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Endo-β-1,4-D-galactanase from *Aspergillus niger* var. *aculeatus*: Purification and some Properties

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ABSTRACT

An endo-1,4-β-D-galactanase from the commercial preparation SP 249 (Novo Industri) originating from Aspergillus niger *var.* aculeatus *was purified using affinity chromatography on laboratory cross-linked Sepharose 6B with divinyl sulfone, gel filtration on Sephadex G 75, and ion-exchange chromatography on DEAE-Sephadex A 50. Galactanase-apparent specific activity was increased 114-fold after purification and the enzyme is devoid of endopolygalacturonase, pectin-, pectate-lyase, arabinanase, β-D-galactosidase, and α-L-arabinofuranosidase activities. It migrates as a single band in sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis (PAGE) and has a molecular weight of 38 000. It is an acidic enzyme of pHi 2·8 containing a low amount of a putative isoenzyme of pHi 2·7 which also migrates as a minor band on PAGE without SDS. Enzymatic activity is optimum at pH 3·5–4·0 and at 50–55°C and the enzyme is most stable between ~ pH 4·0–7·0 and below 30°C. It does not attack other galactans such as agarose, kappa- and iota-carrageenan nor type II arabinogalactans. Activity is inhibited by excess substrate and dissociation constants* K_D *(0·6 mg* ml^{-1}*) and* K'_D *(10·7 mg* ml^{-1}*), and maximal velocity V (8500 nkat) were obtained from Lineweaver–Burk and Khun plots. Endo-galactanase specific activity is 17·0 mkat* mg^{-1}*. Galactose and galactobiose are the end products of galactan hydrolysis.*

INTRODUCTION

Arabinogalactans and galactans occur widely in plant primary cell walls and often contain rhamnose or galacturonic acid residues and proteins

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(Dey & Brinson, 1984). They are essentially side chains attached to the rhamnogalacturonan backbone of pectins and some are galactose-enriched polymers that most likely arise from degradation during pectin extraction. Their close association with pectic polymers and with hydroxyproline-rich proteins suggests that they may play a structural role as interlinking agents in the cell walls as well as some other biological functions (Dey & Brinson, 1984).

Arabinogalactans have been classified according to the linkages between the galactose residues (Dey & Brinson, 1984). Type I consists of a backbone of 1,4-linked β-D-galactopyranose ramified at places by α-L-arabinofuranose residues. The main feature of type II consists of a backbone of 1,3-linked β-D-galactopyranose that can be branched by 1,6-linked β-D-galactopyranose residues. These two types of arabinogalactan have been found in cell walls rich in pectins. However, the exact structure of these polymers and their respective location in the cell wall need further studies.

Enzymes are highly valuable tools in the structural elucidation of polysaccharides (McCleary & Matheson, 1986). They have been particularly useful in confirming the chemical structure of pectic polysaccharides and in distinguishing unbranched from highly branched galacturonic regions (McCleary & Matheson, 1986). Recently, polysaccharidases helped in the determination of the complex structure of grape berries pulp (Saulnier & Thibault, 1987), carrot root (Massiot & Thibault, 1989) and 'hairy' fragments from sugar-beet pectins (Guillon *et al.*, 1989).

As a prerequisite to the study of the fine chemical structure of branched regions of pectin which are enriched in type I arabinogalactan, the purification of an endo-1,4-β-D-galactanase was carried out. Galactanases have already been obtained in enriched or purified preparations from several organisms (Emi *et al.*, 1971; Emi & Yamamoto, 1972; Dekker & Richards, 1976; Labavitch *et al.*, 1976; Bauer *et al.*, 1977; Nakano *et al.*, 1985). However, endo-1,4-β-D-galactanases (EC 3.2.1.89) have only been purified to homogeneity from *Bacillus subtilis* (Emi *et al.*, 1971; Emi & Yamamoto, 1972) and from *Penicillium citrinum* (Nakano *et al.*, 1985) and used in the characterization of pectin side branches (McCleary & Matheson, 1986). In contrast, endo-1,3-β-D-galactanase has been reported only once from the fungus *Rhizopus niveus* (Hashimoto *et al.*, 1969; Hashimoto, 1971). It degrades type II arabinogalactan from coffee bean into galactose and arabinose indicating the presence of several enzymic activities in this preparation.

