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MODES OF ACTION OF β-MANNANASE ENZYMES OF DIVERSE ORIGIN ON LEGUME SEED GALACTOMANNANS

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Key Word Index—*Leucaena leucocephala*; Leguminosae; β-mannanase; affinity chromatography; SDS-polyacrylamide electrophoresis; Driselase; Cellulase.

Abstract—β-Mannanase activities in the commercial enzyme preparations Driselase and Cellulase, in culture solutions of *Bacillus subtilis* (TX1), in commercial snail gut (*Helix pomatia*) preparations and in germinated seeds of lucerne, *Leucaena leucocephala* and honey locust, have been purified by substrate affinity chromatography on glucomannan-AH-Sepharose. On isoelectric focusing, multiple protein bands were found, all of which had β-mannanase activity. Each preparation appeared as a single major band on SDS-polyacrylamide gel electrophoresis. The enzymes varied in their final specific activities, *Km* values, optimal pH, isoelectric points and pH and temperature stabilities but had similar MWs. The enzymes have different abilities to hydrolyse galactomannans which are highly substituted with galactose. The preparations Driselase and Cellulase contain β-mannanases which can attack highly substituted galactomannans at points of single unsubstituted D-mannosyl residues if the D-galactose residues in the vicinity of the bond to be hydrolysed are all on only one side of the main chain.

INTRODUCTION

β-D-Mannanase (EC 3.2.1.78) enzymes are endohydrolyases cleaving randomly within the D-mannan backbone [1]. Hydrolysis of D-galacto-D-mannans is limited by the degrees of D-galactose substitution on the D-mannan backbone [2–4]. In general, D-galacto-D-mannans highly substituted with D-galactose are resistant to β-D-mannanase attack. Two D-galacto-D-mannans which are hydrolysed by β-D-mannanase to greater extents than would be expected based on their D-galactose contents are those from soybean [2, 5] and *Leucaena leucocephala* seeds [5]. It has been proposed that this is due to the disposition of D-galactosyl residues along the D-mannan backbones of these D-galacto-D-mannans rather than to the presence of branches containing more than one D-galactosyl residue [5].

β-D-Mannanase enzymes with apparently different modes of action on D-galacto-D-mannans and D-manno-oligosaccharides have been reported. A highly purified β-D-mannanase enzyme from *Bacillus subtilis* [2] was shown to hydrolyse coffee bean D-galacto-D-mannan to mannobiose, mannotriose and mannotetraose plus two mixed oligosaccharides containing both D-galactose and D-mannose, i.e. a tetrasaccharide with a D-mannose to D-galactose ratio of 3:1 ($GalMan_3$), and a pentasaccharide with a D-mannose to D-galactose ratio of 4:1 ($GalMan_4$). Traces of a trisaccharide with a D-mannose to D-galactose ratio of 2:1 ($GalMan_2$) were found in soybean and guar D-galacto-D-mannan hydrolysates. These results were consistent with those previously obtained by Courtois and Le Dizet [6, 7] using a partially purified preparation of β-D-mannanase (devoid of α-galactosidase) from *Bacillus subtilis*. The release of $GalMan_2$ as a reaction product suggests that this β-D-mannanase has some ability to cleave β-D-mannosyl bonds directly adjacent to D-mannosyl units containing a D-galactose branch. A β-D-mannanase enzyme from *T. spiralis* [8] (devoid of α-galactosidase and β-mannosidase) did not release the trisaccharide $GalMan_2$ on hydrolysis of carob galactomannan, but significant quantities of the tetrasaccharide $GalMan_3$, as well as large quantities of Man_2 and Man_3, were produced.

Like the β-mannanase enzyme from *Bacillus subtilis*, β-mannanases from *Streptomyces* sp. [9], germinated fenugreek [10] and lucerne seeds [11] can only degrade β-D-manno-oligosaccharides which have a degree of polymerization (DP) greater than 3. However, enzymes from *Aspergillus niger* [12, 13] and *Basidiomycetes* sp. [5] can also hydrolyse mannotriose. *Rhizopus niveus* [14] β-D-mannanase is unable to degrade D-manno-oligosaccharides of DP less than or equal to 4.

