polysaccharides and a few glycoproteins (Aspinall, 1980). Pectins are complex polysaccharides composed of a rhamnogalacturonan backbone to which side-chains of neutral sugars are linked; arabinose and galactose are the main neutral sugars. Hemicelluloses consist of different polysaccharides which vary with plant variety and cell type; xyloglucan is a major hemicellulosic component, others are xylans, arabinoxylans and glucomannans (McNeil *et al.*, 1979).

Arabinans, associated with galactans and arabinogalactans, are regarded as forming part of the neutral pectic fraction of the cell wall (Aspinall, 1980). Structurally, all of the arabinans that have been investigated so far appear to have similar structures, consisting of an α-1,5 linked L-arabinofuranosyl (Ara *f*) main backbone and a varying number of side chains of other α-L-Ara *f* residues, linked by α-1,2 and/or α-1,3 bonds to the main chain (McNeil *et al.*, 1979; Aspinall, 1980; Villettaz *et al.*, 1981). An almost linear 1,5-α-L-arabinan has been isolated from apple-juice concentrate (Voragen *et al.*, 1982; Churms *et al.*, 1983).

Structural information concerning the interconnections of cell wall polysaccharides has been deduced from analyses of the oligosaccharides produced by purified polysaccharide degrading enzymes. The advantage of using highly purified, specific enzymes lies in their ability to degrade the complex polymers present in the cell wall to a limited set of identifiable oligomers (Matheson & McCleary, 1985), whereas the classical acidic or basic extraction procedures result in the simultaneous cleavage of a number of different types of bonds present in the cell wall (Aspinall, 1982). The purified enzymes required to perform such structural analyses are generally isolated from microbial sources, using different

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combinations of column chromatographic (CC) separation methods.

Arabinanases are hydrolytic polysaccharases capable of degrading arabinans as well as arabinose side-chains, which occur in arabinoxylans and arabinogalactans. Three different types of arabinanases have been reported so far (Kaji, 1984; Kaji & Shimokawa, 1984; Rombouts *et al.*, 1988; Van der Veen *et al.*, 1991; Beldman *et al.*, 1993; Lerouge *et al.*, 1993): (1) *endo*-1,5-α-L-arabinanases (*endo*-ABA; EC 3.2.1.99), which cleave arabinans at α-1,5 positions in a random mechanism; (2) α-L-arabinofuranosidases (EC 3.2.1.55), which hydrolyse both 1,3- and 1,5-α-L-Ara*f* linkages, remove arabinose units from the non-reducing end of an arabinan chain; and (3) *exo*-arabinanase, which releases arabinotriose from the non-reducing end of 1,5-α-L-arabinans and shows no activity towards synthetic α-L-arabinosides.

Endo-ABAs have been isolated from several fungal and bacterial sources (Kaji, *et al.*, 1967; Kaji & Saheki, 1975; Weinstein & Albersheim, 1979; Yoshihara & Kaji, 1983; Karimi & Ward, 1989; Van der Veen *et al.*, 1991; Ramon *et al.*, 1993) as well as from commercial pectic enzyme (pectinase) preparations derived from *Aspergillus niger* (*A. niger*) (Rombouts *et al.*, 1988; Shöpplein, 1989; Beldman *et al.*, 1993; Lerouge *et al.*, 1993). The latter preparations generally contain a variety of enzymes other than pectic enzymes (Waibel *et al.*, 1980; Schöpplein, 1989). Within these enzyme cocktails, the *endo*-ABA is considered to be a minor component, but is nevertheless great value not only in structural studies of plant cell walls (Matheson & McCleary, 1985), but also in the food industry (Churms *et al.*, 1983; Voragen *et al.*, 1982; Whitaker, 1984).

This paper describes the elaboration of a new combination of CC separation methods for the isolation of an *endo*-ABA from a complex *A. niger* pectinase preparation. Furthermore, the major physico-chemical properties of the enzyme are characterised.

EXPERIMENTAL

Substrates and chemicals

Unless explicitly specified, all chemicals used were of analytical grade and supplied by Merck AG (Darmstadt, Germany) or Fluka Chemie AG (Buchs, Switzerland). The water used was of Nanopure quality (Barnstead NANOpure, Skan AG, Basle, Switzerland).

Polysaccharides

Branched arabinan A (sugar beet) and debranched arabinan (purified, branched sugar beet arabinan, completely debranched with α-L-arabinofuranosidase, EC 3.2.1.55) were purchased from MegaZyme Pty. Ltd (North Rocks, NSW, Australia). The linear 1,5-α-L-haze-arabinan (haze arabinan from apple juice concentrate) was a gift from Novo Nordisk Ferment AG (Dittingen, Switzerland).

Extraction and purification of branched arabinan B from sugar beet

Crude branched arabinan B was extracted from dried, ground sugar beet pellets (Zuckerfabrik Frauenfeld, Switzerland) according to Tagawa & Kaji (1988). The isolated arabinan was further purified by CC at room temperature (RT) over a Q-Sepharose Fast Flow anion exchange gel (Pharmacia Biosystems, Uppsala, Sweden), packed in a XK 26/40 column (340 mm × 26 mm i.d., Pharmacia) and equilibrated at 80 ml/h with 900 ml 0·5 M Na-phosphate, pH 5·5 (plus 0·02% (w/v) NaN_3) followed by 900 ml of water (plus 0·01% (v/v) toluene). After application of 25 ml crude arabinan B solution (4% (w/v) in water) on the column, the neutral polysaccharide fraction was eluted at 90 ml/h with 500 ml of water. The acidic fractions were subsequently eluted with a 600 ml linear Na-phosphate gradient (0–0·5 M, pH 5·5 + 0·01% (v/v) toluene). The pooled neutral fractions were freeze-dried. CC eluates were monitored with a refractive index detector 1037 A (Hewlett-Packard, Avondale, PA, USA).

Arabinoxylan (wheat flour), galactan (lupin), mannan (ivory nut) and curdlan (1,3-β-glucan, *Alcaligenes faecalis* var. myxogenes 10C3K) were purchased from MegaZyme. Arabinogalactan (larch wood) was acquired from Serva GmbH (Heidelberg, Germany). Carboxymethylxylan (CMX) was prepared according to Batzer (1986). Galactomannans (carubin and guar type) came from Carl Roth GmbH (Karlsruhe, Germany). Glucomannan (tuber salep) was prepared as described by Bänziger (1992). Starch (potato, Zulkowsky type), carboxymethylcellulose Na-salt (CMC) and dextran (*Leuconostoc mesenteroides*, $M_r \sim 35\,000$) were from Fluka. Lichenin was from our laboratory collection. Polygalacturonic acid Na-salt (orange) and gum arabic (acacia tree) were from Sigma Chemical Co. (St. Louis, MO, USA). Pectins (apple) with degrees of esterification (DE) of 35 and 75% were obtained from Obipektin AG (Bischofszell, Switzerland).

para-*Nitrophenyl derivatives* (p-*NP-*)

p-NP-α-L-arabinofuranoside (*p*-NP-α-L-Ara *f*), *p*-NP-α-L-arabinopyranoside (*p*-NP-α-L-Ara*p*), *p*-NP-β-D-glucopyranoside (*p*-NP-β-D-Glu *p*) and *p*-NP-β-D-xylopyranoside (*p*-NP-β-D-Xyl *p*) were from Sigma; *p*-NP-β-L-arabinopyranoside (*p*-NP-β-L-Ara *p*) and *p*-NP-β-D-galactopyranoside (*p*-NP-β-D-Gal *p*) were from Senn Chemicals AG (Dielsdorf, Switzerland). *p*-NP-α-D-galactopyranoside (*p*-NP-α-D-Gal *p*) was from Serva, *p*-NP-α-D-glucopyranoside (*p*-NP-α-D-Glu *p*) was from Merck and *p*-NP-α-D-mannopyranoside (*p*-NP-α-D-Man *p*) was from Carl Roth.

Carbohydrate analyses

Neutral sugar composition/determination of galacturonic acid content/glycosidic-linkage analysis (methylation analysis)
The neutral sugar composition and the amount of galacturonic acid residues in the polysaccharide substrates were determined as described previously (Dunkel & Amadò, 1994). Galacturonic acid quantifications in CC eluate fractions were performed with a Skalar 5100 dual-channel segmented flow analyser (Skalar Analytical, B.V., Breda, The Netherlands) with a *m*-phenylphenol concentration of 0·04% (w/v). Glycosidic-linkage analysis was performed by GC–MS of the partially methylated alditolacetates (Dunkel & Amadò, 1994).

Total neutral sugar determination
Total amounts of neutral sugars were determined according to Bailey (1958) with the following modifications: 0·5 ml samples were mixed with 5 ml of acidic anthrone reagent (0·015% (w/v) anthrone in 72·5% (v/v) sulphuric acid), boiled for 8 min and cooled in an ice bath. The absorbance was measured at 610 nm (Uvikon 940 spectrophotometer, Kontron Instruments, Zürich, Switzerland) after 30 min; arabinose was used as standard. Determinations in CC eluate fractions were performed with the Skalar 5100 analyser as above (0·02% (w/v) anthrone in 72·5% (v/v) sulphuric acid).

Enzyme preparations

Pectinex AR KPG 027, an enzyme preparation from *A. niger*, was kindly provided by Novo Nordisk Ferment AG. Rapidase KE 8810, a non-commercial *A. niger* enzyme preparation, was generously supplied by Gist-Brocades (Séclin, France). The *A. niger* enzyme preparation Rohapect DA 3L (lot 465 240) was a gift from Röhm GmbH (Darmstadt, Germany). Partially purified *A. niger endo*-ABA (lot MAR00301) was purchased from MegaZyme.

Enzyme activity assays

Enzyme activities were expressed in International Units (U). One U was the amount of enzyme that released 1 μmol/min of monosaccharide from linear 1,5-α-L-haze-arabinan. All buffers contained 0·02% (w/v) NaN_3 to prevent microbial growth.