The purification and some properties of an endo-1,4-β-D-galactanase from *Aspergillus niger* var. *aculeatus* are now reported.

MATERIALS AND METHODS

Materials

Sepharose 6B, Sephadex G 75, DEAE-Sephadex A 50, and agarose IEF were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden). Divinyl sulfone, β-mercaptoethanol and amyloglucosidase from *A. niger* were obtained from Merck (Darmstadt, FRG). Potato pulp was kindly provided by Roquette Frères (Lestrem, France).

Enzyme

Endo-1,4-β-D-galactanase was purified from the commercial enzyme preparation SP 249 obtained from *A. niger* var. *aculeatus* and kindly provided by Novo Industri (Bagsvaerd, Denmark).

Miscellaneous assays

Reducing power was determined by the method of Nelson (1944). Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Neutral sugars were analysed by GLC as their alditol acetates after hydrolysis with sulfuric acid (Blakeney *et al.*, 1983). Uronic acids (as galacturonic acid) were determined by the automated 3-hydroxybiphenyl method (Thibault, 1979).

HPLC

HPLC of galactan enzymic hydrolysate was performed on two Merck Superspher C18 cartridges (125 × 0·4 mm) eluted with water at 1 ml min^{-1}. Elution was monitored by a differential refractive index detector.

Polyacrylamide-gel electrophoresis and isoelectric focusing

Polyacrylamide-gel electrophoresis (PAGE) with and without sodium dodecyl sulfate (SDS) was performed by using a 10–20% acrylamide gradient gel at pH 8·3. Gels were fixed in aqueous trichloroacetic acid (12·5%) and stained with Coomassie brilliant blue G (0·3% in methanol). Molecular weights were determined from SDS–PAGE using high-molecular-weight (MW) calibration kit (Pharmacia).

Isoelectric focusing was performed on agarose IEF in pH ranges of 3·0–10·0 and 2·5–5·0 (Pharmacia). The isoelectric points were determined from calibration kits (Pharmacia).

Preparation of 1,4-linked β-D-galactan

Potato cell-walls are known to contain 1,4-linked β-D-galactan and thus potato pulp was chosen as a source for this polysaccharide (Wood & Siddiqui, 1972). The extraction and purification is as follows.

Potato pulp (66 × 30 g) was suspended in sodium hydroxide solution (0·5 M, 66 × 1 litre), autoclaved for 1 h (120°C), cooled to room temperature and adjusted to pH 5 with 6 M HCl. Sodium azide (0·02% w/v) was added and starch was hydrolysed by amyloglucosidase (200 mg enzyme 90 g^{-1} autoclaved potato pulp) for 2 h at 40°C followed by 60 h at 20°C. The insoluble particles were removed using a Westfalia separator (model SA7-06, Château-Thierry, France) and the crude galactan in the liquid phase was passed through a 1-mm pure polypropylene filter (Ouest Anti-corrosion, Richelieu, France), concentrated by ultrafiltration on an OM-141 Pellicon cassette system equipped with a PTGC membrane (nominal MW limit 10 kDa, Millipore Corp. Bedford, MA., USA), precipitated by four volumes of absolute ethanol and dehydrated by solvent exchange (yield: 7·5%).

Crude galactan (145·92 g) was dissolved in a trifluoroacetic acid solution (TFA, 50 mM, 1·5 litres), heated in a boiling water-bath for 1 h, dialysed extensively against deionized water (nominal MW limit 8 kDa), filtered through sintered glass G4 (porosity about 10 μm) and added to 3 litres DEAE-Sephadex A 50 in deionized water. The unbound material was recovered by filtration on sintered glass G4, combined to the 7·3 litres deionized water used to wash the ion exchanger and concentrated to 600 ml *in vacuo* at 40°C. In order to remove arabinosyl residues from the galactan as much as possible, TFA was added to make 50 mM and the acidified polysaccharide solution was heated in a boiling water-bath for 1 h, dialysed extensively against deionized water, concentrated *in vacuo* at 40°C, precipitated by four volumes of absolute ethanol and dehydrated by solvent exchange (yield from starting potato pulp: 1·9%).