Recent results of the author and co-workers [5, 15] have indicated that β-D-mannanase enzymes from different sources, e.g. from honey locust seeds [15] and from Driselase preparations [5], have different abilities to act at points of single unsubstituted D-mannosyl residues in the D-galacto-D-mannan backbone. It has been concluded that Driselase β-D-mannanase can readily act at points of single unsubstituted D-mannosyl residues when the D-galactoses in the vicinity of the bond to be hydrolysed (i.e. 2–3 D-mannosyl residues on either side) are positioned on only one side of the D-mannan backbone. Honey locust seed enzyme, in contrast, has only a very limited ability to hydrolyse at such points. Both enzymes can readily act at sites of two or more contiguous unsubstituted D-mannosyl residues.

Since such a comparison of the modes of action of β-D-mannanase enzymes on D-galacto-D-mannans has not previously been reported in any detail, the aim of the current work was to examine the modes of action of a range of β-D-mannanase enzymes on these polysaccharides.

RESULTS AND DISCUSSION

Purification and properties of β-mannanases

The final purification steps in the preparation of each of the β-mannanases was substrate affinity chromatography on glucomannan-AH-Sepharose [16]. The degree of retardation of different β-mannanases on this column material varied. Thus on addition of 2 μkat of β-mannanase to an affinity column 1.5 × 15 cm, the β-mannanase from Cellulase preparation was only slightly retarded, and to obtain a relatively pure preparation the enzyme had to be re-chromatographed at least 3 times. Driselase β-mannanase eluted just behind the initial protein peak and lucerne seed β-mannanase was highly retarded. Snail gut and *Bacillus subtilis* β-mannanases, when added at a level of 2 μkat, bound very firmly and could only be eluted by the addition of substrate. If much higher levels of the last two β-mannanases were applied to the column (i.e. *ca* 40 μkat) the enzyme eluted in the absence of substrate. Columns have been re-used up to 10 times with only slight changes in the elution patterns. All β-mannanase enzymes recovered from the affinity column, except that from Cellulase preparation, were completely devoid of α-galactosidase, β-mannosidase, β-glucosidase and cellulase activities. The β-mannanase from Cellulase preparation still contained a minute trace of α-galactosidase contamination after purification by substrate affinity chromatography. This was due to the low degree of retardation of this β-mannanase enzyme on the affinity column.

Some properties of the β-mannanases are shown in Table 1. The enzymes varied significantly in their final specific activities, *Km* values, optimal pH for activity and isoelectric points. The enzymes from Cellulase preparation and from *Bacillus subtilis* had very similar physicochemical properties to similar enzymes previously purified and characterized by Eriksson and Winell [12] and Emi *et al.* [2].

On isoelectric focusing, Cellulase preparation β-mannanase appeared as a single protein band (p*I* 4.0). Driselase preparation β-mannanase showed two major protein bands (p*I*'s 5.0 and 5.5) whereas *Bacillus subtilis* β-mannanase was a single major protein band (p*I* 5.1) with two minor bands (p*I*'s 5.0 and 5.6). Snail gut preparation β-mannanase consists of a series of proteins with three major bands (p*I*'s 7.7, 7.4 and 7.0) and numerous minor bands (p*I*'s 7.2–5.0). However, as far as could be determined, all protein bands had β-mannanase activity. On SDS-polyacrylamide gel disc electrophoresis, a single major protein band was detected in each preparation except the Boehringer snail gut preparation which had a minor protein band (MW = 40000) as well as the major band (MW = 37000). The major protein bands on isoelectric focusing of snail gut preparation (after affinity chromatography) have been separated and their comparative modes of β-mannanase action studied. With each of the above β-mannanases the final specific activity on carob galactomannan was *ca* twice that obtained on *Livistona australis* mannan (soluble).

