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Purification and Characterization of *Endo*-1,4-β-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: Use in Combination with Arabinanases from *Aspergillus niger* in Enzymic Conversion of Potato Arabinogalactan

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ABSTRACT

Two galactanases, purified from experimental enzyme preparations derived from Aspergillus niger *and* Aspergillus aculeatus *were found to be similar in a number of properties. They had similar molecular weights (M_r = 42–43 kD) and both showed highest activity on 1,4-β-D-galactan. Optimal activity was measured at 50–55°C and pH 4·00–4·25; optimal stability was observed in the pH range of 5–7 at 30°C. A sodium acetate buffer was found to be the best incubation buffer. Activity and stability were affected by Pb^{2+} (*endo-*galactanase from* A. aculeatus *was inhibited completely) and to a lesser extent by Ag^{+} and Zn^{2+} ions. Digestion of (arabino)-1,4-β-D-galactan resulted initially in a big shift in the M_w value of the bulk of (arabino)-1,4-β-D-galactan and formation of low galacto-oligomers, mainly tetra- and trimers of galactose. In the final stage of the reaction mono- and dimer accumulated as end products. Therefore a multiple attack mechanism was suggested for the* endo-*1,4-β-D-galactanases. The galactanases did not hydrolyse arabino-1,3/6-β-D-galactan. The effect of the temperature on their stability, their specific activities and their affinity for potato arabinogalactan differed; in the absence of substrate the* A. niger *and* A. aculeatus endo-*galactanase were stable up to 60°C and 35°C, respectively. The specific activities on potato arabinogalactan in a sodium acetate buffer pH 5·0, 30°C were found to be*

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158 and 244 u/mg, respectively. The K_m values estimated for potato arabinogalactan were 0·77 and 0·31 g/litre, respectively.

For an optimal breakdown of potato arabinogalactan combined action of arabinanases from A. niger *and* endo-*galactanase was required. Arabinofuranosidase B did not stimulate the degradation of potato arabinogalactan by the* endo-*galactanases under the conditions used, whereas* endo-*1,5-α-L-arabinanase had an immediate synergistic effect with both* endo-*galactanases, indicating the presence of linear 1,5-α-L-arabinan side chains.*

INTRODUCTION

1,4-β-D-Galactanases have been shown to occur in several micro-organisms (Dekker & Richards, 1976). Purification of galactanases to a homogeneous state has only been reported for various strains of *Bacillus subtilis* (Emi *et al.*, 1971; Labavitch *et al.*, 1976; Yamamoto & Emi, 1988) and one fungus, *Penicillium citrinum* (Nakano *et al.*, 1985). Interaction between galactanases and arabinanases during breakdown of arabinogalactans has not been studied up till now.

In plant cell walls two types of arabinogalactans are present. Type I (L-arabino-)1,4-β-D-galactans and type II L-arabino-1,3/6-β-D-galactans which have a branched backbone (Clarke *et al.*, 1979; Stephen, 1983). The (arabino)galactans are considered to be involved in the interconnections between components of the primary cell wall, as indicated in a recently updated tentative model of the primary cell wall (Fry, 1986).

Studies have been carried out on the role of galactanases in the processing of fruit and vegetables in order to facilitate juice and colour extraction tissue maceration and tissue liquefaction. According to Chesson (1980) galactanases do not play a role in maceration of vegetables. Studies on the action of cell-wall-degrading enzymes on an apple cell wall preparation performed by Renarde *et al.* (1990) and Voragen *et al.* (1980, 1982) indicate that 1,4-β-D-galactanase action does not appear to be very important in tissue liquefaction. However, it may be required to achieve complete saccharification of solubilised cell wall polysaccharides. Monogastric animals lack enzymes which can split β-glycosidic bonds (except for β-galactosidase). Treatment of feed by galactanases may facilitate digestion of animal feed (Beudeker *et al.*, 1988).

The objective of this study was to isolate and characterize *endo*-1,4-β-D-galactanases from fungal enzyme preparations derived from *Aspergillus niger* and *Aspergillus aculeatus* and to investigate the

enzymes with regard to substrate specificity, mode of action and interaction with arabinanases in the degradation of arabinogalactans.

MATERIALS AND METHODS

Substrates

Arabino-1,4-β-D-galactans were isolated from high methoxy apple pectin (Obi Pectin Ltd, Bischofzell, Switzerland), citrus pectin NF (Sunkist Growers Inc., California, USA) and potato fibre (kindly provided by Avebe, Veendam, The Netherlands) according to Labavitch *et al.* (1976). Prior to arabinogalactan extraction from potato fibre, residual starch was removed enzymatically (Rombouts *et al.*, 1988). Tetragalactose was obtained by partial enzymic degradation of citrus arabinogalactan by 1,4-β-D-galactanase of *B. subtilis* as described by Labavitch *et al.* (1976). A linear 1,4-β-D-galactan was obtained by removal of arabinose residues from potato arabinogalactan with arabinofuranosidase B. Other substrates used were 1,5-α-L-arabinan and arabinan-rich apple juice ultrafiltration retentate (UFR) (Rombouts *et al.*, 1988), larch wood arabino-1,3/6-β-D-galactan ('Stractan', St Regis Paper company, Tacoma, Washington, USA), arabino-1,4-β-D-xylan ex oats spelts (Koch and Light, Colnbrook, Bucks, UK), polygalacturonic acid (ICN, Cleveland, Ohio, USA) and CM-cellulose (Akucell AF type 0305, Akzo, Arnhem, The Netherlands). Highly esterified pectin (DE 93%) was from the laboratory collection.

p-Nitrophenyl derivatives of α-L-arabinofuranose (Sigma, Chemical Company, St Louis, Missouri, USA), α/β-D-galactopyranose, β-D-glucopyranose and α/β-D-xylopyranose (Koch & Light) were also used.