Quantitative determination of glycanase activities
Substrate solutions consisted of 0·01 to 0·2% (w/v) polysaccharide in 50 mM Na-acetate buffer, pH 4·8. Glycanase activities were determined in a reaction mixture containing 200 μl of substrate solution and 25 μl of appropriately diluted enzyme solution in 50 mM Na-acetate buffer, pH 4·8. A standard incubation time of 20 min at 40°C was used. To detect residual glycanases activity of the purified enzyme towards substrates other than arabinans, an incubation time of 7 h at 40°C was used. The concentration of reducing end-groups was determined according to the Nelson–Somogyi method, with the modifications described by Dunkel & Amadò (1994). Different monosaccharides were used as standards.

Quantitative determination of glycosidase activities
Substrate solutions consisted of 0·05% (w/v) *p*-NP derivative in 50 mM Na-acetate buffer, pH 4·8. Glycosidase activities were determined in a reaction mixture containing 500 μl of substrate solution and 50 μl of appropriately diluted enzyme solution in 50 mM Na-acetate buffer, pH 4·8. Determinations of all glycosidase activities in the starting enzyme preparation were performed after incubation for 10 min at 35°C. Glycosidases activity of the purified enzyme were determined after incubation for 7 h at 35°C. In every case, the reaction was stopped by adding 500 μl of 1 M Na_2CO_3. The concentration of liberated *p*-nitrophenol was immediately determined at 405 nm using *p*-nitrophenol as standard.

Semi-quantitative endo-*1,5-α-L-arabinanase activity measurement in column chromatography eluate fractions*
Semi-quantitative determinations of *endo*-ABA activity in CC eluate fractions were performed with a cup-plate assay, using dye-labelled arabinan as substrate (Dunkel & Amadò, 1994).

Protein determination

Determination of the total protein content was performed with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA), according to the supplier's indications. Bovine serum albumin (Serva) was used as standard.

Column chromatographic methods

Unless specified otherwise, all gels, columns and apparatus were from Pharmacia. Columns were connected to two P-500 FPLC pumps controlled by a liquid chromatography controller LCC-500, and elution profiles were monitored at 280 nm (single path monitor UV-1). Simultaneous recording of conductivity in CC eluates was performed with a flow-through electrode LDM/S connected to a conductometer LF 537 (WTW, Weilheim, Germany). Protein absorbance and conductivity were recorded on a two-channel recorder REC-482. All buffers for CC and dialysis contained 0·02% (w/v) NaN_3 to prevent microbial growth and the separations were performed at RT. Concentration and dialysis of protein solutions were carried out at 4°C.

Column chromatographic purification procedure of endo-*1,5-α-L-arabinanase*

The crude enzyme preparation Pectinex AR KPG 027 was centrifuged (20 min, 20 000 *g*, 4°C) and the supernatant was filtered through a 0·22 μm membrane filter (Millipore Corp., Bedford, MA, USA). Aliquots of the filtered solution were stored at −20°C. As summarised in Fig. 1, the following CC separation steps were used for the purification of the *endo*-ABA:

1. Anion exchange on Q-Sepharose Fast Flow (Fig. 2)

Aliquots of filtered and pretreated Pectinex AR KPG 027 solutions were dialysed at 4°C (stirred cell 8400, PM 10 membrane cut-off 10 000 Da, Amicon Inc., Beverly, MA, USA) against 50 mM Na-acetate buffer (pH 4·5) and subsequently concentrated (from 24·1 to 74·3 mg/ml). The separation of the concentrated enzyme preparation was performed on a Q-Sepharose Fast Flow gel packed in an XK 26/40 column (170 mm × 26 mm i.d.) and equilibrated with 0·15 M NaCl, 50 mM Na-acetate buffer, pH 4·5 (7 ml/min). A 5 ml aliquot of concentrated enzyme solution (74·3 mg/ml of protein in 50 mM Na-acetate buffer, pH 4·5) was applied through an MV-7 valve on the equilibrated column (1 ml/min), and the elution was performed with a stepwise increasing NaCl gradient (0·15 M for 660 ml; 0·2 M for 280 ml; 1 M for 300 ml) in 50 mM Na-acetate buffer (final pH 4·5) at a flow rate of 3 ml/min. Fractions of 10 ml were collected and the column was washed with 2 M NaCl before re-equilibration with the starting buffer.

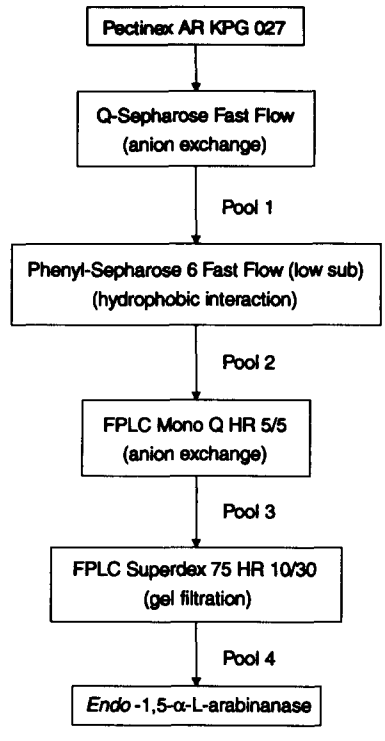

Fig. 1. Flow sheet of the purification of *endo*-1,5-α-L-arabinanase from Pectinex AR KPG 027 (*A. niger*).

2. Hydrophobic interaction on Phenyl-Sepharose 6 Fast Flow (low sub) (Fig. 3)

The gel Phenyl-Sepharose 6 Fast Flow (low sub) was packed in an XK 26/40 column (280 mm × 26 mm i.d.) and equilibrated with 1·8 M ammonium sulphate, 50 mM

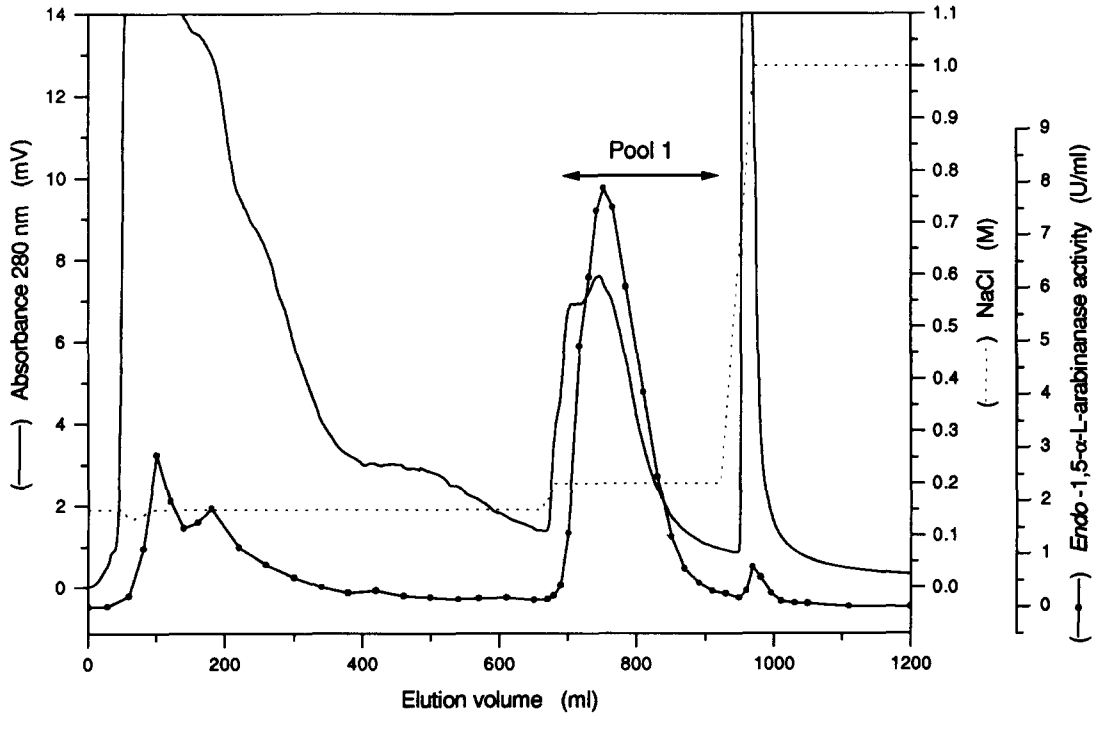

Fig. 2. Anion exchange column chromatography on Q-Sepharose Fast Flow of Pectinex AR KPG 027 (*A. niger*). Complete experimental details as well as *endo*-ABA activity determination procedures are described in the text.

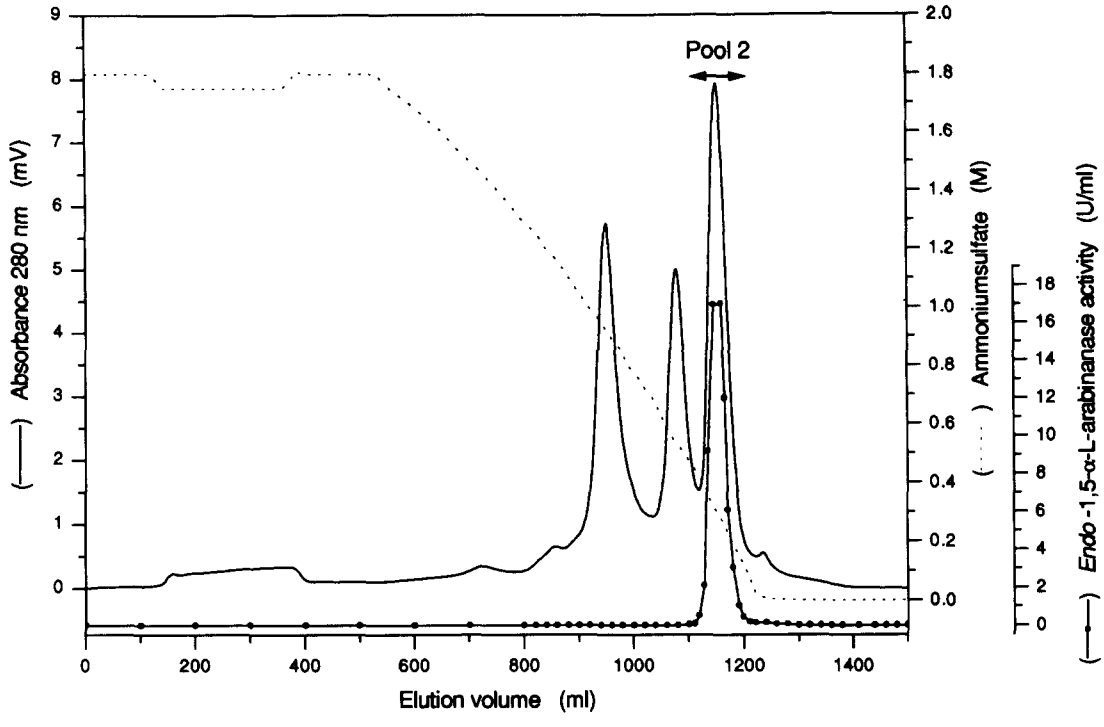

Fig. 3. Hydrophobic interaction column chromatography on Phenyl-Sepharose 6 Fast Flow (low sub) of partially purified *endo*-1,5-α-L-arabinanase. Pooled *endo*-ABA activity containing fractions of the previous purification step (Fig. 2, pool 1) were applied on a Phenyl-Sepharose 6 Fast Flow (low sub) gel. Complete experimental details as well as *endo*-ABA activity determination procedures are described in the text.