The purified galactan is composed of 2% (w/w) protein, 88·6% neutral sugars (0·2% rhamnose, 1·6% arabinose, 0·4% mannose and 86·4% galactose) and 1·4% uronic acids.

The β-1,4-linkages between the galactose residues was confirmed by ^{13}C-NMR spectroscopy using a Bruker WM 250 operating at 62·89 MHz. Galactan that had been treated only once with TFA (150 mg) was dissolved in D_2O (2 ml) and its NMR spectrum recorded at 50°C, using a recycling time of 1 s. Chemical shifts are expressed in parts per million related to internal dimethylsulfoxide and converted to values relative to tetramethylsilane (conversion constant: 39·6). Chemical shifts of C1–6 are as follows: 105·2, 72·8, 74·2, 78·5, 75·4, 61·7 ppm, respectively. They

are in agreement with those reported by Messer *et al.* (1980). Minor signals were also observed at 108·4, 81·7, 77·7, 83·2 and 67·8 ppm and were assigned to C1–5 of 1,5-linked α-L-arabinofuranose by comparison with values published by Joseleau *et al.* (1977).

Assays for enzymic activities

Enzymic activities are expressed in nanokatal (nanomol equivalent saccharide or galacturonic acid released per second) and were determined from the initial velocity of the reaction.

Galactanase activity was determined using a 1% (w/v) solution of purified potato galactan in acetate buffer (100 mM, pH 4·5) filtered through 3-μm membrane (Millipore Corp.).

Endo-polygalacturonase activity was determined using a 1% (w/v) solution of polygalacturonic acid from orange (Sigma, St Louis, MI., USA) in acetate buffer (50 mM, pH 4·5).

Pectin- and pectate-lyase activities were measured at 235 nm on 75% esterified apple pectin (Obipectin, Bischofszell, CH) and citrus polygalacturonic acid (ICN Pharmaceutical Inc., Cleveland, OH, USA), respectively (0·5% w/v), in acetate buffer (100 mM, pH 4·5) at 40°C using molar absorption coefficients of 5500 (Van Houdenhoven, 1975) and 4800 M^{-1} cm^{-1} (Rombouts *et al.*, 1978), respectively.

β-D-galactosidase and α-L-arabinofuranosidase activities were measured at 400 nm after incubation with 2 mM *p*-nitrophenylglycosides (Sigma) in acetate buffer (100 mM, pH 4·5) at 40°C and after addition of 0·6 ml of 1 M sodium carbonate to 0·2 ml of reaction mixture. For calculation of both activities, a molar absorption coefficient of 18 350 M^{-1} cm^{-1} was used.

Arabinanase activity was determined using a 2% (w/v) solution of sugar-beet araban in acetate buffer (10 mM, pH 4·0). The preparation of the arabinan from sugar-beet pulp follows a similar protocol as that of the galactan.

Activity on other galactans were measured by the increase in reducing power after incubation with solutions containing 0·5% (w/v) agarose, kappa-carrageenan, iota-carrageenan, or 1% (w/v) type II arabinogalactan (larch, Serva, Heidelberg, FRG, and Koch-light Laboratories, Coinbrook, UK) in acetate buffer (100 mM, pH 4·5) at 40°C.

Preparation of the affinity chromatography column

Sepharose 6B was cross-linked by divinyl sulfone according to the method of Porath *et al.* (1975). To 50 g of gel (wet weight) were added 50

ml of sodium carbonate buffer (0·5 M, pH 11·0) and 2·0 ml of divinyl sulfone (DVS). The suspension was shaken in a water-bath at room temperature for 2 h and at 45°C for 2 h and then washed extensively with deionized water until a neutral pH was reached. The wet cross-linked Sepharose was resuspended in 50 ml of acetate buffer (20 mM, pH 4) and unreacted vinylic groups were inactivated by addition of β-mercaptoethanol (0·6 ml). The cross-linked gel was left for 17 h and then was extensively washed with deionized water. Because agarose is the constituent of Sepharose, the cross-linked gel is referred to in the text as agarose/DVS.