Carbohydrate analysis of substrates

Neutral sugar composition of arabinogalactans, 1,4-β-D-galactan and UFR was determined by Seaman hydrolysis and conversion to their alditol acetates as described by Englyst & Cummings (1984) or by the method of Albersheim *et al.* (1967), as indicated. Neutral sugars and galacturonic acid were determined colorimetrically by an automated orcinol sulphuric acid (Tollier & Robin, 1979) and *m*-hydroxydiphenyl assay (Thibault, 1979), respectively. Correction was made for the interference of neutral sugars in the determination of galacturonic acid. Methylation analysis was carried out according to the procedure of Talmadge *et al.* (1973).

Enzyme preparations

Pectinase 29, an experimental enzyme preparation derived from *A. niger* was kindly provided by Gist-Brocades (Seclin, France). Pectinex Ultra-SP-L, a technical enzyme preparation derived from *A. aculeatus*, was a gift from Novo-Ferment AG (Basel, Switzerland).

Enzyme assays

All enzyme activities were expressed in international units (U). One unit of enzyme activity is defined as the amount which liberates 1 μmol reducing sugars per minute. The protein content was measured according to Sedmak & Grossberg (1977). Bovine serum albumin was used as standard.

Endo-galactanase activities were measured by incubating 0·1 ml of 0·5% (w/v) potato arabinogalactan solution in distilled water with 0·4 ml diluted enzyme solution in 0·05 M sodium acetate buffer, pH 5·0. The increase in reducing end groups was measured after 1 h incubation at 30°C by the method of Nelson–Somogyi (Somogyi, 1952), unless stated otherwise. Galactose was used as standard.

Activities on *p*-nitrophenyl derivatives, UFR, 1,5-α-L-arabinan, polygalacturonic acid, CM-cellulose and arabinoxylan were measured as described by Rombouts *et al.* (1988).

Column chromatography of *endo*-galactanases from *A. niger* and *A. aculeatus*

The original chromatographic procedure consisted of gel filtration chromatography on Bio-gel P10 (100–200 mesh, column 30 × 950 mm) for desalting, anion exchange chromatography on DEAE Bio-gel A (column 30 × 200 mm) and adsorption chromatography on Bio-gel HTP (column 20 × 140 mm) (Biorad Laboratories, Richmond, California, USA) as described by Rombouts *et al.* (1988). In the optimisation of this procedure an additional step — gel filtration on Bio-gel P100 (100–200 mesh, column 20 × 900 mm) (Biorad Laboratories, Richmond, California, USA) — was added.

Purification of *endo*-galactanase from *A. aculeatus* was performed by identical chromatographic steps. Chromatography on HTP and Bio-gel P100 could be effectively replaced by anion exchange chromatography on the Mono Q column HR 5/5 with the FPLC system of Pharmacia (Uppsala, Sweden). Gradient elution was carried out as described by Rombouts *et al.* (1988). All purification steps were carried out at 4°C.

All buffers contained 0·01% (w/v) sodium azide to prevent microbial growth.

SDS–gel electrophoresis and isoelectric focussing

SDS–gel electrophoresis, isoelectric focussing and titration curves were performed with the Pharmacia Phastsystem. Experimental details are given by Rombouts *et al.* (1988).

Influence of pH, temperature, various buffers and chemicals on activity and stability of *endo*-galactanases

Effect of pH on *endo*-galactanase activity was measured with a series of McIlvaine buffers under standard conditions. The pH stability was investigated similarly by pre-incubation of the enzyme for 1·5 h before adding the substrate.

The optimum temperature was measured at various temperatures under standard conditions. The temperature stability was studied similarly by pre-incubation of the enzyme for 1·5 h before adding the substrate. The influence of various 0·05 M buffers and chemicals (1 mM) on activity and stability was studied at 55°C, pH 5·0. Activities are expressed as a percentage of the activity in sodium acetate buffer. Stability is calculated as a percentage of the activity in sodium acetate buffer.

Analysis of reaction products by HPLC

Reaction products formed during incubation of (arabino)-1,4-β-D-galactans, tetragalactose and other substrates with *endo*-galactanase were analysed by HPLC. For the determination of the shift in the M_w distribution of polymeric substrates digests were analysed by high performance gel permeation chromatography (HPGPC). HPGPC was performed on a SP 8700 HPLC (Spectra Physics, San Jose, California, USA) equipped with three gel permeation columns (each 7·5 × 300 mm) in series, Bio-gel TSK-40 XL, −30 XL and −20 XL (Biorad Laboratories, Richmond, California, USA) in combination with a TSK XL guard column (6 × 40 mm) at 30°C using 0·4 M sodium acetate buffer (pH 3·0) as eluent at a flowrate of 0·8 ml/min. The eluate was monitored using a Shodex SE-61 refractive index detector. A mixture of dextrans was used as standard. Mono- and oligomeric reaction products were analysed by an Aminex HPX 87P (Voragen *et al.*, 1986) or by an

Aminex HPX 42A column (Biorad Labs, Richmond, California, USA)., as indicated. Analysis using the HPX 42A column was carried out as described for the HPX 87P column; oligomers of glucose were used as standards.

Kinetic properties

Lineweaver–Burk plots of *endo*-galactanase action on potato and citrus arabinogalactan were determined by incubation with *endo*-galactanase in 0·05 M sodium acetate, pH 4·0, for 1 h at 55°C.