Na-acetate buffer, pH 4·5 (5 ml/min). Pooled *endo*-ABA activity containing fractions of the previous purification step (pool 1 of Fig. 2, 226 ml, 124 μg/ml of protein) were brought to a final concentration of 1·8 M ammonium sulphate, filtered (0·22 μm) and applied in several steps through a 50 ml superloop (connected to an MV-7 valve) on the equilibrated column at 3 ml/min. After elution with 150 ml of equilibration buffer (3 ml/min), a linear, decreasing ammonium sulphate gradient (1·8–0 M) in 50 mM Na-acetate (pH 4·5) was applied over 700 ml. Fractions of 10 ml were collected and the column was washed with water (5 ml/min) before re-equilibration with the starting buffer.

3. FPLC anion exchange on Mono Q HR 5/5 (Fig. 4)

Pooled *endo*-ABA activity containing fractions of the hydrophobic interaction CC separation (pool 2 of Fig. 3) were dialysed against 20 mM piperazine/HCl buffer (pH 5·0), until a constant conductivity was achieved, and concentrated (stirred cell 8400, PM 10 membrane cut-off M_r 10 000, Amicon). The anion exchange CC was performed on an FPLC Mono Q HR 5/5 column equilibrated with the same buffer. Repeated injections of 1 ml of dialysed and concentrated samples (307 μg/ml of protein) were performed automatically through a 50 ml superloop at 0·3 ml/min. After 1 ml elution with equilibration buffer (5 ml/min), the NaCl concentration was raised to 0·2 M and a linear, increasing NaCl gradient (0·2–0·25 M) in 20 mM piperazine/HCl (pH 5·0) was applied over 15 ml. Fractions of 0·5 ml were collected and the column was washed with 1 M NaCl in 20 mM piperazine/HCl, pH 5·0 (1 ml/min) before re-equilibration with the starting buffer.

4. FPLC gel filtration on Superdex 75 HR 10/30 (Fig. 5)

Pooled *endo*-ABA activity containing fractions of the FPLC Mono Q CC (pool 3 of Fig. 4, 61 μg/ml of protein) were applied on an FPLC gel filtration Superdex 75 HR 10/30 column as 200 μl aliquots through a superloop at 0·3 ml/min. Isocratic elution was performed with 0·15 M NaCl in 50 mM Na-acetate buffer (final pH 4·5) at 0·5 ml/min. *Endo*-ABA activity containing fractions of 0·25 ml were collected, pooled and stored at 4°C.

Polyacrylamide gel electrophoretic analyses

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

All chemicals for SDS–PAGE were of electrophoretic purity and supplied by Bio-Rad. Purification of the *endo*-ABA was monitored using the Mini-Protein II slab cell (Bio-Rad). Staining for protein was performed with the silver stain method as described by Merril *et al.* (1984). Detailed electrophoretic conditions are described in the caption to Fig. 6.

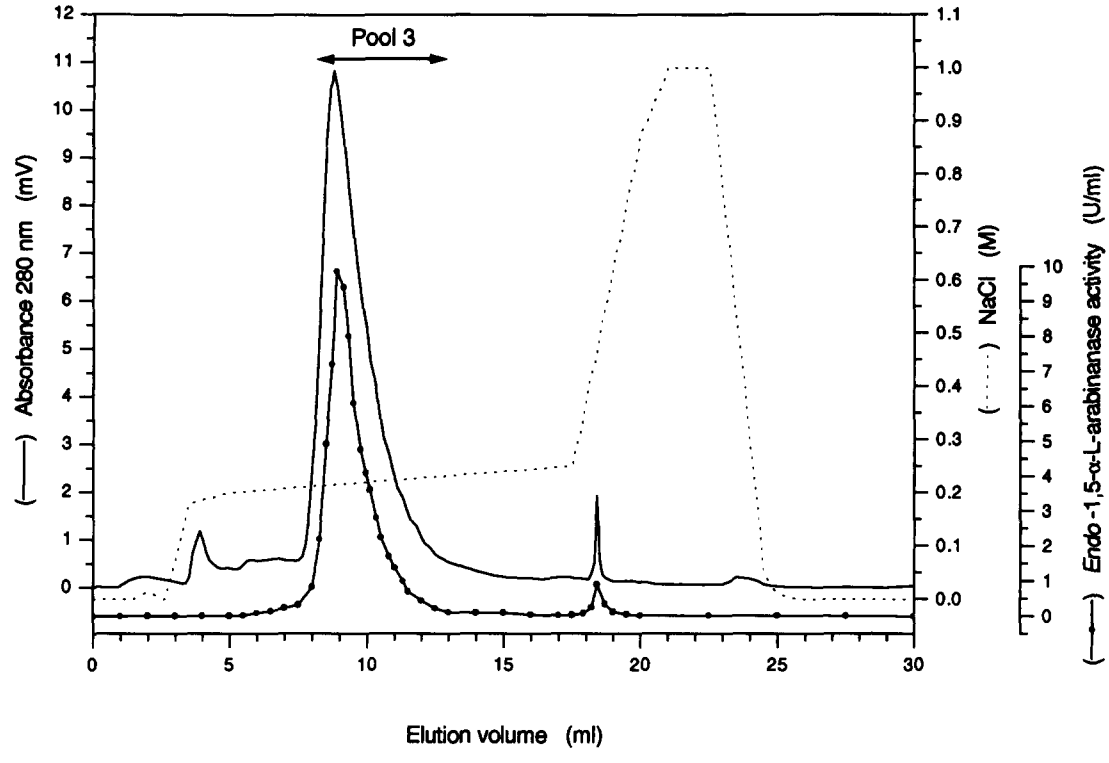

Fig. 4. FPLC anion exchange column chromatography on Mono Q HR 5/5 of partially purified *endo*-1,5-α-L-arabinanase. Pooled *endo*-ABA activity containing fractions of the hydrophobic interaction CC separation (Fig. 3, pool 2) were dialysed and applied on an FPLC Mono Q HR 5/5 column. Complete experimental details as well as *endo*-ABA activity determination procedures are described in the text.

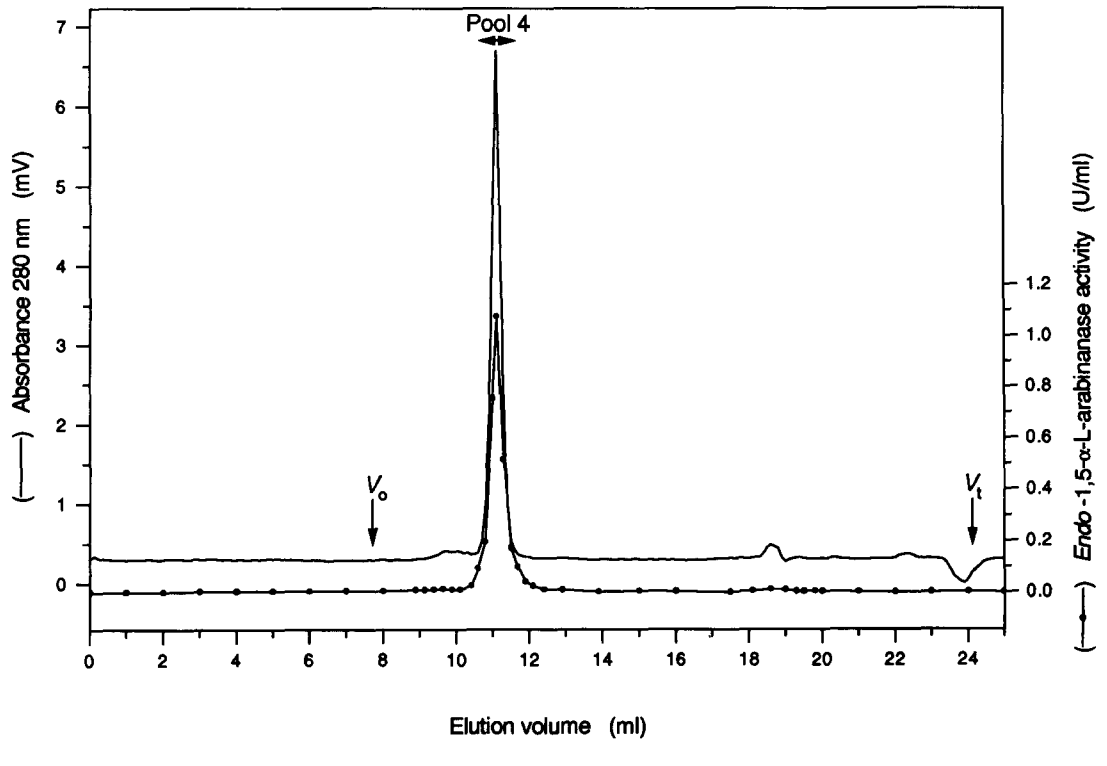

Fig. 5. FPLC gel filtration column chromatography on Superdex 75 HR 10/30 of partially purified *endo*-1,5-α-L-arabinanase. Pooled *endo*-ABA activity containing fractions of the FPLC Mono Q CC (Fig. 4, pool 3) were applied on an FPLC gel filtration Superdex 75 HR 10/30 column. Complete experimental details as well as *endo*-ABA activity determination procedures are described in the text.