RESULTS AND DISCUSSION

All operations were performed at 4°C. SP 249 (31 ml) was dialysed against deionized water (3 × 1 litre) and against acetate buffer (20 mM, pH 4·0, 2 × 1 litre and 50 mM, pH 4·0, 2 × 1 litre) with a change of the dialysis tubing every 2 h. No loss in total galactanase activity was observed after dialysis and a slight increase in specific activity was obtained (× 1·2).

Affinity chromatography

Affinity chromatography has been used by Labavitch *et al.* (1976) and Nakano *et al.* (1985) in the purification of *B. subtilis* and *P. citrinum* endo-1,4-β-D-galactanases, respectively. The former authors complexed β-1,4-galacto-tetraose to Sepharose while the latters used surprisingly arabinose as ligand on Sepharose 6B. Our studies showed that no ligand was required for the binding of *A. niger* var. *aculeatus* galactanase to Sepharose 6B. Indeed, in the preparation of an affinity gel for galactanase purification, galactan was added to the Sepharose 6B during cross-linking by DVS, but galactanase activity was observed to be also retained on agarose/DVS without galactan (Fig. 1). Although a weak affinity was observed for Sepharose 6B alone, it was greatly enhanced after the gel was cross-linked by DVS (Figs 1 and 2). Thus, in the subsequent affinity column, galactan was omitted and best galactanase recovery was obtained for a ratio of 4ml DVS for 100 g wet Sepharose 6B (Fig. 2).

The presence of salts was required for a reproducible fixation of the enzyme to the agarose/DVS column. Dialysed SP 249 was brought to 1 M ammonium sulfate prior to application to the affinity column.

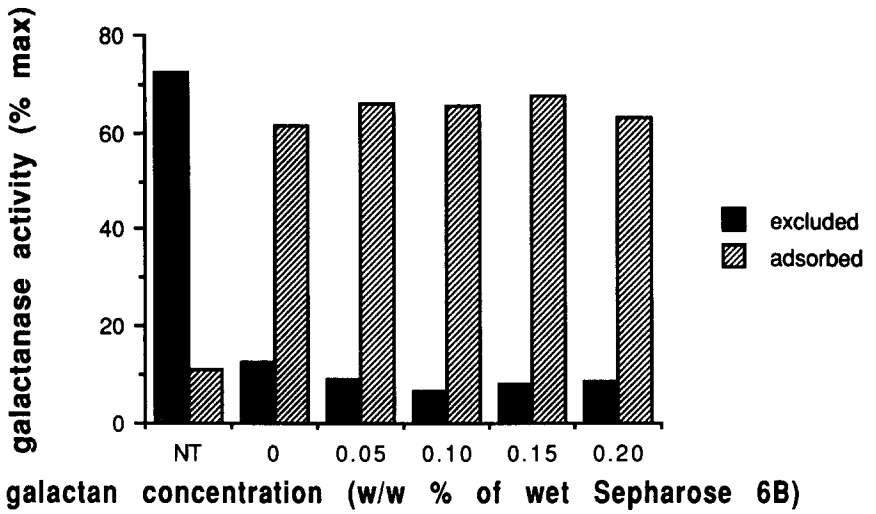

Fig. 1. Effect of galactan concentration added during the cross-linking reaction of Sepharose 6B with divinyl sulfone on the binding of galactanase. NT, untreated Sepharose 6B; percentage of galactanase activity represents percentage of activity excluded or adsorbed and eluted with acetate buffer (1 M, pH 4·0) from total galactanase activity applied to the column.