RESULTS

Sugar composition of substrates

Characterization of the various substrates was required for a proper interpretation of the action of the enzymes. Their sugar composition is

TABLE 1
Sugar Composition of Substrates[a]

Sugar	*Linearised*[b] *potato arabino-galactan*	*Potato*[b] *arabino-galactan*	*Citrus*[b] *arabino-galactan*	*Apple*[b] *arabino-galactan*	*UFR*[c] *arabinan*
Rhamnose/fucose	0·4	0·5	0·3	0	5·6
Arabinose	2·5	12	20	32	55
Xylose	0·3	2·2	0	0·5	8·4
Mannose	0	0·3	1·9	0	0
Galactose	94	77	74	47	9·9
Glucose	0	4·9	4·5	21	0
Uronic acid	2·9	2·6	nd	nd	21
Total sugar content (% w/w)	89	87	69[d]	69[d]	90
Gal/ara ratio	38	6·4	3·7	1·5	0·18

[a]The sugar composition is expressed in mol percentages with the total sugars set at 100 mol %. nd = not determined.
[b]Analysis according to Englyst & Cummings (1984).
[c]Analysis according to Albersheim *et al.* (1967).
[d]Total content of neutral sugars.

given in Table 1. 1,4-β-D-Galactan and potato arabinogalactan were fairly pure polysaccharides with total sugar contents of 87–89% (w/w) and a molar galactose:arabinose ratio of 38 and 6·4, respectively. Citrus and apple arabinogalactan both had a neutral sugar content of 69% (w/w) and a molar galactose:arabinose ratio of 3·7 and 1·5, respectively. UFR arabinan had a total sugar content of 90% (w/w) and a molar galactose:arabinose ratio of 0·18. Methylation analysis revealed that potato arabinogalactan had a backbone of 1,4-linked galactopyranose residues substituted at C_6 by 1,5-arabinans. Linearised citrus arabinogalactan was substituted to a low extent at C_6 with 1·5-linked arabinose residues. Citrus arabinogalactan was a linear 1,4-galactan substituted with short arabinose side chains or highly branched side chains (results not shown).

Purification of *endo*-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*

The procedure used initially for the isolation of *endo*-galactanase from *A. niger* was based on the procedure used for the isolation of arabinanases by Rombouts *et al.* (1988) and is shown in Fig. 1. Fractions containing galactanase activity were obtained by chromatography on DEAE Bio-gel A. They were combined in pools II_5, III_1, III_4 and III_5 and were further purified on a Bio-gel HTP column. Contaminating arabinofuranosidase, *endo*-arabinase and a substantial amount of polygalacturonase activity could be removed in this last step. The various fractions (F, J, L and M) obtained were found to be very similar in their properties.

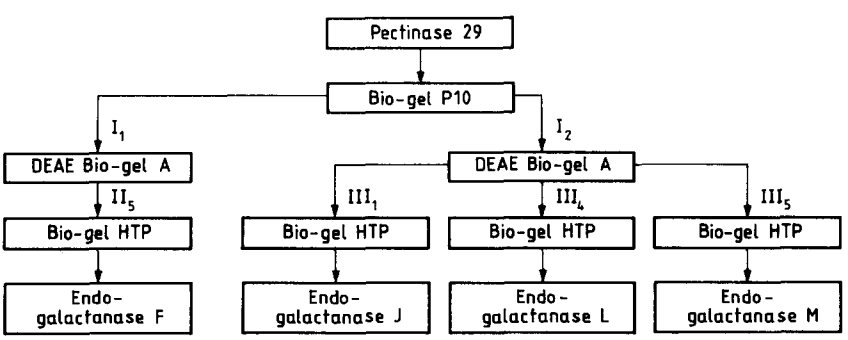


Fig. 1. Flow sheet of the purification of endo-galactanases F, J, L and M from an enzyme preparation of *Aspergillus niger*. The Roman numerals with Arabic subscripts refer to enzyme pools.

The galactanase isolation could be simplified and optimised by a similar procedure consisting of four steps (Fig. 2(a)). HPLC analysis of incubation mixtures of enzyme fractions and potato arabinogalactan revealed that all *endo*-galactanase activity could be found in pool II_6. Contaminating protein in pool III_4 could be removed by gel filtration chromatography on Bio-gel P100.

The purification of *endo*-galactanase from *A. aculeatus* is summarised in Fig. 2(b). *Endo*-galactanase in pool II_3 was eluted at a similar ionic strength as *A. niger endo*-galactanase (pool II_6, Fig. 2)a)). The enzyme was further purified by anion exchange chromatography on Mono Q. In this step contaminating polygalacturonase and CMC-ase were separated from *endo*-galactanase.

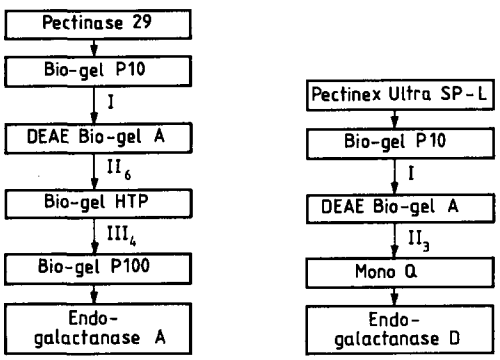


Fig. 2. Flow sheet of the optimised purification of endo-galactanase A and D from an enzyme preparation of *Aspergillus niger* (a) and *Aspergillus aculeatus* (b). The Roman numerals with Arabic subscripts refer to enzyme pools.

