Purification and Properties of β -Mannanases I and II from the Germinated Seeds of *Trifolium repens*

MODE OF GALACTOMANNAN DEGRADATION *IN VITRO*

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Two β -mannanases (β -mannosidases, EC 3.2.1.25) purified from the germinated seeds of *Trifolium repens* by a procedure that included chromatography on hydroxyapatite, gel filtration on acrylamide/agarose (Ultrigel 5/4) and preparative polyacrylamide-gel electrophoresis. The final purification step completely resolved two β -mannanases with distinct specificities, which were termed β -mannanase I and β -mannanase II. β -Mannanase I was purified 1400-fold and β -mannanase II 1000-fold. The purified enzymes showed a single protein band when examined by polyacrylamide-gel disc electrophoresis. β -Mannanase I, apparent mol.wt. 43 000, accounted for 49% of the total activity recovered from the final step of purification. β -Mannanase II, apparent mol.wt. 38 000, accounted for the remaining 51% of activity. Molecular-weight determinations were by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and by the electrophoretic method of Hendrick & Smith [(1968) *Arch. Biochem. Biophys.* **126**, 155–164]. The substrate specificities of both enzymes were examined with the galactomannans of *T. repens* and of *Medicago sativa*, as well as with manno-oligosaccharides. The pH optimum was between pH 5.1 and 5.6 for both enzymes.

β -Mannanases (EC 3.2.1.25, β -mannosidase, β -D-mannoside mannohydrolase) have been found to be widely distributed in Nature (Dekker & Richards, 1976). Relatively few β -mannanases have been isolated from tissues of plant origin: from plant seeds (McCleary & Matheson, 1975), from the tubers of *Amorphophallus konjac* (Shimahara *et al.*, 1975), from the germinating seeds of *Medicago sativa* (Villarroya & Petek, 1976) and *Lactuca sativa* (Halmer *et al.*, 1976). β -Mannanases of microbial origin have been reported to be both inductive (Lyr, 1963; Ritter, 1964; Reese & Shibata, 1965; Eriksson & Winell, 1968) and constitutive enzymes (Innami, 1961; Hashimoto, 1970; Bailey & Gaillard 1969), usually being secreted extracellularly into the medium in which the micro-organism is cultured. Exceptions are the enzymes produced by *Sporocytophaga myxococcoides* (Eriksson & Winell, 1968) and *Aerobacter mannolyticus* (Inone & Inone, 1958), which were found to be intracellular.

The highly purified enzyme preparations from *Bacillus subtilis* (Emi *et al.*, 1972) and *Aspergillus*

niger (Tsujisaka *et al.*, 1972) have been shown to be capable of the hydrolysis of the D-gluc-D-mannans of *A. konjac* and *Orchis intacta*. In germinating seeds, such as those of *Trifolium pratense* and *M. sativa*, the cells of the aleurone layer (Reid & Meier, 1972) have been implicated in the synthesis *in vivo* and secretion into the endosperm of the extracellular β -mannanase (and α -galactosidases) responsible for the metabolism of the galactomannan storage reserve. The present paper describes two forms of β -mannanase, I and II, isolated and purified from the germinating seeds of *T. repens*.

Experimental and Results

Materials

The seeds of *Trifolium repens* (white clover, variety Trefle nain blanc mira) were obtained from Vilmorin, Paris, France. Acrylamide/agarose was of the type Ultrigel 5/4 from L.K.B., Bromma, Sweden. The sources of other materials have been given previously (Williams *et al.*, 1977).

Preparation of polysaccharides

The galactomannan of *Trifolium repens* T₀ was

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extracted from dormant seeds and purified as described in the preceding paper (Williams *et al.*, 1978). This galactomannan, which has an initial galactose/mannose ratio of 1:1.1, was used to produce, by hydrolytic attack with α -galactosidase II (Williams *et al.*, 1978), galactomannans of much smaller galactose contents. The assay medium for these preparations consisted of 7.5ml of galactomannan *T. Repens* T₀ (1.6%), 2.5ml of McIlvaine (1921) buffer, pH4.2, and 2ml of α -galactosidase from *T. repens*. Incubation was at 37°C and the liberation of galactose followed by the Nelson-Somogyi technique (Nelson, 1944; Somogyi, 1945). Portions (100 μ l) were withdrawn from the incubation mixture and dosed by the standard technique (see under 'Assay of enzyme activity'). Four galactomannans of lower galactose content were produced in this way, from which 40, 62, 76 and 77% of the galactopyranosyl residues had been removed. These galactomannans were termed T₁, T₂, T₃ and T₄ respectively.