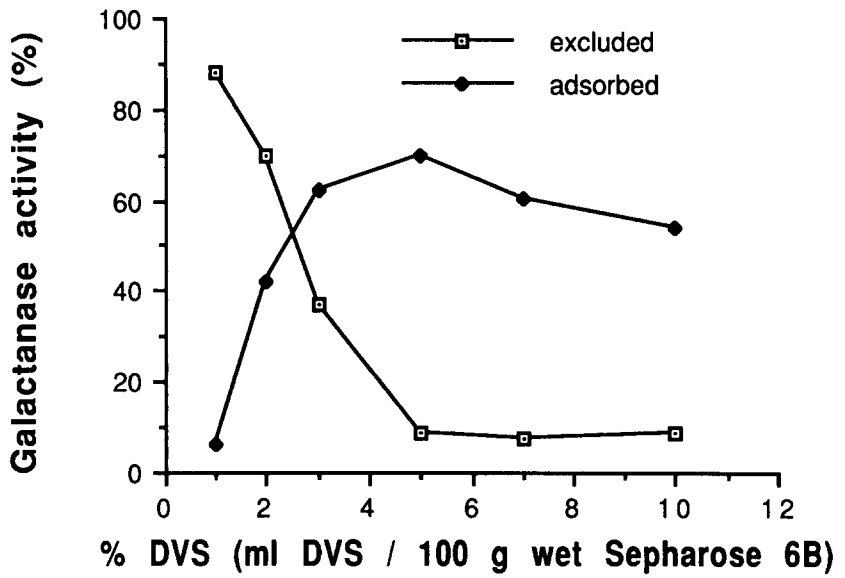

Fig. 2. Effect of divinyl sulfone concentration added during the cross-linking reaction of Sepharose 6B on the binding of galactanase. Percentage of galactanase activity represents percentage of activity excluded or adsorbed and eluted with acetate buffer (1 M, pH 4·0) from total galactanase activity applied to the column.

Protein concentrations applied to the agarose/DVS column for optimal galactanase adsorption were up to 800 mg/100 ml of affinity gel. Ionic strength, pH, and type of salt are particularly critical for desorption. The enzyme was eluted with 1·0 M acetate during a gradient of 0·05 to 1·0 M acetate buffer at pH 4·0. Galactanase was not desorbed by 1·0 M acetate buffer at pH 5·0 even with a gradient of NaCl (0–2 M) in 1·0 M acetate nor by a NaCl gradient (0–2 M) in 50 mM acetate or citrate buffer at pH 4·0.

The nature of the affinity site and mechanism of binding are not clear but local conformation of DVS cross-linked agarose, probably involving the β-1,4-galactose linkages present in this polysaccharide (Araki, 1966), must be mimicking that of galactan and recognized by the binding site of the enzyme. Adsorption and desorption of galactanase from agarose/DVS with buffers of high ionic strength suggest that the binding cannot be ascribed to ionic or hydrophobic interactions between the enzyme and the derivatized agarose.

Purification of endo-1,4-β-D-galactanase

Dialysed SP 249 (10 ml) was brought to 1 M $(NH_4)_2SO_4$ and applied to a column of agarose/DVS (16 × 1·6 cm) equilibrated with acetate buffer (50 mM, pH 4·0) and eluted at a flow rate of 55 ml h^{-1} by 7 bed volumes of the same buffer followed by 4 bed volumes of acetate buffer 1 M, pH 4·0. Fractions eluted with the latter buffer containing galactanase activity were pooled, dialysed against citrate phosphate buffer (10 mM, pH 3·5) and freeze-dried.

The pool of enzyme resulting from two agarose/DVS fractionations was dissolved in a minimal amount of citrate phosphate buffer (10 mM, pH 3·5) and applied to a column of Sephadex G 75 (85 × 2·2 cm) equilibrated and eluted with citrate phosphate buffer (10 mM, pH 3·5) at a flow rate of 20·4 ml h^{-1}. Fractions having galactanase activity were pooled and applied to a DEAE-Sephadex A 50 column (17 × 3·2 cm) equilibrated with citrate phosphate buffer (10 mM, pH 3·5). The unbound material was eluted with 1 bed volume of the same buffer. Fractions having galactanase activity were eluted with an NaCl gradient (0·3–1·0 M, 2 bed volumes) in the same buffer, pooled, dialysed against acetate buffer (5 mM, pH 4), and freeze-dried.

Finally, the enzyme was redissolved in 3·5 ml of citrate phosphate buffer (10 mM, pH 3·5), reapplied to the Sephadex G 75 column and eluted as above. Galactanase-containing fractions were pooled and freeze-dried.