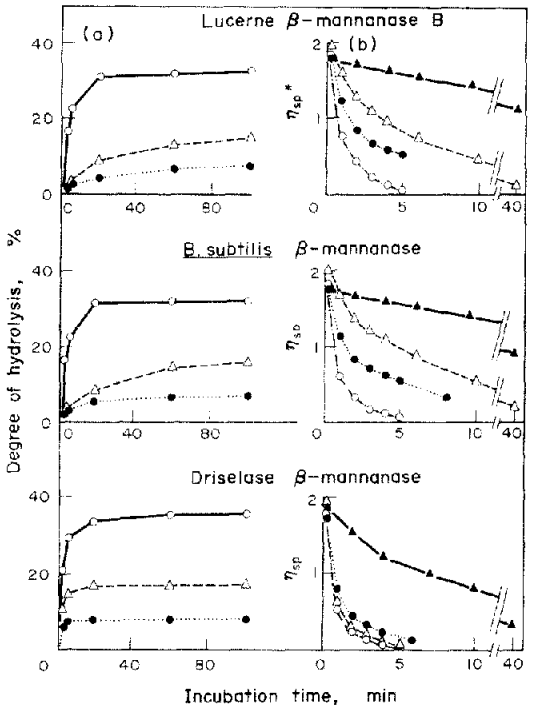

Fig. 1. Increases in reducing sugar levels and decreases in viscosities on hydrolysis of galactomannans by lucerne seed, *B. subtilis* and Driselase β-mannanases. Rate of increase in reducing sugar level (a) was determined by incubating β-mannanase (0.1 ml, 1 nkat on carob galactomannan) with galactomannan solutions (0.2 ml, 0.1 %). Viscometric assays (b) were performed by incubating β-mannanase (0.1 ml, 0.5 nkat on carob galactomannan) with galactomannan solutions (15 ml, 0.1 %) in an Ubbelohde suspended level viscometer. Galactomannans hydrolysed were from carob (○); guar (●); *Leucaena leucocephala* (△); and, lucerne seeds (▲). Specific viscosity (*).

Table 1. Properties of β-mannanese enzymes

Source of β-mannanase	MW*	p*I*†	Km‡ % w/v	V_{max}‡ nkat/mg	pH optima	Stability pH§	Stability Temp.‖
Lucerne seed (B)	41000	4.5	0.09	2160	4.5	4–8	< 50°
Driselase (*Basidiomycetes* sp.)	53000	5.0, 5.5	0.03	987	3.0	3–8	< 60°
Cellulase (*Aspergillus niger*)	45000	4.0	0.01	500	3.0	3–8	< 70°
Snail gut	37000	7.7, 7.0	0.03	733	4.5–5.5	5–8	< 50°
Bacillus subtilis (TX1)	37000	5.1	0.11	8600	5.0–6.0	5–8	< 50°

* Determined using SDS-polyacrylamide gel disc electrophoresis.
† Major protein bands only.
‡ With carob galactomannan as substrate.
§ On storage at 40° for 1 hr.
‖ On storage at pH 5 for 15 min.

Modes of action

The modes of action of β-mannanases on galactomannans were studied by a number of techniques. Initially, the rates of release of reducing sugars and rates of decrease in viscosities of galactomannan solutions by each of the β-mannanase enzymes, were compared. In Fig. 1. results obtained with β-mannanases from lucerne seed, *Bacillus subtilis* and Driselase preparation are shown. β-Mannanases from honey locust seeds and from snail gut preparation gave similar results to lucerne seed and *Bacillus subtilis* β-mannanases. Cellulase β-mannanase gave results similar to those obtained using the Driselase enzyme.

All β-mannanases gave a rapid decrease in viscosity of carob galactomannan solutions. This galactomannan has a low galactose to mannose ratio of 23:77 [5]. Driselase β-mannanase had a greater ability to hydrolyse highly substituted galactomannans than did β-mannanases from lucerne seed, honey locust seed, snail gut preparation or *Bacillus subtilis* preparation. It decreased the viscosity of guar (Gal to Man = 38:62) [5] and *Leucaena leucocephala* galactomannan (Gal to Man = 38:62) [5] solutions at a similar rate to that for solutions of carob galactomannan, whereas the other β-mannanases hydrolysed guar and *Leucaena leucocephala* galactomannans at a much lesser rate than carob galactomannan. Driselase enzyme also gave a more rapid decrease in viscosity of lucerne galactomannan (Gal to Man = 47:53) [5] than did the other β-mannanases. The relative rates of decrease in viscosity of various galactomannan solutions with a given β-mannanase correlated with the initial relative rates of increase in reducing sugar levels (Fig. 1). In no instance did any enzyme give a decrease in viscosity of galactomannan solutions without a concurrent increase in the mannose reducing sugar equivalent of the solution as was reported for a 'galactomannan-depolymerase' enzyme from *Leucaena leucocephala* seeds [17]. In fact, in the current studies a single enzyme capable of *endo*-depolymerizing carob galactomannan was purified from germinated *Leucaena leucocephala* seeds and this appeared as a single protein band on isoelectric focusing (p*I* = 4.5). This enzyme was shown to be a typical β-mannanase with an action pattern almost identical to that of honey locust seed β-mannanase [15]. Cellulase β-mannanase had a similar action pattern to Driselase β-mannanase. However, the significance of the viscometric results is masked by the presence of trace amounts of contaminating α-galactosidase activity in this enzyme preparation (Fig. 3).