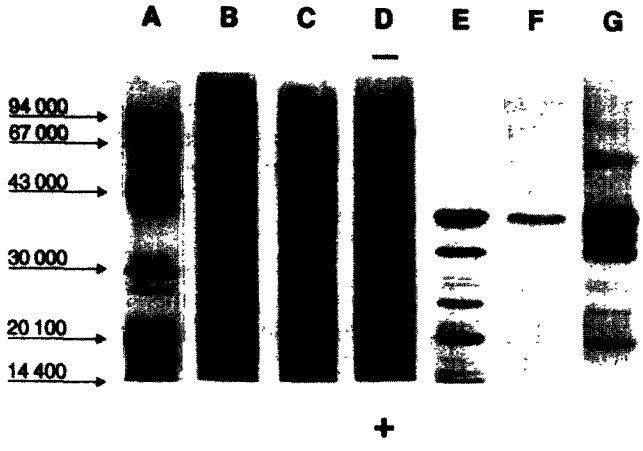

Fig. 6. SDS–PAGE of *endo*-1,5-α-L-arabinanase at different purification stages. Gel dimensions: 70 mm × 80 mm; 1 mm thick SDS polyacrylamide separating gels (T = 12%; C = 2·67%) and sampling gels (T = 4%; C = 2·67%) were cast according to the Laemmli method as described in the supplier's manual (Bio-Rad). Samples were diluted twice with sample buffer (62·5 mM Tris/HCl, pH 6·8; 15% (v/v) glycerol; 2% (w/v) SDS; 0·5% (w/v) dithiothreitol (DTT); 0·005% (w/v) bromphenol blue) and boiled for 8 min. After cooling to RT, DTT was added to a total final concentration of 26 mM. Electrode buffer consisted of 25 mM Tris, 192 mM glycine and 0·1% (w/v) SDS, pH 8·3. Sample volumes: 25 μl. Running conditions: 150 V constant setting for 15 min followed by 200 V constant setting until the end of the run. Detection of proteins with silver staining. Marker proteins: phosphorylase B (M_r 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400). For further details see text.

		protein (ng)/25 μl
lane A:	Low M_r electrophoretic calibration kit	—
lane B:	Pectinex AR KPG 027 (raw material)	1863
lane C:	Pool 1 from Q-Sepharose Fast Flow separation (Fig. 2), dialysed in water	1163
lane D:	Pool 2 from Phenyl-Sepharose 6 Fast Flow (Fig. 3), dialysed in water	834
lane E:	Pool 3 from FPLC Mono Q HR 5/5 (Fig. 4)	420
lane F:	Purified *endo*-1,5-α-L-arabinanase (Fig. 5, pool 4)	191
lane G:	*endo*-1,5-α-L-arabinanase, commercial preparation (MegaZyme)	177

Analytical isoelectric focusing (IEF)

All apparatus and gels were from Pharmacia. Analytical IEF was performed with the PhastSystem according to the instructions of the supplier, using precast polyacrylamide gels in the pH range 3–9. Samples were treated and separations were carried out as described in the PhastSystem separation technique file No. 100. Silver staining of the gels was done according to the instructions of the PhastGel instruction manual.

Analytical IEF in presence of urea

IEF studies under denaturating conditions were performed with reswollen precast CleanGels (code 80–5067–50) from Pharmacia Biosystems GmbH (Freiburg, Germany) on the PhastSystem. Sample preparation, electrophoretic separation and silver staining were performed following the instructions of the supplier. The last electrophoretic separation step was carried out with a maximum of 1250 V instead of the recommended 1500 V to avoid burning the gels. Detailed electrophoretic conditions are described in the caption to Fig. 7.

Antibodies and Western blot analyses

The antiserum against *endo*-ABA was a generous gift from Professor Visser (Department of Genetics of the Agricultural University Wageningen, The Netherlands). It had been raised in Balb/c mice as described by Van der Veen *et al.* (1991). Cross-reactivity between the antiserum and the purified *endo*-ABA was tested by Western blotting, using a Mini trans blot electrophoretic transfer cell (Bio-Rad). Resolved SDS–PAGE gels were blotted on nitrocellulose (NC) sheets BA 83 (0·2 μm, Schleicher & Schuell GmbH, Dassel, Germany). The membranes were incubated with 1:5000 diluted antiserum and subsequently developed using alkaline phosphatase conjugated anti-mouse goat IgG (H + L), as described by the manufacturer (Bio-Rad). Staining for total protein on the NC blots was performed with Fast Green (Westermeier, 1990).

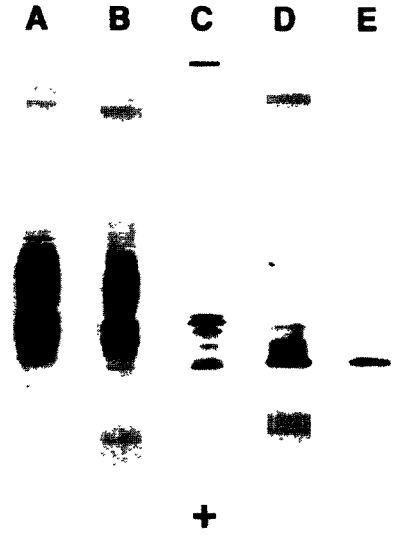

Fig. 7. Denaturating analytical IEF of *endo*-1,5-α-L-arabinanase at different purification stages in presence of 8 M urea. Dry CleanGels were reswollen in a GelPool with the following buffer system (quantities apply for one gel): 67·5 μl Servalyt 2–4, 7·5 μl Servalyt 2–11, 480 mg urea and 925 μl water. Servalytes and urea were from Serva GmbH. Detection of proteins with silver staining. Further experimental details are described in the text. Sample volumes: 1 μl. Lane A: Pectinex AR KPG 027 (raw material); lane B: Pool 1 from Q-Sepharose Fast Flow separation (Fig. 2); lane C: Pool 2 from Phenyl-Sepharose 6 Fast Flow (Fig. 3); lane D: Pool 3 from FPLC Mono Q HR 5/5 (Fig. 4); lane E: purified *endo*-1,5-α-L-arabinanase (pool 4, Fig. 5).

Characterisation of the purified *endo*-ABA

Influence of pH on activity and stability

The *pH optimum* of *endo*-ABA activity on linear 1,5-α-L-haze-arabinan was investigated at pH values ranging from 2·0 to 8·0, with 0·5 unit increments. HCl/KCl buffers were used for pH 1·0–2·0, and McIlvaine buffers (Perrin & Dempsey, 1974) for pH 2·5–8·0. Substrate solubilisation (0·2% (w/v)) and enzyme dilutions were performed in the same series of buffers. Activity determinations were performed as described before. The *pH stability* was studied by appropriate dilution of the enzyme solutions in different buffers ranging from pH 1·0 to 12·0, with 0·5 unit increments. The same buffers as above were used for the pH range 1·0–8·0 and $NaHCO_3/Na_2CO_3$ buffers for pH 9·0–12·0. After storage of the diluted enzyme solutions for 67 h at RT, the residual activities were determined at pH optimum as described above.

Influence of temperature on activity and stability

T optimum determinations of *endo*-ABA activity were performed with 0·2% (w/v) linear 1,5-α-L-haze-arabinan, dissolved in 50 mM Na-acetate buffer at pH optimum. Enzyme solutions (in 50 mM Na-acetate buffer, pH optimum) were incubated with substrate solutions for 20 min at *T* ranging from 25 to 70°C. *endo*-ABA activity was quantitatively determined as described above. *T stability* was investigated after incubating enzyme solutions for 3 h (in 50 mM Na-acetate buffer, pH optimum) at *T* ranging from 25 to 70°C. Long-term stability investigations at 40°C were performed by incubating enzyme solutions in 50 mM Na-acetate buffer at pH optimum for up to 27 h. Residual activities were determined under standard conditions as described above.

Kinetic properties

The K_m and V_{max} values of the *endo*-ABA were determined using 0 to 0·8% (w/v) solutions of linear 1,5-α-L-haze-arabinan as substrate at pH and *T* optimum conditions.

Isoelectric point (pI) determinations

With isoelectric focusing. Determinations were performed on the PhastSystem as described before. The Pharmacia isoelectric focusing calibration kit (broad range, pH 3–10) was used as standard.

With column chromatographic chromatofocusing. Chromatofocusing studies were performed on an FPLC Mono P HR 5/20 column (Pharmacia) at RT, pH values in the different eluate fractions were determined using an Orion Ross combination pH electrode (Müller + Krempel AG, Bülach, Switzerland). Purified *endo*-ABA fractions were buffer-exchanged in 25 mM piperazine/HCl buffer (pH 5·4) (without NaN_3) using Econo-Pac 10DG columns (Bio-Rad). The column was equilibrated with 25 mM piperazine/HCl buffer (pH 5·4) (without NaN_3) at 0·5 ml/min. Samples were applied as 500 μl aliquots. Reasonably linear, decreasing pH gradients were generated in the column by washing the gel with 80 ml of 1:40 diluted Polybuffer 74 (Pharmacia, titrated with HCl to pH 2·8) at 0·5 ml/min.

Relative molecular weight determinations

With gel filtration. CC conditions were identical to those described in the purification scheme. The following standards (Pharmacia), dissolved in elution buffer, were used: ferritin (M_r 440 000), catalase (232 000), adolase (158 000), bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000), ribonuclease A (13 700), cytochrome C (12 400, Serva), vitamine B12 (1355·4, Serva) and cytidin (243·2, Serva). Standard samples were applied as 200 μl aliquots on the column.