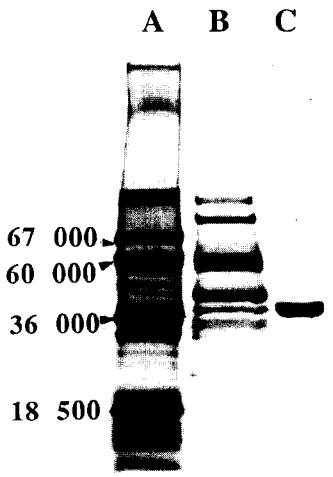

Fig. 3. Sodium dodecyl sulfate/polyacrylamide-gel electrophoresis of: (A) molecular weight standards; (B) dialysed SP 249; and (C) purified galactanase.

The purified enzyme was free from β-D-galactosidase, α-L-arabinofuranosidase, arabinanase, pectin- and pectate-lyase, and endopolygalacturonase activity and migrated as a single band by SDS–PAGE (Fig. 3). However, two bands (a major and a minor) were observed by isoelectric focusing and by PAGE without SDS. These were assumed to be isoenzymes of similar MW but differing only in their charge density.

Table 1 shows the recovery of galactanase activity during purification. The protein yield was 0·13% which represented 14·3% of the initial galactanase activity. The latter figure is only an apparent value because: (1) the original SP 249 preparation contained several galactanase activities which were not all recovered; and (2) the purified galactanase is inhibited by excess substrate (see below) and galactan concentration in the enzymatic assay may not have been in the proper range for accurate galactanase activity measurement. Galactanase specific activity is 17·0 mkat mg^{-1} at galactan concentration giving the maximum reaction velocity.

Properties of galactanase

The enzyme can be freeze-dried and redissolved in buffer without loss of activity. From SDS–PAGE, its molecular weight is 38 000 and thus is

TABLE 1
Purification of Galactanase from *Aspergillus niger* var. *aculeatus*

Step	*Volume (ml)*	*Protein (mg)*	*Total apparent activity (nkat)*	*Apparent specific activity (nkat mg^{-1})*	*Purification (fold)*	*Apparent activity yield (%)*
Dialysed SP 249	20	954	59 060	61·9	1	100
Agarose/DVS	6·4	31·2	22 123	708	11·4	37·5
Sephadex G75	62	6·1	18 483	3 010	48·6	31·3
DEAE-Sephadex A 50	48	2·5	13 379	5 352	86·4	22·7
Sephadex G75	50	1·2	8 459	7 049	113·9	14·3

close to those observed for *P. citrinum* (Nakano *et al.*, 1985) and *B. subtilis* (Emi *et al.*, 1971; Emi & Yamamoto, 1972; Labavitch *et al.*, 1976) galactanases.

In contrast, it has an unusual acidic pHi (pHi 2·8) since those from *B. subtilis* are at about 6·9–8·9 (Emi *et al.*, 1971; Emi & Yamamoto, 1972; Labavitch *et al.*, 1976), *S. sclerotinium* at 8·3 (Bauer *et al.*, 1977), and from *P. citrinum* at about 4·2 (Nakano *et al.*, 1985). Another protein assumed to be an isoenzyme of pHi 2·7 is present in low concentration in the purified preparation.

The optimum activity occurs at a lower pH (3·5–4·0) than those reported for bacterial and fungal endo-β-1,4-D-galactanases (6·0–7·0 and 4·5–5·0, respectively; Emi *et al.*, 1971; Emi & Yamamoto, 1972; Labavitch *et al.*, 1976; Bauer *et al.*, 1977; Nakano *et al.*, 1985) and the enzyme is stable over a narrower range of pH (between ~pH 4·0–7·0) than those reported in the literature, pH 4–10 for *P. citrinum* (Nakano *et al.*, 1985) and pH 5·5–10 for *B. subtilis* galactanases (Emi *et al.*, 1971; Emi & Yamamoto, 1972; Labavitch *et al.*, 1976). The optimal temperature for activity, at about 50–55°C, is in the range reported for other microbial endo-β-1,4-D-galactanases (Emi *et al.*, 1971; Emi & Yamamoto, 1972; Labavitch *et al.*, 1976; Bauer *et al.*, 1977; Nakano *et al.*, 1985). However, maximum stability of the enzyme is below 30°C and the enzyme activity is markedly reduced after exposure for 30 min at temperatures above 30°C.