Table 2. Relative initial rates* of hydrolysis of *Leucaena leucocephala* and carob galactomannans by β-mannanase enzymes

Source of enzyme	Ratio of initial rates of of hydrolysis of *Leucaena leucocephala*/carob galactomannans
Honey locust seed (B)	11
Leucaena leucocephala seeds	13
Bacillus subtilis (TX1)	15
Lucerne seed (B)	22
Snail gut whole preparation	60
p*I* 7.7 fraction	37
p*I* 7.4 fraction	50
p*I* 7.0 fraction	45
p*I* 6.5 fraction	100
p*I* 5.6 fraction	100
Driselase (*Basidiomycetes* sp.)	82
Cellulase (*Aspergillus niger*)	100

* Determined by incubating dilute β-mannanase enzyme preparation with galactomannan substrate (1 ml, 0.2%) in 0.1 M acetate buffer (pH 5). The reaction was terminated after 0, 2, 5 and 10 min intervals when less than 2% of the galactomannan had been hydrolysed.

This difference in the modes of action of β-mannanases on galactomannans highly substituted with galactose can most readily be demonstrated by comparing the initial rate of hydrolysis of a galactomannan highly substituted with galactose (i.e. *Leucaena leucocephala* galactomannan; Gal to Man = 38:62) to that for a galactomannan substituted to a low degree with galactose (i.e. carob galactomannan; Gal to Man = 23:77). *Leucaena leucocephala* galactomannan has large sections of the chain consisting of the repeating unit —(Man(Gal)-Man)$_n$— with insignificant proportions of the chain composed of adjacent unsubstituted D-mannosyl residues [5]. Thus, by comparing the initial rates of hydrolysis of this and of carob galactomannan, some information can be obtained on the relative ability of the enzyme to hydrolyse at points of single unsubstituted D-mannosyl residues (in chain sections where all D-galactosyl branch points are on only one side of the main chain) compared to its ability to hydrolyse at relatively unsubstituted sections of the chain. It is apparent from results in Table 2 that β-mannanases from Cellulase and Driselase preparations can readily act at some points of single unsubstituted D-mannosyl residues, whereas honey locust β-mannanase cannot. Snail gut preparation contains some β-mannanases which depolymerize galactomannan polysaccharides in a similar fashion to Driselase β-mannanase (Table 2). However, other β-mannanases from this preparation act more like the *Bacillus subtilis* enzyme preparation.

Further information on the comparative modes of action of the β-mannanase enzymes has been obtained by studying the oligosaccharides produced on extended incubation with carob galactomannan. The patterns of amounts of oligosaccharides produced on extended incubation of carob galactomannan with honey locust and *B. subtilis* β-mannanases (Fig. 2), and Driselase and Cellulase preparations β-mannanases (Fig. 3), show there are differences in the modes of action of these enzymes. The patterns of amounts of the oligosaccharides formed on incubation of lucerne seed or whole snail gut preparation β-mannanase with carob galactomannan were almost identical to those formed with *B. subtilis* β-mannanase.

The fungal β-mannanases (Driselase and Cellulase) can readily attack highly substituted galactomannans at points of single unsubstituted D-mannosyl residues if the D-galactosyl residues in the vicinity of the bond to be hydrolysed are all on only one side of the main chain. This is reflected in the rapid release of the trisaccharide $GalMan_2$ on hydrolysis of carob and *Leucaena leucocephala* [5] galactomannans, and suggests the ability of these enzymes to hydrolyse D-mannosyl linkages directly adjacent to D-galactose branch points. This trisaccharide is also formed on the hydrolysis of the pentasaccharide $GalMan_4$ (mannose to galactose = 4:1). The β-mannanase from Cellulase preparation rapidly hydrolyses $GalMan_4$, as shown by the lack of accumulation of this