TABLE 2
Comparison of Purification of *Endo*-galactanases from *A. niger* and *A. aculeatus*

Enzyme	*Specific activity pH 5·0, 30°C (U/mg)*	*Yield (%)*	*Total activity*
From *A. niger*			
F	95·2	5·2	178
A	158	21	404
From *A. aculeatus*			
D	244	22	391

Some characteristics of *endo*-galactanase fractions isolated from *A. niger* and *A. aculeatus* are presented in Table 2. The optimised procedure for the *A. niger* preparation also resulted in an *endo*-galactanase fraction with a high specific activity on potato arabinogalactan. The *endo*-galactanase was obtained in a higher yield. *Endo*-galactanase D from *A. aculeatus* showed a higher specific activity (255 U/mg versus 95·2–158 U/mg at pH 5·0, 30°C). Enzymes A and F showed the same mobility on SDS–gel electrophoresis. Enzyme D also moved as one band and showed a similar mobility on SDS–gel electrophoresis.

Physico-chemical properties

The *A. niger* (M_r = 43 kD) and *A. aculeatus* (M_r = 42 kD) *endo*-galactanases differed with respect to their specific activities, especially at pH 5·0 and 30°C and to their temperature stability (Tables 2 and 3). *A. niger endo*-galactanase was stable up to 60°C as opposed to the *A. aculeatus* galactanase which was only stable below 35°C. Both enzymes showed highest activity at pH 4·00–4·25 and 50–55°C.

TABLE 3
Physico-chemical Properties of *Endo*-galactanases from *A. niger* and *A. aculeatus*

	A	*D*
Specific activity (pH 4·0, 55°C) on potato arabinogalactan	223 i.u./mg	287 i.u./mg
M_r (SDS-electrophoresis)	43 000 D	42 000 D
Glycoprotein	—	nd
Isoelectric point	4–6	4–6
Optimum pH	4·00	4·25
pH stability	5–7	5–7
Optimum temperature	50–55°C	50°C
Temperature stability	≤60°C	≤35°C
Kinetic parameters (potato arabinogalactan, 0·005 μg protein/ml)		
V_{max} ($\times 10^3$ min^{-1})	55·5	58·4
K_m (g/l)	0·77	0·31
V_{max}/K_m ($\times 10^3$ litres/min/g)	72	188
Freezing and thawing (5×)	93%	
Freezing and thawing (10×)	78%	
0·1% (w/v) Thiomersal in buffer	80%	
0·01% (w/v) Thiomersal in buffer	108%	

The isoelectric points of these enzymes could not be determined. Titration curves of *endo*-galactanase A and D showed that in the pH range 4–6 the enzymes did not migrate but remained at the site of application. This aberrant behaviour was also found by Rombouts *et al.* (1988) for *A. niger* arabinanases.

The enzyme from *A. niger* was fairly resistant to freezing and thawing. In the presence of 0·1 and 0·01% (w/v) thiomersal in the reaction mixture, 80% and 108% of the activity under standard conditions was measured (i.e. in the presence of 0·01% (w/v) NaN_3). The deviation of the latter figure is within experimental error.

The effects of various incubation buffers and chemicals on the activities and stabilities of both enzymes are listed in Table 4. Both enzymes displayed high activities (and stability in case of the *A. niger* galactanase) in a sodium acetate buffer. Similar activities were found using a citric acid/NaOH buffer; because the stability of the *A. niger* galactanase was decreased the sodium acetate buffer is preferred. *A. aculeatus* galacta-

TABLE 4

Effects of Various Incubation Buffers and Chemicals on the Activity and Stability of *Endo*-galactanases from *A. niger* and *A. aculeatus*[a]

Chemical/buffer	*A*		*D*
	Activity	*Stability*	*Activity*
NaAc	100	100	100
Nasuccinate/HCl	81	75	78
Oxalic acid/NaOH	77	69	68
Citric acid/NaOH	103	62	102
Nasuccinate/oxalic acid	95	93	93
KCl	97	92	93
$AgNO_3$	41	2·1	9·0
$MgCl_2$	88	79	100
$CaCl_2$	97	93	98
$MnCl_2$	103	84	77
$CoCl_2$	80	74	89
$NiCl_2$	73	57	70
$ZnSO_4$	52	43	58
$BaCl_2$	90	86	93
$Pb(NO_3)_2$	14	0	0
DTT	105	101	109
EDTA	102	99	106

[a]Concentration of EDTA and $CaCl_2$, present in reaction mixtures: 2 mM. Digests were also analysed on HPLC using an Aminex HPX 87P column.

nase was not stable in both buffers. Due to analytical problems with the Nelson–Somogyi assay of digests in citric acid/NaOH buffer, these digests were analysed by HPLC and compared with digests in sodium acetate buffer. Pb^{2+} and, to a lesser extent, Ag^{+} and Zn^{2+} ions in the sodium acetate buffer had a negative effect on the activities of both enzymes and stability of the *A. niger* enzyme. Ca^{2+} ions and EDTA did not have any effect on activity at optimum temperature and thermal stability of both enzymes.