With SDS–PAGE. Electrophoretic conditions were identical to those described before. The Pharmacia low M_r electrophoresis calibration kit was used as standard.

With matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS). MALDI-TOF-MS experiments were performed by Dr M. Schär (Ciba-Geigy Ltd., Basle, Switzerland) on an LDI-1700 TOF mass spectrometer (Linear Scientific Inc., Reno, NV, USA). The matrix consisted of 0·1 M sinapinic acid dissolved in 0·1 M acetonitrile. As no internal standard was used, the precision of the determination was ±0·1%. For experimental details, see Schär *et al.* (1991).

Effect of specific inhibitors and metal ions

Solutions of different inhibitors and metal ions were prepared in 50 mM Na-acetate buffer, pH 4·8. *Endo*-ABA activities in the presence of effectors were determined in a reaction mixture containing 150 μl of substrate solution (0·2% (w/v) linear 1,5-α-L-haze-arabinan), 50 μl of effector solutions and 25 μl of appropriately diluted enzyme solution in 50 mM Na-acetate buffer, pH 4·8. The concentration of reducing end groups was determined after 20 min incubation at 40°C.

RESULTS AND DISCUSSION

Purification studies of the *endo*-1,5-α-L-arabinanase

Different enzyme preparations from *A. niger* were compared for their specific *endo*-ABA activities. As the pH optimum of all preparations was between 4·0 and 5·0, the *endo*-ABA activity measurements were performed at pH 4·8. The pectinase preparation Pectinex AR KPG 027 (4·14 U/mg) was selected as starting material for the following *endo*-ABA purification as it

contained the highest specific *endo*-ABA activity (other enzymic preparations examined: Rohapect DA 3L: 0·72 U/mg; Rapidase KE 8810: 0·51 U/mg).

The purification of the *endo*-ABA was accomplished by using a combination of different low and medium (FPLC) pressure CC separation methods. To avoid the use of too many conditioning steps (e.g. dialysis and/or buffer exchange), in which no effective purification could be attained, but the yields decreased, special attention was attributed to the sequence of the CC steps. In the purification scheme outlined in Fig. 1, only one conditioning step (ultrafiltration preceding FPLC Mono Q CC) was, therefore, required. Crude Pectinex AR KPG 027 had to be clarified by centrifugation before starting the CC purification procedure. Removal of small organic molecules ($M_r < 10\,000$) from the clarified solution was achieved by concentration and simultaneous dialysis in Na-acetate buffer. The removal of the major part of the brown colour from the enzyme solution was a positive side effect of this preliminary purification step.

The first CC separation step was performed on an anion exchange gel (Fig. 2). During this separation, the NaCl concentration was found to be most important as *endo*-ABA elution could be observed at different salt concentrations. To avoid dilution of the pooled samples the application of a stepwise increasing NaCl gradient was preferred over a linear gradient. Already at 0·15 M NaCl, a significant amount of *endo*-ABA co-eluted with large amounts of UV absorbing material (proteins and most of the initial sample's residual colour). The majority of *endo*-ABA activity eluted at 0·2 M NaCl, although a small part was still tightly bound to the column. Up to 5 mg of total protein/ml gel could be applied on the gel so as to obtain reproducible separations. The column could be used for at least 100 separations without significant loss in resolution as long as it was extensively washed with high salt concentrations (e.g. 2 M NaCl). For the next separation step, only fractions from pool 1 (Fig. 2), which represented 50% of the total *endo*-ABA activity (Table 1), were used.

Using hydrophobic interaction as a second CC purification step had the advantage that the previously pooled fractions, which contained a non-negligible amount of salt, did not require any yield-lowering conditioning pretreatment. Ammonium sulphate was added to pool 1 to a final concentration of 1·8 M. This solution was applied on a Phenyl-Sepharose 6 Fast Flow column (see Experimental section). Application of a linear decreasing ammonium sulphate gradient over approximately five column volumes allowed the elution of three distinct peaks. *Endo*-ABA activity was located in the last peak (pool 2, Fig. 3).

Several methods of CC purification were examined to purify further the *endo*-ABA. A substrate affinity CC method for arabinan degrading enzymes has been described by Waibel *et al.* (1980). Substrate affinity CC separations of polysaccharases have also been reported by Batzer (1986) and Rozie *et al.* (1992). A first trial of *endo*-ABA substrate affinity CC purification was performed with a commercial gel: epoxy-activated Sepharose 6B (Pharmacia). Linear 1,5-α-L-haze arabinan was directly coupled with the gel, and separations were carried out at pH 7·0 to avoid too rapid an enzymic degradation and the concomitant loss of the gel matrix specificity. This step did not yield any significant enrichment in *endo*-ABA activity (data not shown). The use of a less specific lectin affinity medium, Concanavalin A Sepharose (Pharmacia), was also examined. Again, no sufficient separation could be achieved. As all *endo*-ABA activity was bound to the column before elution with methyl-α-D-Man *p* solutions (data not shown), it could nevertheless be concluded that the *endo*-ABA is probably a glycoprotein. Finally, adsorption chromatography on hydroxyapatite proved to be inefficient for the purification of the *endo*-ABA, as all activity eluted in a single major peak together with other proteins (data not shown).

Table 1. Summary of the purification of *endo*-1,5-α-L-arabinanase (*A. niger*)

Purification step	Total volume (ml)	Protein concentration (μg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Concentrated Pectinex AR KPG 027	5·0	74 280	371·40	309·80	1549	4·17	100	- -
Q-Sepharose Fast Flow anion exchange chromatography	226·0	124	28·02	3·35	757·1	27·02	48·88	6 5
Phenyl-Sepharose 6 Fast Flow hydrophobic interaction chromatography	98	89	8·72	6·53	639·94	73·39	41·31	17·6
FPLC Mono Q anion exchange chromatography	123·5	61	7·53	4·68	577·98	76·76	37·31	18·4
FPLC Superdex 75 gel filtration chromatography	260·0	7·54	1·96	0·68	176·80	90·20	11·41	21·6

Considering the unsuccessful separation trials mentioned above, the third separation step, therefore, consisted of an anion exchange CC on a high resolution media, using an FPLC Mono Q HR 5/5 column. A typical elution profile consisted of a major, tailing peak eluting at approximately 0·2 M NaCl and containing most *endo*-ABA activity (Fig. 4, pool 3). Even if it seems that this Mono Q purification step did not result in a considerably higher purification factor (Table 1), it was nevertheless integrated in the overall purification scheme, because it allowed the removal of two types of proteins (located in the small peak eluting at approximately 18·5 ml, Fig. 4) having a slightly lower molecular weight than the *endo*-ABA (as shown by SDS–PAGE analysis in Fig. 6, lanes D and E).

Final purification of *endo*-ABA to electrophoretic homogeneity as shown by SDS–PAGE (Fig. 6, lane F) was obtained by applying aliquots of pool 3 directly on an FPLC Superdex 75 HR 10/30 gel filtration column (Fig. 5). This last purification step allowed the removal of residual contaminating proteins, mostly of smaller molecular weights (Fig. 6, lane E). *Endo*-ABA activity eluted as a symmetrical peak. All details of recoveries and purification results of each separation step are summarised in Table 1.

Carbohydrate composition of substrates

Carbohydrate analyses of the different substrates used in the determinations of specific enzymic activites were relevant for two reasons: (1) elucidation of possible residual contaminating activities in the purified enzyme preparation, and (2) proper interpretation of action patterns of the purified enzyme. Detailed carbohydrate analyses were performed only on those substrates where a residual activity of $\geq$0·05% (compared to the most actively degraded substrate) could be detected after 7 h incubation with the purified *endo*-ABA. Table 2 summarises the sugar composition of these relevant substrates.

All arabinans contained a definite majority of arabinose residues (64·1–98·6%). Arabinans from sugar beet displayed relatively high amounts of galactose (4–16%) as well as some galacturonic acid residues (1·1–14·5%). As arabinans are part of the side-chains of pectins, only totally pure arabinan preparations would be completely free of galactose and/or galacturonic acid. The galactose contents are, therefore, probably due to arabinogalactan and/or galactan portions, which are known to occur frequently in sugar beet preparations (Aspinall, 1980). Arabinose could also be detected in various amounts in all other substrates analysed. Arabinoxylan (35·2%), arabinogalactan (16·7%) and galactan (7·7%) contained the highest amounts. As expected, the two acidic substrates (75% esterified pectin and polygalacturonic acid) contained mainly galacturonic acid residues (71·7 and 96% respectively).

Methylation analyses (Table 3) were performed to evaluate the linkage type between the arabinose residues. Analysis patterns of the substrates showed that the haze-arabinan was primarily a linear 1,5-α-L-arabinan. The debranched arabinan from sugar beet was also predominantly an 1,5-α-L-arabinan, containing more 1,2- than 1,3-α-L-arabinose ramifications. The two branched arabinans displayed the same 1,5-α-arabinose backbone, but were much more branched. Branched arabinan B contained a greater portion of linear 1,3-α-L-arabinose side chains, linked through the C-3 to the main backbone; ramifications on these side-chains at C-2 could also be expected. Branched arabinan A had much less 1,3-α branchings on the backbone, but showed a larger amount of terminal arabinose residues. As discussed before, the terminal arabinose residues might derive from small portions of arabinogalactan associated to the arabinan. Most of the arabinose residues in arabinogalactan were 1,3-α and terminally linked. Small portions of 1,5-α-L-arabinan were also present. Arabinoxylan contained more 1,5-α than 1,3-α linked Ara *f* residues and, as expected, a large majority of terminal residues. Considerable amounts of linear 1,5-α-L-arabinan were also present in the galactan preparation

Table 2. Neutral sugar and galacturonic acid composition of arabinose containing substrates. All values are expressed in mol %; Σ = 100

	Linear 1,5-α-L-haze-arabinan (apple)	Debranched arabinan (sugar beet)	Branched arabinan A (sugar beet)	Branched arabinan B (sugar beet)	Arabino-galactan (larch wood)	Galactan (lupin)	Arabino-xylan (wheat)	Poly-galacturonic acid, Na salt (orange)	Pectin DE 75% (apple)
Rhamnose/ fucose	0·7	5·4	3·2	1·2	1·3	3·0	0·7	1·1	1·7
Arabinose	98·6	64·1	75·5	93·2	16·7	7·7	35·2	0·7	5·0
Xylose	n.d.[a]	n.d.	n.d.	n.d.	n.d.	1·2	61·2	n.d.	1·6
Mannose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glucose	n.d.	0·1	0·2	0·5	0·2	1·5	1·0	n.d.	14·2
Galactose	n.d.	15·9	13·0	4·0	81·3	84·4	0·8	2·2	5·8
Galacturonic acid	0·7	14·5	8·1	1·1	0·5	2·2	1·1	96·0	71·7

[a]Not detected.