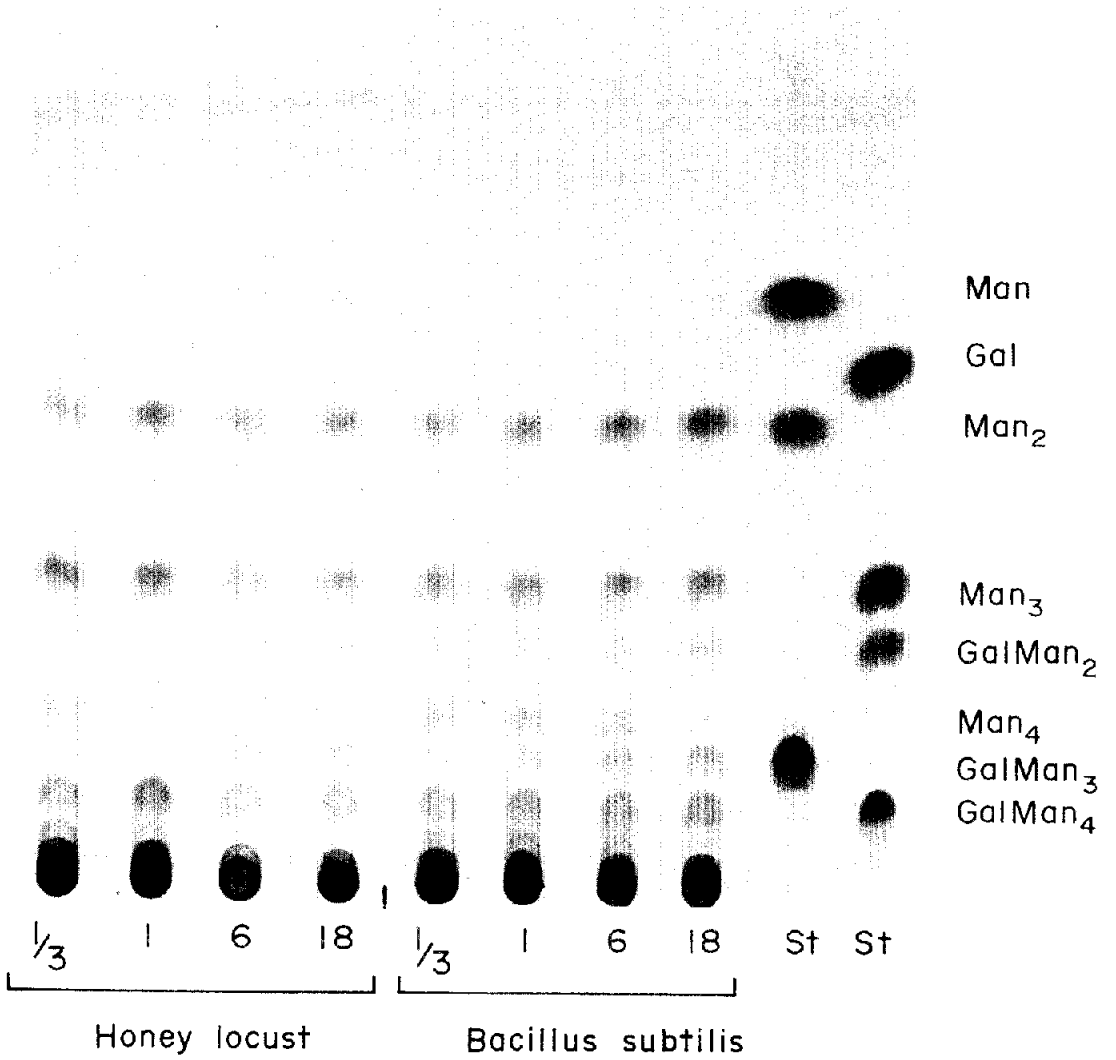

Fig. 2. TLC of the hydrolysis products of carob galactomannan by honey locust seed and *Bacillus subtilis* β-mannanases. Galactomannan (20 ml, 0.5% w/v, unbuffered) was incubated with β-mannanase (0.8 μkat on carob galactomannan) and aliquots removed at 1/3–18 hr for chromatography.

oligosaccharide at any stage during the hydrolysis of carob galactomannan (Fig. 3). Driselase β-mannanase can also rapidly hydrolyse this pentasaccharide, but not as readily as Cellulase β-mannanase (Fig. 3). Lucerne seed, *B. subtilis* and snail gut β-mannanases are less effective in producing $GalMan_2$, suggesting a lesser ability to act as points of single unsubstituted D-mannosyl residues in the galactomannan polysaccharide and also a lesser ability to hydrolyse $GalMan_4$ to $GalMan_2$ plus Man_2. Honey locust seed β-mannanase has negligible ability to release $GalMan_2$ from carob galactomannan. Snail gut β-mannanase p*I* 6.5 acts somewhat like Driselase β-mananase, whereas the p*I* 7.0 and 7.7 fractions act more like honey locust seed β-mannanase. All the β-mannanase enzymes can readily act at points of two or more contiguous unsubstituted mannosyl residues.