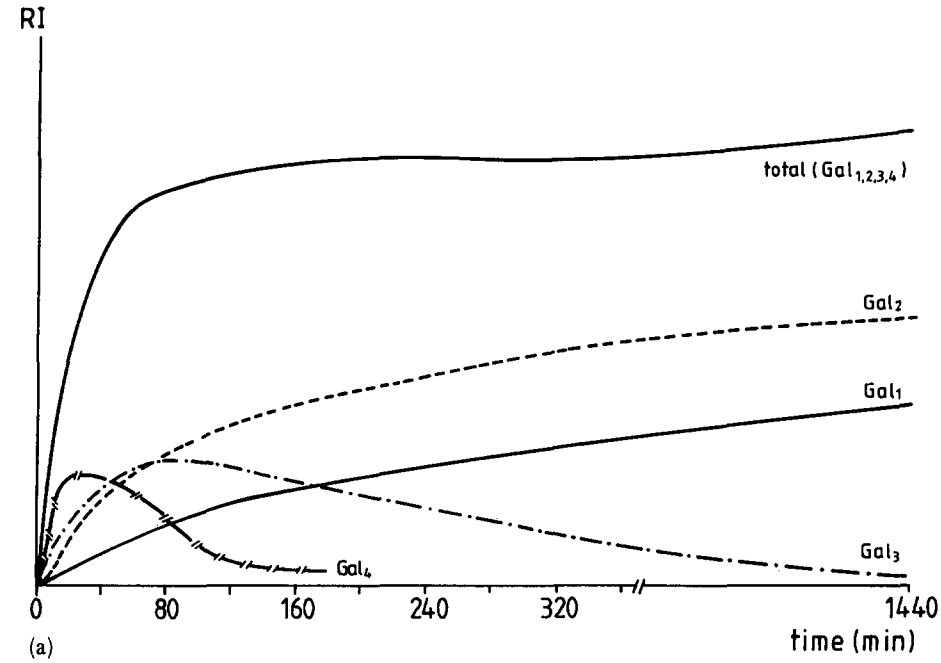
Substrate specificity and mode of action

The data presented in Table 5 and Fig. 3 clearly show that enzymes A, D and F are only active on substrates with a backbone of 1,4-β-D-galactopyranose residues. Small amounts of pectin lyase in fractions A and F and polygalacturonase in fractions A and D could be detected. Fraction F showed some activity on polygalacturonic acid. HPLC analysis of reaction products, however, showed that galactose and galactobiose were released, presumably from galactan impurities in the polygalacturonic acid preparation.

TABLE 5
Substrate Specificity of *Endo*-galactanases from *A. niger* and *A. aculeatus*[a]

Substrate/activity	*Activities expressed as percentage of main activity*		
	Enzymes A. niger		*Enzyme* A. aculeatus
	A	*F*	*D*
PNP-α-D-Gal	—	—	—
PNP-β-D-Gal	—	—	—
PNP-α-L-Ara	—	—	—
PNP-α-D-Xyl	—	—	—
PNP-β-D-Glc	—	—	—
PNP-β-D-Xyl	—	—	—
Arabinoxylan	—	—	—
1,5-α-L-arabinan	—	—	—
UFR arabinan	—	—	—
Stractan	—	—	—
Polygalacturonic acid	0·4	0·06	0·4
Pectin lyase	0·11	0·16	—
Pectate lyase	—	—	—

[a]Incubation: 10 μg enzyme (6·0 μg of F) protein per millilitre 0·05 M sodium acetate buffer pH 5·0, 20 h at 30°C.



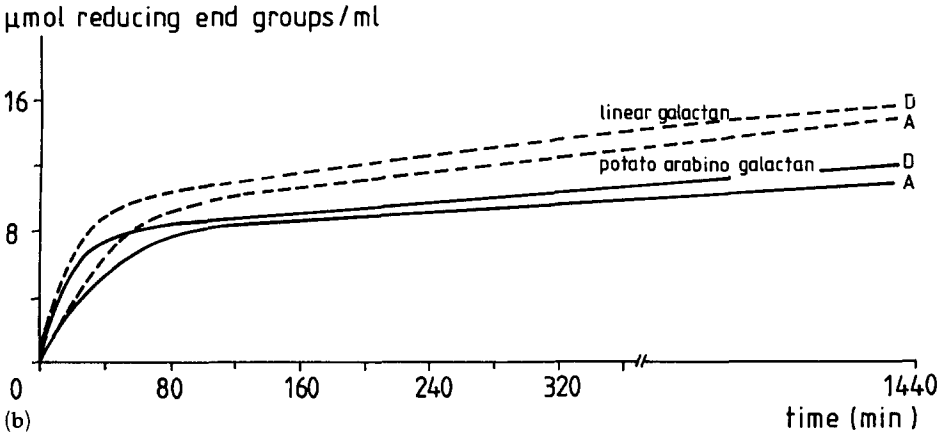


Fig. 3. Time course studies of the degradation of potato arabinogalactan by *endo*-galactanase from *A. niger* (a) analysed by HPLC using an HPX 42A column. Time curves of action of *A. niger* and *A. aculeatus endo*-galactanases on potato arabinogalactan and linearised potato arabinogalactan (b). Incubation: 0·60 µg protein and 4 mg substrate/ml 0·05 M sodium acetate buffer, pH 5·0 at 30°C. Reaction products released from tetragalactose by *endo*-galactanase from *A. niger* and *A. aculeatus* (c) analysed by HPLC using an HPX 87P column. Incubation: 1 µg protein and 2 mg substrate/ml 0·05 M sodium acetate buffer, pH 5·0, 0·5 h at 30°C.