Table 3. Arabinosidic linkage analysis of relevant substrates. 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. Data are expressed as mol % of arabinose residues; Σ = 100

	Linear 1,5-α-L-haze-arabinan	Debranched arabinan	Branched arabinan A	Branched arabinan B	Arabino-galactan	Galactan	Arabino-xylan	Poly-galacturonic acid, Na salt	Pectin DE 75%
1-Ara *f* (terminal)	3	3·1	49·3	32·1	58·2	tr.[a]	88·8	43·5	37·3
1-Ara *p* (terminal)	n.d.[b]	n.d.	n.d.	0·7	n.d.	5·5	n.d.	n.d.	n.d.
1-Ara *p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1,2-Ara *f*	n.d.	n.d.	n.d.	2·7	n.d.	n.d.	5·5	n.d.	n.d.
1,3-Ara *f*	3	0·4	1·2	8·4	38·8	n.d.	1·4	n.d.	5 9
1,5-Ara *f*	91	93·6	38·9	35·1	3·0	94·5	4·0	56·5	38·8
1,2,3-Ara *f*	n.d.	n.d.	n.d.	0·7	n.d.	n.d.	n.d.	n.d.	n.d.
1,2,5-Ara *f*	1	1·9	2·1	2·9	n.d.	n.d.	n.d.	n.d.	n.d.
1,3,5-Ara *f*	2	1·0	3·2	15·5	n.d.	n.d.	n.d.	n.d.	9·0
1,2,3,5-Ara *f*	n.d.	n.d.	5·3	1·9	n.d.	n.d.	0·3	n.d.	9·0

[a]Traces.
[b]Not detected.

as 94·5% of the arabinose fragments were in the 1,5-α-form. 1,5-α-L-Arabinan was also the main type of the arabinose containing polysaccharide in highly esterified pectin as well as in polygalacturonic acid.

Except for arabinogalactan, the 1,5-α linked L-Ara *f* residues found in substrates other than arabinans could be attributed to impurities rather than to structural characteristics. This may, therefore, be a likely explanation for the low activities of the purified *endo*-ABA observed towards these substrates.

Characterisation of the *endo*-1,5-α-L-arabinanase

Since, depending on the monosaccharide used, quantifications with calibration curves could significantly diverge, the most appropriate monosaccharide was used as standard for each substrate (e.g. arabinose for arabinans, glucose for starch, galacturonic acid for polygalacturonic acid, etc). Specific activity determinations in the initial enzyme preparation Pectinex AR KPG 027 are summarised in Table 4. The specific *endo*-ABA activity (4·5 U/mg, measured on linear-1,5-α-L-haze-arabinan) corresponded to only 1% of *endo*-polygalacturonase activity (432 U/mg), whereas the activity of *exo*-arabinanase (17·81 U/mg, measured on *p*-NP-α-L-Ara *f*) was almost four times superior. Due to the synergistic activities of *endo*- and *exo*-arabinanase, branched arabinans yielded higher amounts of reducing sugar equivalents than the linear/debranched ones (Voragen *et al.*, 1987; Beldman *et al.*, 1993). Other significant activities in Pectinex AR KPG 027 were detected on differently esterified pectins and on lupin galactan. These results confirmed that the Pectinex AR KPG 027 was a preparation optimised for pectic enzymes. The *endo*-ABA proportion could, therefore, be considered to have been only a minor component in the crude starting material.

To evaluate the enrichment of *endo*-ABA, the same broad selection of substrates was tested on the purified enzyme (Table 5); 7 h of incubation was chosen to be sure to detect even very small quantities of contaminating activities. The best substrate for the pure *endo*-ABA was the linear 1,5-α-L-haze-arabinan with a specific activity of 90·2 U/mg, followed by debranched arabinan from sugar beet (81·1 U/mg). The activity on both types of branched arabinans was almost approximately six times less. These results suggest that the degree of branching is closely correlated to the enzyme's activity: a progressive reduction of *endo*-ABA activity was observed as the 1,5-α-L-Ara *f* sequences became shorter and/or more ramified (Voragen *et al.*, 1987; Rombouts *et al.*, 1988; Beldman *et al.*, 1993). Residual activities of ≥0·05% on substrates other than arabinans were detected only on galactan (<0·7%), polygalacturonic acid (<0·17%), 75% esterified pectin (<0·09%), arabinoxylan (<0·06%) and arabinogalactan (<0·05%). As described above, these residual activities could probably be attributed to arabinan contaminations in the substrates analysed rather than to impurities in the purified enzyme. No *exo*-arabinanase activity could, furthermore, be observed.

Determination of the pI and SDS–PAGE analyses

Chromatofocusing experiments (Fig. 8) located the pI of the purified *endo*-ABA around pH 2·9. This value was confirmed by analytical IEF (Fig. 9) and was in good agreement with values previously found for *A. niger endo*-ABAs (Schöpplein, 1989; Van der Veen *et al.*, 1991), whereas *endo*-ABAs from bacterial sources (Weinstein & Albersheim, 1979) are reported to have much higher pIs (9·3). Using precast gels (IEF 3–9), the resolution on the PhastSystem was only satisfactory when samples were applied on the anodic side, but even then, smearing effects represented a major problem. The purified enzyme accumulated as a single, broad band at the very bottom of the gel (anodic side, Fig. 9). Satisfactory purity assertions were, therefore,

Table 4. Specific glycanases and glycosidases activities in the crude enzyme preparation Pectinex AR KPG 027 (*A. niger*). (Abbreviations of substrates are explained in the text)

Substrate (%, w/v)	Specific activity (U/mg)	U% with regard to polygalacturonic acid
Linear 1,5-α-L-haze-arabinan (0·2)	4·49	1·04
Debranched arabinan (0·2)	4·20	0·97
Branched arabinan A (0·2	9·13	2·11
Branched arabinan B (0·2)	12·78	2·96
Arabinogalactan (0·2)	0·47	0·11
Arabinoxylan (0·2)	1·54	0·36
CMX (0·2)	4·04	0·94
Galactan (0·2)	45·35	10·50
Gum arabic (0·2)	0·04	0·01
Galactomannan carubin type (0·1)	0·14	0·03
Galactomannan guarin type (0·1)	0·01	~0
Mannan (0·01)	0·01	~0
Glucomannan (0·2)	2·40	0·55
Starch (0·2)	1·72	0·40
Curdlan (0·1)	0·16	0·04
Lichenin (0·05)	0·49	0·11
CMC (0·2)	0·42	0·10
Dextran (0·2)	tr.[a]	< 0·01
Polygalacturonic acid, Na salt (0·2)	431·97	100
Pectin DE 35% (0·2)	88·42	20·47
Pectin DE 75% (0·2)	46·58	10·78
p-NP-α-L-Ara *f* (0·05)	17·81	3·77
p-NP-α-L-Ara *p* (0·05)	0·32	0·07
p-NP-β-L-Ara *p* (0·05)	0·60	0·13
p-NP-α-D-Gal *p* (0·05)	2·59	0·55
p-NP-β-D-Gal *p* (0·05)	4·99	1·06
p-NP-α-D-Glu *p* (0·05)	tr.	< 0·001
p-NP-β-D-Glu *p* (0·05)	1·42	0·30
p-NP-α-D-Man *p* (0·05)	n.d.[b]	~0
p-NP-β-D-Man *p* (0·05)	0·22	0·05
p-NP-β-D-Xyl *p* (0·05)	0·27	0·06

[a]Traces.
[b]Not detected.

impossible, as impurities possessing lower pIs could not be detected. To circumvent these serious limitations, trials were undertaken with dried polyacrylamide gels (CleanGels). These gels had two advantages: (1) urea denaturation of proteins led to a well-resolved separation pattern without smearing, and (2) the creation of a pH gradient well below 3·0 was possible by reswelling the gel in an appropriate buffer. The major disadvantage of this method, however, was the impossibility of determining exact pIs. Figure 7 illustrates the electrophoretic patterns of the different CC separation steps, after denaturation in the presence of 8 M urea. The purified *endo*-ABA was detected as a single sharp band.

SDS–PAGE analyses proved to be most useful as they were the most sensitive method of monitoring the separation successes and purity claims. Figure 6 displays the electrophoretic patterns of the different CC separation steps. The pure *endo*-ABA (lane F) was resolved as a single major band after silver staining (Coomassie blue stainings revealed the same sole band; data not shown). A first indication of the specificity of the isolated enzyme was obtained after performing comparative SDS–PAGE analysis of a commercially available *A. niger endo*-ABA preparation (Fig. 6, lane G).

Immunoaffinity analyses of the pure *endo*-1,5-α-L-arabinanase

The Western blot of SDS gels, followed by immunoaffinity analysis, allowed the unambiguous assignment of the single band found on SDS–PAGE to *endo*-ABA activity (Fig. 10, lanes D and E). The high *endo*-ABA specificity of the antiserum used has been illustrated both by Van der Veen *et al.* (1991) and Beldman *et al.* (1993). A positive control (Fig. 10, lane C) consisted of the same commercial *endo*-ABA preparation as described above.