These β-mannanases also vary in their ability to hydrolyse mannotetraose. This oligosaccharide is most rapidly hydrolysed by Cellulase β-mannanase; Driselase and honey locust seed β-mannanases also hydrolyse it quite rapidly; whereas lucerne seed, snail gut and *B. subtilis* enzymes act quite slowly.

Although the fungal β-mannanases could readily hydrolyse at some points of single unsubstituted D-mannosyl residues in carob galactomannan, none of the β-mannanases could act at all single unsubstituted D-mannoses as shown by the persistence in hydrolysates of a high $\overline{DP}$ ethanol precipitated fraction containing less than 50% galactose. Independent of the β-mannanase enzyme employed, the galactose to mannose ratio of the high $\overline{DP}$ fraction remaining after incubation of carob galactomannan with a large excess of the enzyme was always *ca* 37:63 (cf. refs. [5] and [6]. A product of similar Gal to Man ratio was obtained by Reese and Shibata [8] on incubation of guar galactomannan with *T. spiralis* β-mannanase. Based on this observation and the fact that the major oligosaccharides released on hydrolysis of carob galactomannan by this enzyme were Man_2, Man_3 and $GalMan_3$ (no $GalMan_2$), these authors

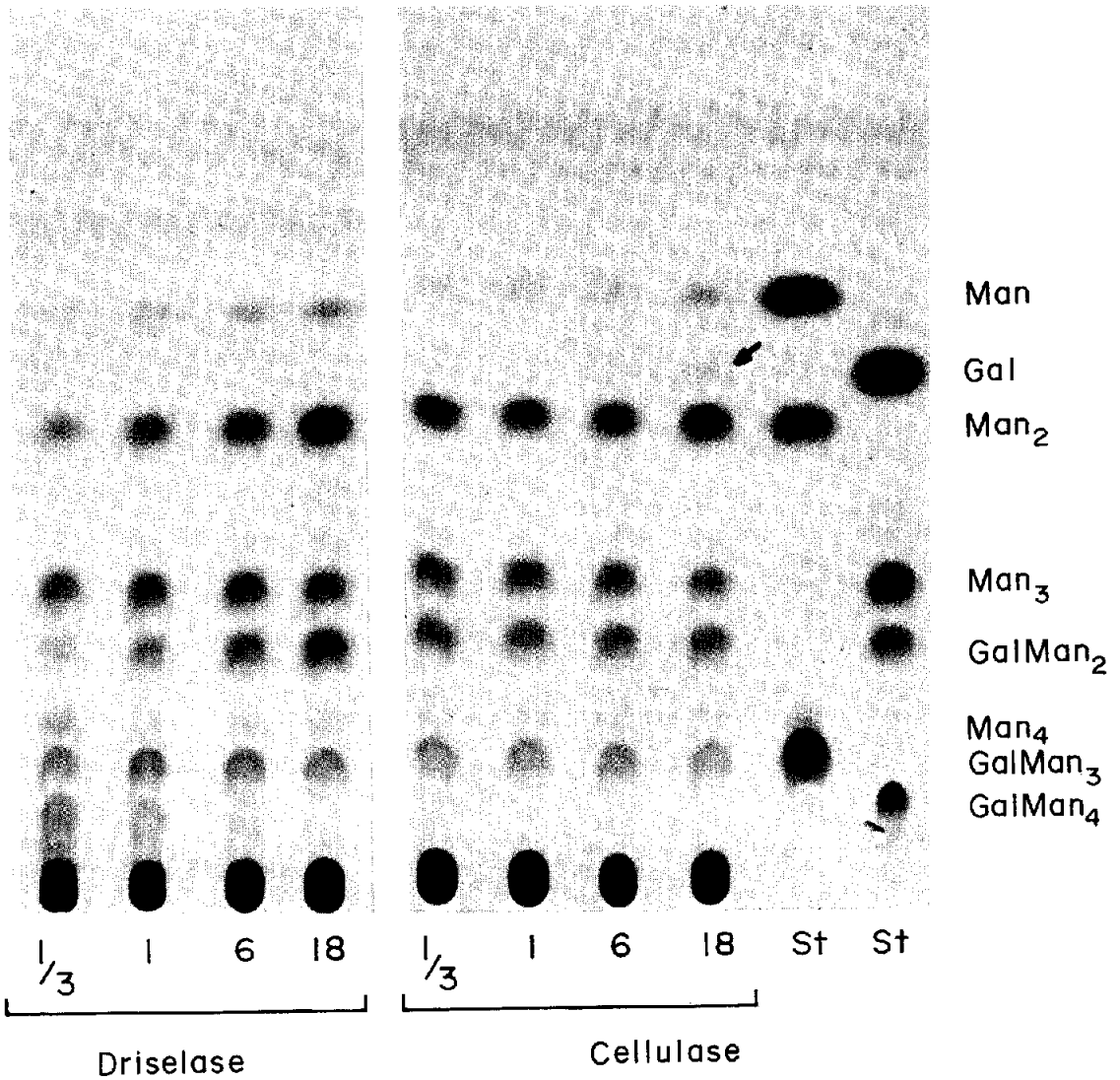

Fig. 3. TLC of the hydrolysis products of carob galactomannan by 'Driselase' and 'Cellulase' β-mannanases. Incubation conditions as in Fig. 2.

concluded that *T. spiralis* β-mannanase is unable to act at points of single unsubstituted D-mannosyl residues in the galactomannan chain. Consequently this enzyme has an action pattern similar to that now proposed for *B. subtilis* and honey locust seed β-mannanases.

Recently, a new substrate for the assay of β-mannanase enzymes has been introduced [18]. This substrate is carob galactomannan dyed with Remazolbrilliant Blue R (RBB) to an extent of one dye molecule per fifty anhydrohexose molecules. In this assay, enzyme units of a particular β-mannanase on carob galactomannan (0.2% w/v) are related to the rate of release of dyed fragments soluble in 66% v/v aqueous ethanol (i.e. the increase in *A* at 590 nm) on hydrolysis of RBB-carob galactomannan (1:50). In the current studies, it was found that for a given level of enzyme activity on undyed carob galactomannan the rate of release of ethanol soluble dyed fragments from RBB-carob galactomannan (1:50) by snail gut, *B. subtilis* and lucerne seed β-mannanases is *ca* twice that by β-mannanases