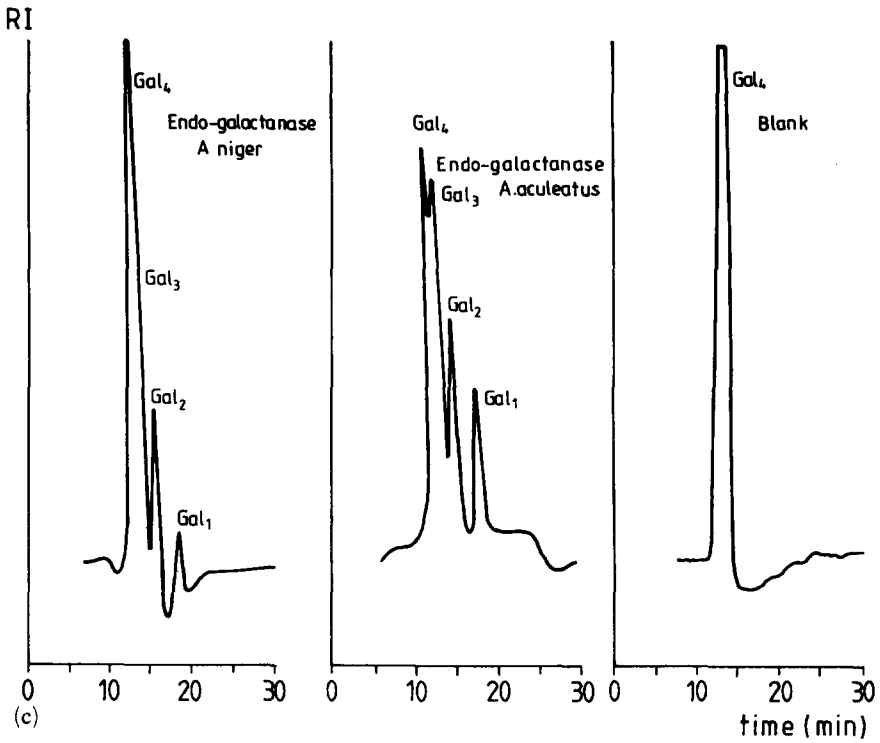


Figure 3 – *continued*

The release of oligomers from potato arabinogalactan by enzyme A is presented in Fig. 3(a). Similar patterns were obtained when apple or citrus arabinogalactan was used as substrate. It can be seen that initially tetramer galactose is formed and to a lesser extent the trimer; maximum amounts were reached after 0·5 h and 1·5 h, respectively. Also dimeric galactose was released from the very beginning. After 2 h its production gradually levelled off. Monomeric galactose accumulated at a low rate in the course of the reaction. After 24 h a level of hydrolysis of 52% was reached. These values were derived from the time curves in Fig. 3(b). This action pattern is typical for all *endo*-galactanase fractions of the *A. niger* preparation. The degradation of tetragalactose by the *A. niger* and *A. aculeatus endo*-galactanase was demonstrated by HPLC analysis of digests of tetragalactose (Fig. 3(c)).

The mode of action of galactanase D differed only very slightly from galactanase A. Enzyme D degraded potato arabinogalactan more rapidly. Very small amounts of oligomers of DP≥9, which were

hydrolysed by enzyme A, were not completely degraded by enzyme D. The ratio of released galactose/galactobiose was found to be higher for enzyme D.

The mode of action of enzymes A and D on linear galactan was similar to the pattern in Fig. 3(a). Linear galactan was degraded more rapidly and to a further extent by both enzymes, viz. to a level of 66% and 71% hydrolysis after 24 h incubation with A and D (Fig. 3(b)).

Kinetic parameters

Kinetic parameters of both enzymes are presented in Table 3 and Figs 4 and 5. Because V_{max}/K_m is considered to be a measure of the catalytic efficiency and specificity, these values were compared. For K_m (in grams per litre) and V_{max} (per minute) the V_{max}/K_m values of *endo*-galactanase F from *A. niger* (0·058 μg protein/ml reaction mixture) were $8{\cdot}46 \times 10^3$ and $13{\cdot}3 \times 10^3$ on citrus arabinogalactan and citrus arabinogalactan linearised with arabinofuranosidase B, respectively. The Lineweaver–Burk plots in Fig. 4 show that lowering the degree of branching did not affect V_{max} but had a clear effect on the K_m values. The Lineweaver–Burk plots in Fig. 5 show that the enzyme from *A. aculeatus* differed

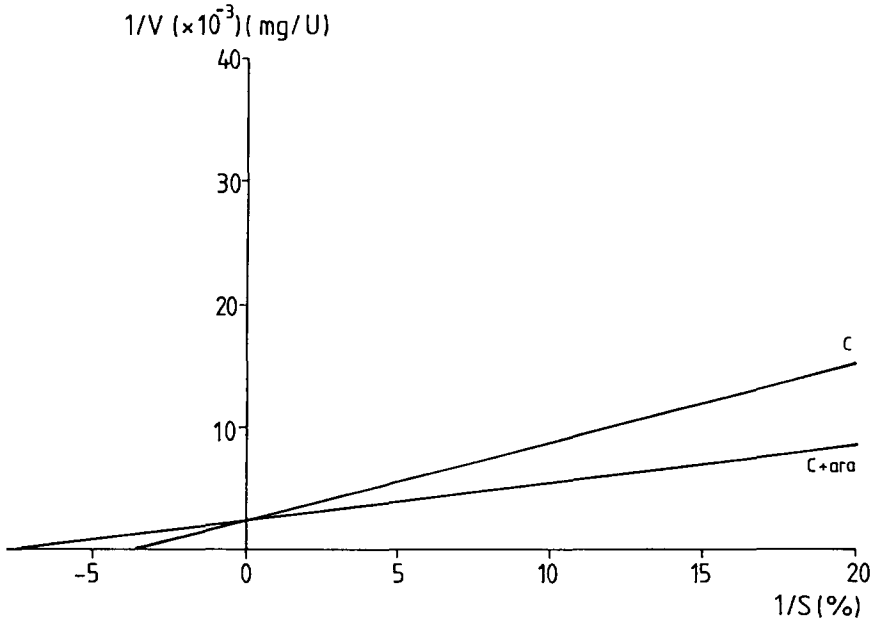


Fig. 4. Lineweaver–Burk plots of *endo*-galactanase F (*A. niger*) on citrus arabinogalactan (C) and linearised citrus arabinogalactan (C + Ara).