This endo-β-1,4-D-galactanase has no activity towards other galactans such as agarose, kappa- and iota-carrageenan, and type II arabinogalactans.

Enzyme kinetics

Particular kinetics was observed for this endo-galactanase. The reaction initial velocity decreases as the substrate concentration increases above a certain concentration (Fig. 4). This phenomenon is typical of inhibition by excess substrate (Penasse, 1974) and two dissociation constants K_D (0·6 mg ml^{-1}) and K'_D (10·7 mg ml^{-1}) were obtained from the Khun plot (Fig. 4). These constants correspond to the two reactions:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \overset{k_{+2}}{\rightarrow} E + P$$

and

$$ES + S \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} ESS' \overset{k'_{+2}}{\rightarrow} E + P$$

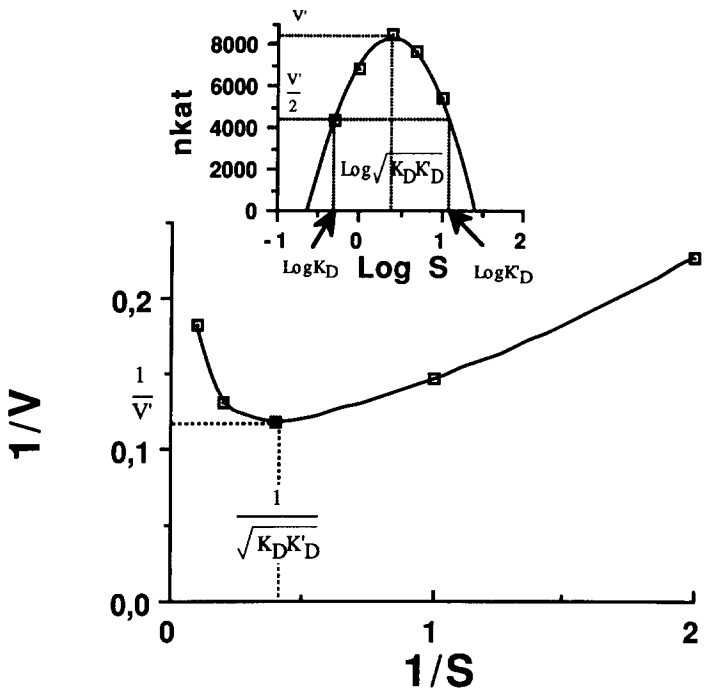

Fig. 4. Lineweaver–Burk plot of galactanase kinetics. Insert: Khun plot of galactanase kinetics.

where E is the enzyme, S and S' are the substrate molecules, ES and ESS' are the enzyme–substrate complexes, and P is the product of the reactions.

These two dissociation constants are:

$$K_m \sim K_D = \frac{k_{-1} + k_{+2}}{k_{+1}} \text{ and } K'_D = \frac{k'_{-1} + k'_{+2}}{k'_{+1}}$$

Good agreement was obtained for the product $K_D K'_D$ (6·3 mg ml^{-1}) and maximal velocity V (8500 nkat) obtained from the Lineweaver–Burk and Khun plots. Inhibition by excess substrate may be interpreted as arising from a dystopic binding in a site that is large enough to accommodate more than one substrate molecule but which is insufficient to carry out the proper reaction on all the molecules. Such a binding site could explain the affinity of the enzyme for the derivatized agarose used in its purification.

Endo- attack was verified by HPLC chromatography by the generation of a series of oligomers in the galactan hydrolysate. The

kinetic of hydrolysis demonstrates that during a first step of degradation galacto-tetraose (DP4) is the limit of oligosaccharide. Prolongation of hydrolysis leads to the accumulation of DP3, DP2, and galactose in the order of decreasing concentration. It is thought that DP4 is split slowly into 2 DP2 and DP3 into DP2 and galactose. On addition of more enzyme and after 70 h hydrolysis, only galactose and galactobiose were recovered.

CONCLUSION

An endo-β-1,4-D-galactanase has been purified in a few steps from an easily available source, to a commercial preparation originating from *A. niger* var. *aculeatus*. It is an acidic protein having an unusually low pHi and particular kinetics demonstrating inhibition by excess substrate.

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