Physico-chemical properties of the purified *endo*-1,5-α-L-arabinanase

The major physico-chemical properties of the purified *endo*-ABA are summarised in Table 6. The relative

Table 5. Specific activities of purified *endo*-1,5,-α-L-arabinanase (*A. niger*) on various substrates. (Abbreviations of substrates are explained in the text)

Substrate (%, w/v)	Specific activity after 7 h incubation (U/mg)	U% with regard to linear 1,5-α-L-haze-arabinan
Linear 1,5-α-L-haze-arabinan (0·2)	90·17[a]	100
Debranched arabinan (0·2)	81·11[a]	89·96
Branched arabinan A (0·2	13·44[a]	14·90
Branched arabinan B (0·2)	15·35[a]	17·02
Arabinogalactan (0 2)	~0·04	< 0·05
Arabinoxylan (0·2)	~0·05	< 0·06
CMX (0·2)	n.d.[b]	~0
Galactan (0·2)	~0 59	< 0·7
Gum arabic (0·2)	n.d.	~0
Galactomannan carubin type (0·1)	n.d.	~0
Galactomannan guarin type (0·1)	n.d.	~0
Mannan (0·01)	n.d.	~0
Glucomannan (0·2)	tr[c]	< 0·001
Starch (0·2)	tr.	< 0·01
Curdlan (0·1)	n.d.	~0
Lichenin (0·05)	tr.	< 0·01
CMC (0·2)	tr.	< 0·01
Dextran (0·2)	tr.	< 0·001
Polygalacturonic acid, Na salt (0·2)	~0·15	< 0·17
Pectin DE 35% (0·2)	~0·03	< 0·04
Pectin DE 75% (0·2)	~0·08	< 0·09
p-NP-α-L-Ara *f* (0·05)	tr.	< 0 01
p-NP-α-L-Ara *p* (0·05)	n.d.	~0
p-NP-β-L-Ara *p* (0·05)	n.d.	~0
p-NP-α-D-Gal *p* (0·05)	n.d.	~0
p-NP-β-D-Gal *p* (0·05)	n.d.	~0
p-NP-α-D-Glu *p* (0·05)	n.d.	~0
p-NP-β-D-Glu *p* (0·05)	n.d.	~0
p-NP-α-D-Man *p* (0·05)	n.d.	~0
p-NP-β-D-Man *p* (0·05)	n.d.	~0
p-NP-β-D-Xyl *p* (0·05)	n.d.	~0

[a]Incubation time of 20 min instead of 7 h.
[b]Not detected.
[c]Traces.

molecular weight was determined by three different methods: (1) SDS–PAGE (M_r of *c.* 42 500, Fig. 6, lane F); (2) gel filtration (M_r of *c.* 41 000); and (3) MALDI-TOF-MS (M_r of 33 900, Fig. 11). The large discrepancies found between the MALDI-TOF-MS measurement (which enables very accurate M_r determinations; Schär *et al.*, 1991) and the two more classical M_r determination methods could probably be explained by the glycoprotein nature of the *endo*-ABA, which was demonstrated (1) by the strong binding of the enzyme to Concanavalin A, and (2) by the typically broad signal obtained in the MALDI-TOF-MS spectra. An out-jut of the glycosidic part of the glycoprotein would considerably decrease the electrophoretic migration rate of the *endo*-ABA in SDS–PAGE and decrease the retention time in the gel filtration analysis. The influence of O-linked glycosylation on M_r determinations of *endo*-ABA has also been confirmed by Flipphi *et al.* (1993), who have cloned the *A. niger endo*-ABA gene. The sole putative N-glycosylation site found in the deduced gene sequence was supposed to be responsible for the *endo*-ABA M_r differences of *c.* 10 kDa found by several authors. Using SDS–PAGE, Van der Veen *et al.* (1991, 1993) (M_r 41 000 resp. 43 000) and Beldman *et al.* (1993) (M_r 45 000) have found similar M_r for *A. niger endo*-ABAs. Other authors reported M_r in the range of 32 000–35 000 (SDS–PAGE or gel filtration) (Rombouts *et al.*, 1988; Schöpplein, 1989).

Investigations of the influence of the pH on the activity of the purified enzyme showed that the pH optimum of the enzyme was quite broad (pH 4·5–5·0). Maximum activity was observed at pH 4·8. This pH optimum value is typical for fungal *endo*-ABAs (4·0–5·5) (Kaji *et al.*, 1967; Rombouts *et al.*, 1988; Van der Veen *et al.*, 1991; Beldman *et al.*, 1993; Van der Veen *et al.*, 1993). After incubation for 67 h at RT, the *endo*-ABA examined retained more than 85% of its initial activity between pH 3·5 and 8·0. Beldman *et al.* (1993) have reported good stability for *A. niger endo*-ABA between pH 5·5 and 6·3.

The *T* optimum was found to be 55°C; the enzyme retained more than 95% of its initial activity up to 45°C,

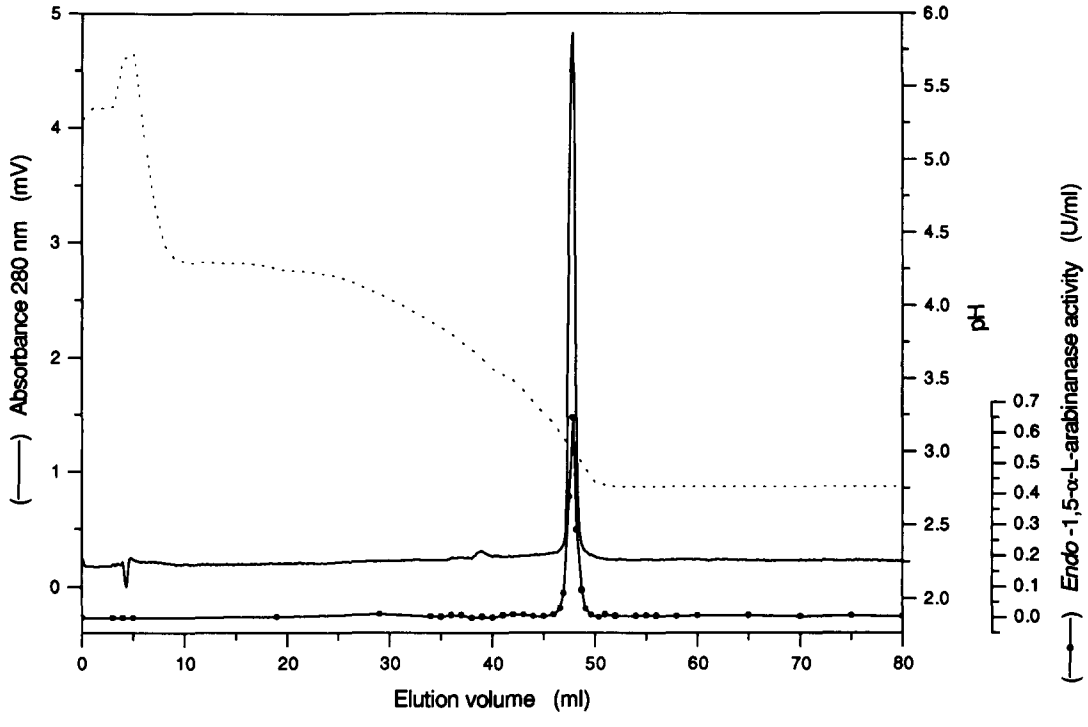

Fig. 8. pI determination of the purified *endo*-1,5-α-L-arabinanase with chromatofocusing on an FPLC Mono P HR 5/20 column. Purified *endo*-ABA fractions were buffer-exchanged and applied on an FPLC Mono P HR 5/20 column. Complete experimental details, principles of *endo*-ABA activity determination and pH measurement in the fractions are as described in the text.

after 3 h of incubation. These values lie in the expected range (Rombouts *et al.*, 1988; Van der Veen *et al.*, 1991). As standard activity determinations were consequently performed at 40°C, long-term stability determinations at this T were most relevant for experiments requiring long incubation times (e.g. substrate degradation analyses). The purified *endo*-ABA was found to retain 90% of its initial activity even after 26·5 h incubation at 40°C, and the Arrhenius energy of activation was determined to be 12·20 kcal/mol.

Using linear 1,5-α-L-haze arabinan as substrate, the kinetic parameters K_m and V_{max} were determined as 0·205 mg/ml and $1{\cdot}77 \times 10^{-4}$ μmol/min, respectively. A comparison of the kinetic parameters could not be made because of the use of different substrates for the determinations (Van der Veen *et al.*, 1991).

Inhibition studies, summarised in Table 7, showed that only $HgCl_2$ at a concentration of 5 mM inhibited the enzyme completely; $ZnSO_4$ was also inhibitory, but to a lesser extent. Arabinose, arabitol, arabino-lactones and EDTA showed no effect. Comparison of inhibitory studies with those on other *A. niger endo*-ABAs was not possible, because no previous studies have been reported. *Bacillus subtilis endo*-ABAs have, nevertheless, been reported to be Hg and Fe sensitive, EDTA and arabono-lactones showing no effect on these enzymes (Kaji & Saheki, 1975; Weinstein & Albersheim, 1979; Yoshihara & Kaji, 1983).

CONCLUSIONS

An *endo*-ABA was purified from a crude pectinase preparation of *A. niger* through a sequence of CC purification steps. In the complex initial enzyme mixture, the *endo*-ABA represented only 1% of the carbohydrate degrading activities. The elaboration of a purification scheme allowing high selectivity without excessive recovery losses was, therefore, most important. A combination of various classical CC methods was used to achieve the desired goal. Even if the use of hydrophobic interaction gel chromatography is a common step after ion exchange chromatographies, it is the first time that this separation method was successfully incorporated in an *endo*-ABA purification scheme. The combination of two low and two medium pressure (FPLC) separation steps allowed an *endo*-ABA purification to homogeneity with a recovery greater than 10% and a purification factor of *c.* 22.