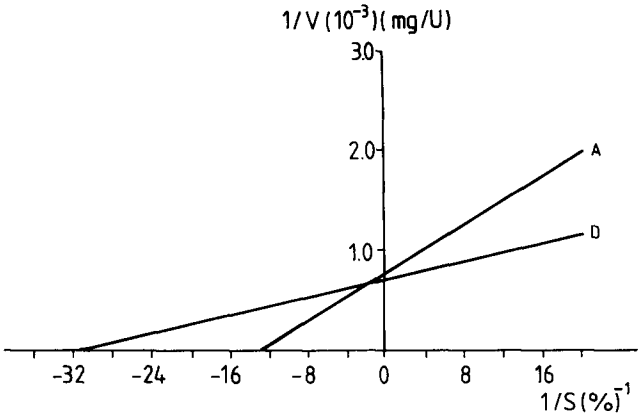


Fig. 5. Lineweaver–Burk plots of *endo*-galactanases A (*A. niger*) and D (*A. aculeatus*) on potato arabinogalactan.

from the enzymes of *A. niger*. Enzymes A and D had similar V_{max} values (Table 3) but differed with respect to their affinity for potato arabinogalactan. Enzymes A and D were characterised by the following V_{max}/K_m values on potato arabinogalactan: $72{\cdot}0 \times 10^3$ and 188×10^3 litres/(min g), respectively.

Analysis of digests of high concentrations of potato arabinogalactan for determination of kinetic parameters could not be performed with the Nelson–Somogyi assay (Somogyi, 1952). The assay gave precipitates with this substrate, resulting in poor reproducibility. The automated neocuproine assay reaction (Stephens *et al.*, 1974) gave more reproducible absorbance readings (Kormelink *et al.*, 1990).

Action of *endo*-arabinanase and *endo*-galactanases from *A. niger* and *A. aculeatus* alone and in combination on potato arabinogalactan

The interaction between galactanases and arabinanases (purified from *A. niger* by Rombouts *et al.* (1988) for an optimal breakdown of potato arbinogalactan was studied. Arabinofuranosidase B (EC 3.2.1.55) showed very little activity on potato arabinogalactan and did not enhance arabinogalactan degradation when it was used together with *endo*-galactanases under conditions used. *Endo*-1,5-α-L-arabinanase (EC 3.2.1.99), however, was found to be active on potato arabinogalactan. This activity was calculated to be 20% of the sum of single activities of *endo*-arabinanase and *endo*-galactanase A. Synergism exerted by *endo*-arabinanase and *endo*-galactanases A and D was

investigated. The results are summarised in Table 6A and from these data it can be seen that *endo*-arabinanase in combination with both *endo*-galactanase preparations accelerates the breakdown of the polymer. The reaction products formed during incubation were also analysed by HPGPC and HPLC for changes in M_w distribution (Fig. 6) and formation of oligomeric reaction products (Table 6B). The chromatograms in Fig. 6 show that arabinogalactan degradation is strongly enhanced by the combined action of *endo*-arabinanase and *endo*-galactanase from *A. niger* and *A. aculeatus*. The data of Table 6B revealed that the combined action of *endo*-arabinanase and *endo*-galactanase from *A. niger* and *A. aculeatus* resulted in an increased release of galactose and galactobiose and galactotriose. An increase in the content of tetramer in the digest could not be detected.

DISCUSSION

Purification of *endo*-galactanases from *A. niger* and *A. aculeatus*

The purification scheme as designed by Rombouts *et al.* (1988) for the isolation of arabinanases from the experimental *A. niger* enzyme

TABLE 6A

Relative Activities[a] of *Endo*-arabinanase and *Endo*-galactanases from *A. niger* and *A. aculeatus* on Potato Arabinogalactan

	A	*D*	Endo-*ara*
Endo-ara	130	121	52
D	88	85	—
A	92	—	—

TABLE 6B

Relative Contents of Galactose, Galactobiose and Galactotriose in Digest of Potato Arabinogalactan[b]

	A	*D*	*A* + Endo-*ara*	*D* + Endo-*ara*
Galactose	100	100	128	119
Galactobiose	100	100	135	132
Galactotriose	100	100	127	132

[a]Activities of combinations are expressed in percentage of sum of activities of single enzymes. Incubation: 0·05 μg galactanase, 1·1 μg *endo*-arabinanase protein and 1 mg substrate/ml 0·05 M sodium acetate buffer, pH 4·0, 1 h at 55°C.

[b]Analysis by HPLC using the HPX 87 P column.