from Cellulase or Driselase preparations. This phenomenon cannot be explained on the basis of the relative abilities of the enzymes to attack polysaccharides, as the opposite result might have been expected, i.e. the fungal enzymes have the greater ability to depolymerize highly branched galactomannans (Fig. 1). A possible explanation is that the lower $\overline{DP}$ manno- and galacto-manno-oligosaccharides, released during initial hydrolysis, act as good substrates for Driselase and Cellulase β-mannanases, but not for the other β-mannanases (Figs. 2 and 3). Hydrolysis of these oligosaccharides would not increase the amount of dyed carbohydrate soluble in 66% v/v aqueous ethanol as these oligosaccharides are already soluble before further hydrolysis. In support of this proposal, it has been found that on addition of a mixture of manno-oligosaccharides ($\overline{DP}$ 3 to 5, 0.5% w/v final concentration) there is a marked decrease (*ca* 50%) in the rate of release of dyed fragments on hydrolysis of RBB-carob galactomannan by Driselase and Cellulase β-mannanases under standard assay conditions. The

release of dyed fragments by snail gut, *B. subtilis* and lucerne seed β-mannanases was decreased by a much lesser degree (*ca* 10–15%).

EXPERIMENTAL

Assay of β-D-mannanase. β-Mannanase was assayed by (a) measuring the decrease in viscosity of galactomannan soln (0.1% w/v) using an Ubbelohde suspended level viscometer [4], (b) measuring the increase in mannose reducing sugar equivalents employing the *p*-hydroxybenzoic acid hydrazide (PAHBAH) method of ref. [19] essentially as previously described [18], and (c) measuring the increase in *A* of dyed material soluble in 66% aq. EtOH on hydrolysis of RBB-carob galactomannan (1:50) [18]. The latter technique was used to assay column eluates during enzyme purification. One unit (kat) is defined as the amount of enzyme that releases 1 mol of mannose reducing sugar equivalent/sec at 40° and pH 5 with carob galactomannan as substrate.

Isoelectric focusing. This was performed using either commercial LKB, PAG(R) plates or slab gels prepared using ampholines made as previously mentioned [5]. Gels were prepared by mixing 3.5 ml of a 0.4% v/v aq. soln of *N,N,N'N'*-tetramethylethylendiamine, 10 ml of an aq. soln of acrylamide (30% w/v) plus *bis*acrylamide (0.8% w/v), 2 ml of ampholine soln (40% w/v), 14.5 ml H_2O and 24 mg ammonium persulphate. Gels were poured immediately and they polymerized within 30 min.