Purity claims of isolated enzymes are often based on dubious arguments. Only few authors have performed sensitive gel staining methods, such as, e.g. silver staining. The widely used Coomassie blue stainings are often not sensitive enough to detect nanogram amounts of impurities. Moreover, long-term incubations with various polysaccharide substrates should always be performed if the purified enzyme has to be used for structural analyses (e.g. of plant cell walls) for which the

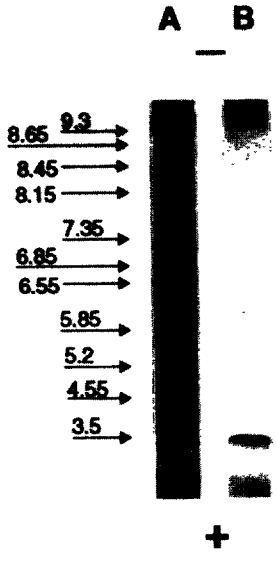

Fig. 9. Analytical IEF of purified *endo*-1,5-α-L-arabinanase on the PhastSystem. Ready-to-use polyacrylamide gels in the pH range 3–9 were used. Experimental details are described in the text. Marker proteins (Pharmacia): amyloglucosidase (pI 3·5), soybean trypsin inhibitor (4·55), β-lactoglobulin A (5·2), bovine carbonic anhydrase B (5·85), human carbonic anhydrase (6·55), horse myoglobin (6·85 and 7·35), lentil lectin (8·15, 8·45 and 8·65) and trypsinogen (9·3). Detection of proteins was with silver staining. Sample volumes: 0·5 μl. Lane A: isoelectric focusing calibration kit (broad range, pH 3–10); lane B: purified *endo*-1,5-α-L-arabinanase (Fig. 5, pool 4).

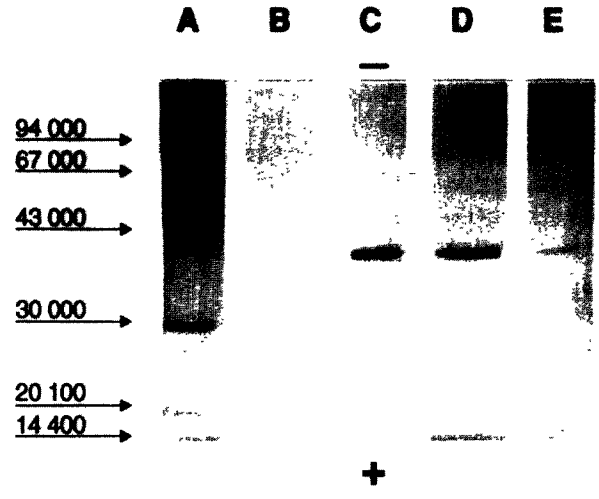

Fig. 10. Western blot of purified *endo*-1,5-α-L-arabinanase. Transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8·3. The low M_r electrophoresis calibration kit was from Pharmacia. Further experimental details are described in the text. Lane A. low M_r electrophoretic calibration kit (stained with Fast Green); lane B: low M_r electrophoretic calibration kit, incubated with antiserum (=negative control); lane C: *endo*-1,5-α-L-arabinanase (MegaZyme) (30 × diluted in water) (=positive control); lane D: purified *endo*-1,5-α-L-arabinanase (Fig. 5, pool 4); lane E: purified *endo*-1,5-α-L-arabinanase (Fig. 5, pool 4) (5 × diluted in water).

specificity of the enzyme is most relevant (Matheson & McCleary, 1985). Such studies are essential for the detection of possible residual, contaminating enzymic activities.

The high purity of the *endo*-ABA isolated in this study was demonstrated by various methods: (1) by using different, sensitive techniques (SDS–PAGE and IEF analysis), the enzyme was homogeneous; (2) 7 h incubations with a broad range of carbohydrates showed almost no other activities than the ones expected on various arabinans; and (3) the identity of the isolated enzyme was demonstrated using high-sensitivity immunoblotting.

It seems very probable that the *endo*-ABA purified from the pectinase preparation Pectinex AR KPG 027 (*A. niger*) and characterised in this paper is similar to the *endo*-ABA described by Rombouts *et al.* (1988), Van der Veen *et al.* (1991, 1993) and Beldman *et al.* (1993). The similarity of the physico-chemical parameters as

Table 6. Some physico-chemical properties and kinetic constants of purified *endo*-1,5-α-L-arabinanase (*A. niger*). (Experimental details of the different determinations and abbreviations are explained in the text)

Physico-chemical parameters and kinetic constants	*endo*-1,5-α-L-arabinanase
M_r (SDS–PAGE, Da)	42 500
M_r (Gel filtration, Da)	41 000
M_r (MALDI-TOF-MS, Da)	33 900
pH optimum	4·8
pH stability (for 67 h at 25°C)	3·5–8·0
Temperature optimum (°C)	55
Temperature stability (for 3 h) (°C)	≤45
Temperature stability at 40°C (h)	~8
IEP (chromatofocusing, pH)	~3·0
IEP (IEF, pH)	2·8–3 0
Chemical nature	Glycoprotein
Arrhenius energy of activation (E_A) (kcal/mol)	12 20
Kinetic parameters on linear 1,5-α-L-haze-arabinan	
K_m (mg/ml)	0·205
V_{max} (μmol min)	$1{\cdot}77 \times 10^{-4}$

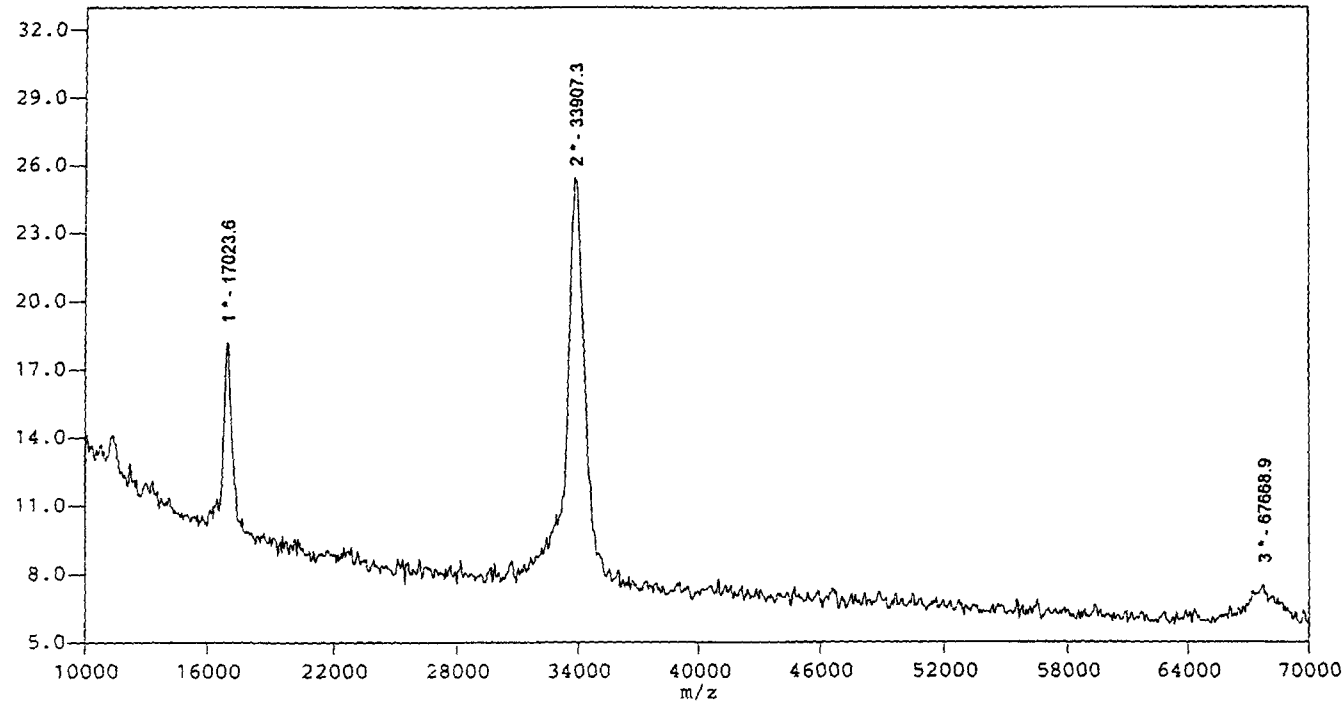

Fig. 11. Negative ion MALDI-TOF-MS mass spectra of the purified *endo*-1,5-α-L-arabinanase. First peak 1* ($m/z \cong 17000$ Da): half molecular weight, double-charged molecule ion $[M+2H]^{2+}$. Second peak 2* ($m/z \cong 33900$ Da: molecule ion $[M+H]^{+}$. Third peak 3* ($m/z \cong 67700$ Da): double molecular weight, dimer-ion $[2M+H]^{+}$. For further experimental details see text.

well as the same immunospecificity support this assertion. However, as shown by Beldman *et al.* (1993), even if immunoaffinity is identical for similar enzymes, the latter could, nevertheless, be quite different. A definitive statement of similitude could, therefore, only be made, after (1) having compared the amino acid sequences of the enzymes, and (2) having compared the specific degradation patterns of these enzymes on well-characterised substrates. To study these degradation patterns, the use of high-performance anion exchange chromatography with pulsed amperometric detection seems most appropriate; such analyses are presently in progress and will be the subject of a future communication.

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Table 7. Effect of some metal ions and inhibitors on the activity of purified *endo*-1,5-α-L-arabinanase (*A. niger*)

Metal ion and inhibitors	Concentration (mM)	Residual activity (%)
$CaCl_2$	0·5	98
	1	98
	5	97
$HgCl_2$	0·5	87
	1	66
	5	4
$MgSO_4$	0·5	100
	1	95
	5	93
$ZnSO_4$	0·5	94
	1	88
	5	62
EDTA	0·5	97
	1	97
	5	95
Iodoacetamide	0·05	97
	0·1	95
	0·5	91
L-(−)-Arabono-γ-lactone	0·5	97
	1	96
	5	95
L-(+)-Arabinose	10	98
	50	97
D-(+)-Arabitol	10	98
	50	98

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