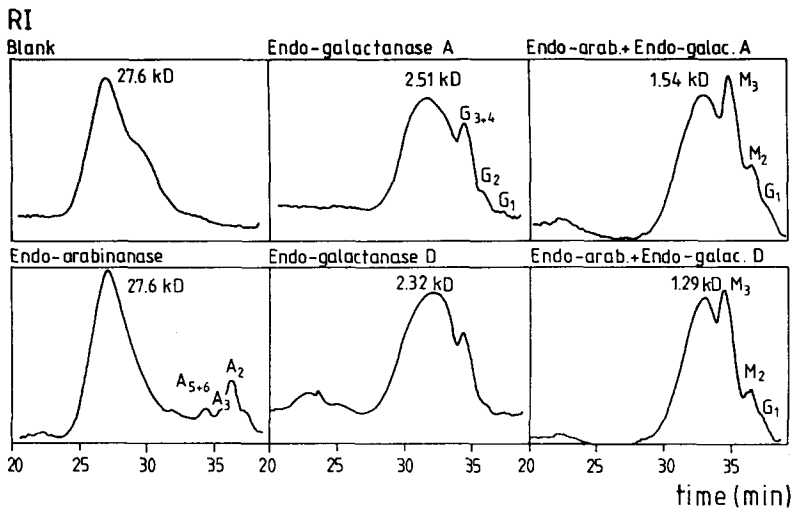


Fig. 6. HPGPC analysis of reaction products of digests of potato arabinogalactan with *endo*-arabinanase and *endo*-galactanases from *A. niger* and *A. aculeatus*. Experimental details in the legend are described in Table 6. G_1, monomeric galactose; G_2, dimeric galactose, etc. A_2, dimeric arabinose; A_3, trimeric arabinose, etc. M_2, mixture of A_2, G_2 and A_3; M_3, mixture of G_3, G_4, A_5 and A_6.

preparation could also be used for the isolation of *endo*-galactanases. It resulted, however, in a number of *endo*-galactanase fractions which were found to have similar properties. The fact that a number of *endo*-galactanase fractions was obtained is obviously caused by the following procedure. This procedure was therefore optimised and successfully applied in the purification of *endo*-galactanases from *A. niger* and *A. aculeatus* enzyme preparations. In the purification procedure of *endo*-galactanase from *A. aculeatus* the last two steps were successfully replaced by chromatography on Mono Q.

Properties of the *endo*-galactanase from *A. niger* and *A. aculeatus*

The two *endo*-galactanases were similar in a number of properties. They only attacked substrates with a backbone of 1,4-β-D linked galactopyranose residues. Linear 1,4-β-D-galactan was the best substrate for these enzymes, as shown in Figs 3(b) and 4. During action of the *endo*-galactanases on (arabino)galactans low galacto-oligomers were formed, mainly of DP≤4 and a very rapid change in the molecular weight distribution of (arabino)galactan was observed. Galactose and galactobiose accumulated as end products. These data suggested that the enzymes from both fungi were *endo*-1,4-β-D-galactanases (EC 3.2.1.89) which hydrolysed according to a multiple attack mechanism (Robyt &

French, 1967). Galactanases from various strains of *B. subtilis* differ in their action pattern from these *endo*-galactanases. *Endo*-galactanases from *B. subtilis* var. *amylosacchariticus* released mainly galactotriose and, to a lesser extent, galactobiose and galactose as final hydrolysis products (Yamamoto & Emi, 1988). Galactanases from two other strains, viz. K-50 (Emi *et al.*, 1971) and WT 168 (Labavitch *et al.*, 1976), showed both *exo*- and *endo*-activity; galactobiose and galactotetraose were formed respectively as major reaction products. The action pattern of the *endo*-galactanases from *P. citrinum* was very similar to the *A. niger* and *A. aculeatus* enzymes (Nakano *et al.*, 1985).

The galactanases did not show glycosyl transferase activity, as described for *endo*-galactanases I and II from *P. citrinum* (Nakano *et al.*, 1985, 1986, 1988). *Endo*-galactanases purified from *A. niger* and *A. aculeatus* showed optimal activity at 50–55°C and pH 4·00–4·25, which is similar to previously reported data for the enzyme complex of *A. niger* (Tavobilov *et al.*, 1986) and the *endo*-galactanases from *P. citrinum* (Nakano *et al.*, 1985). The *endo*-galactanase from *A. niger* was most stable in a sodium acetate buffer, therefore this buffer was used in the experiments. The *endo*-galactanases from *A. niger* and *A. aculeatus* differed in their thermal stability, kinetic properties and specific activity. *Endo*-galactanase from *A. niger* was stable up to 60°C; the *endo*-galactanase from *A. aculeatus*, however, was only stable up to 35°C. EDTA or Ca^{2+} ions did not have any effect on the activity at 55°C and thermal stability of both enzymes. This is in contrast to the *endo*-galactanases of *B. subtilis* var. *amylosacchariticus* which were found to be sensitive to EDTA and could be stabilised by Ca^{2+} ions (Yamamoto & Emi, 1988). The Lineweaver–Burk plots show that the *A. aculeatus* *endo*-galactanase was found to have a higher affinity for potato arabinogalactan than the *A. niger* enzyme. Determination of kinetic properties is strongly influenced by the methods (Kormelink *et al.*, 1990) and substrates used. Therefore these properties could not be compared with values reported in the literature (Nakano *et al.*, 1985; Yamamoto & Emi, 1988). The specific activities of *A. niger* and *A. aculeatus* on potato arabinogalactan were found to be 158 and 244 U/mg, respectively, in a sodium acetate buffer pH 5·0 at 30°C.

Optimal degradation of potato arabinogalactan

Combined action of arabinanases and *endo*-galactanase was studied for an optimal degradation of potato arabinogalactan. *Endo*-arabinanase exerted synergistic effects with both *endo*-galactanases. The action of

these enzyme combinations resulted in an acceleration of the enzymic hydrolysis of the arabinogalactan. Also an increase in the shift of the molecular weight distribution of the digest was observed and galactose, galactobiose and galactotriose were formed in increased amounts. The results also revealed that galactanase action was hindered by the presence of arabinan side chains. Arabinofuranosidase B showed very little activity on the substrate and therefore did not exert a detectable synergistic effect with *endo*-galactanase under conditions used. These data indicated that the substrate is an arabinogalactan with linear 1,5-α-L-arabinans substituted on the galactan backbone. This was also indicated by methylation analysis. A study of synergistic effects exerted by galactosidases, *endo*-galactanases, *endo*-arabinanase and arabinofuranosidases will be the subject of a future communication.

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