Purification of β-D-mannanase. Lucerne seed β-mannanase B[16] and Driselase β-mannanase were purified as previously described. A procedure similar to that used for the purification of lucerne seed β-mannanase B was employed to purify honey locust and *Leucaena leucocephala* β-mannanases. Cellulase (Sigma C7052 from *Aspergillus niger*) β-mannanase (6 μkat/g crude powder) was prepared essentially according to the procedure employed for Driselase β-mannanase [5]. Crude snail gut (*Helix pomatia*) prepn was obtained from both Sigma Chemical Co. (β-Glucuronidase Type H-2, Crude soln G0876) and from Boehringer Mannheim (β-Glucuronidase/arylsulphatase; cat. No. 127698). Each prepn had *ca* 3.5 μkat of β-mannanase activity per ml of crude soln. The crude soln (10 ml, 35 μkat on carob galactomannan) was applied directly to a glucomannan-AH-Sepharose affinity column (1.5 × 15 cm) [16] and eluted with a soln of M KCl in 0.1 M acetate buffer (pH 5). β-Mannanase enzyme eluted without the addition of substrate, well after all the other protein had passed through the column. A pure prepn of β-mannanase was obtained within 2 hr. The overall recovery was essentially 100% and the purification was 15-fold. The major protein bands on isoelectric focusing were separated by slicing a preparative gel. The gel was crushed in a glass Tenn–Broeck homogenizer and extracted with H_2O. The pH and β-mannanase activity of each fraction were measured. Aliquots of each extract were dialysed, lyophilized and isoelectric focused to check purity.

Bacillus subtilis was induced to produce β-mannanase essentially according to the procedure of ref. [8]. The bacteria were cultured for 5 days at room temp. with carob galactomannan (0.5% w/v) as the carbon source. The culture soln (6 l) containing *ca* 8 nkat of β-mannanase activity per ml was centrifuged (3000 *g*, 30 min) to remove insoluble material and the supernatant dialysed 18 hr against flowing tap H_2O. The soln was then adjusted to pH 8 by addition of Tris buffer to a conc of 20 mM and passed through a bed of pre-equilibrated DEAE-cellulose (10.5 × 7 cm) in a sintered glass funnel. The enzyme was recovered by washing with 0.2 M KCl (800 ml) and concd by rotary evapn (below 40°) to a vol. of 100 ml. This soln was further concd (to 10 ml) by dialysis against polyethylene glycol 4000 and then dialysed against 1 M KCl in 0.1 M acetate buffer (pH 5). The enzyme prepn (10 ml, 33 μkat) was applied to a glucomannan-AH-Sepharose affinity column (2.0 × 20 cm) which was washed with 1 M KCl in 0.1 M acetate buffer (pH 5). The enzyme eluted without the addition of soluble substrate, well behind the initial protein peak. Recovery at each step was greater than 80%.

Substrate affinity chromatography. Glucomannan-AH-Sepharose, prepared as previously described [16] was used. Glucomannan was extracted from seeds of *Dracaena draco* in a 40% yield.

Products of hydrolysis of galactomannans by β-mannanase. Identical incubation conditions were used with each β-mannanase [5]. Carob galactomannan (20 ml, 0.5% w/v, unbuffered) was incubated with β-mannanase (0.8 μkat on carob galactomannan; 0.4 μkat on soluble mannan). Aliquots (4 ml) were removed at intervals of 20 min, 1, 6 and 18 hr, heated to denature the β-mannanase activity, lyophilized and readjusted to 2% w/v carbohydrate. Aliquots (20 μl) were applied to Merck DC-Alufolien Kieselgel 60 (0.2 mm) prepared plates which were developed twice with *n*-PrOH–EtOH–H_2O (7:1:2). Spots were visualised by spraying with 5% H_2SO_4 in EtOH and heating to 110° for *ca* 10 min. Sugars identified were mannose (Man); galactose (Gal); mannobiose (Man_2); mannotriose (Man_3); a trisaccharide with a galactose to mannose ratio of 1:2 ($GalMan_2$); a tetrasaccharide with a galactose to mannose ratio of 1:3 ($GalMan_3$); and a pentasaccharide with a galactose to mannose ratio of 1:4 ($GalMan_4$).

Properties of β-mannanase enzymes. Kinetic parameters, MWs (SDS-polyacrylamide gel disc electrophoresis [20]) and isoelectric points were determined as previously described [4, 5, 15].

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