The glucomannan of *O. intacta* was extracted and purified by the procedure described in the preceding paper for the galactomannan of *T. repens* (Williams *et al.*, 1978). This glucomannan is a linear molecule composed of D-glucose and D-mannose in the proportions 1:3.

Preparation of manno-oligosaccharides from the mannan of Phoenix canariensis

The mannan of *Phoenix canariensis* (LeDizet, 1962) was subjected to partial hydrolysis with 50mM-H₂SO₄ during 4h at 100°C. After filtration, the filtrate was neutralized by a sufficient quantity of CaCO₃ and the precipitate so formed, as well as excess CaCO₃, removed by filtration. The clear solution obtained was freeze-dried and the resulting powder dissolved in 2ml of water, which was applied to a column (130cm $\times$ 2cm) of Sephadex G-15. This column was eluted with water (3ml/h) and permitted the separation of pure mannose, mannobiose (Manp β 1 $\rightarrow$ 4Man) and mannatriose (Manp β 1 $\rightarrow$ 4Manp β 1 $\rightarrow$ 4Man), as well as mannopentaose, -hexaose and -hexaose containing slight impurities. These last three oligosaccharides were subsequently purified by chromatography on Whatman no. 3 paper. The solvent was butan-1-ol/pyridine/water (9:5:4, by vol.). Mannosides were located by treatment with β -naphthylamine (Petek, 1962) and recovered by elution with cold water. The solutions were left to dry in a desiccator in the presence of CaCl₂ and conc. H₂SO₄.

Assay of enzyme activity

The standard assay medium consisted of 50 μ l of glucomannan of *O. intacta* (1%, w/v), 25 μ l of McIlvaine buffer, pH5.4 (0.03M), and 25 μ l of enzyme. Incubation was for 10min at 37°C with

either the glucomannan of *O. intacta* or the galactomannan of *T. repens* (T₀, T₁, T₂, T₃, T₄) as substrate. Hydrolysis of these substrates was estimated by the Nelson-Somogyi technique (Nelson, 1944; Somogyi, 1945). The original method was used when the oligosaccharides liberated exceeded the equivalent of 50 μ g. Under conditions where the quantity of mannose-equivalent liberated was between 10 and 50 μ g a micro-version of this technique was used. The enzymic reaction was stopped by the addition of 0.5ml of solution A (Somogyi, 1945) diluted to 50% (v/v). After incubation for 20min at 100°C the tubes were allowed to cool and 0.25ml of solution B (Nelson, 1944) was added, followed by the addition of 3ml of water. After 10min the stabilized blue coloration was read spectrophotometrically at 650nm against suitably prepared blanks. Specific activity is expressed as μ mol of mannose equivalent liberated/min per mg of protein at 37°C, with the glucomannan of *O. intacta* as substrate. Protein was determined by the method of Zamenhof and Chargaff (Zamenhof, 1957) with bovine serum albumin as the protein standard.

Separation and identification of oligosaccharides produced by hydrolysis of the galactomannan (T₀, T₁, T₂, T₃, T₄) of T. repens by β -mannanases I and II

The hydrolysis products were examined by t.l.c. on silica-gel-coated plastic sheets by comparison with marker spots of authentic samples of manno-oligosaccharides. The solvent system was propan-1-ol/ethanol/ethylacetate/pyridine/water (7:4:3:2:4, by vol.). Samples (2 μ l) were removed after 1, 2, 4, 6 and 24h of incubation at 37°C.

To identify these oligosaccharides further they were purified by paper chromatography (Whatman no. 3) with the solvent butan-1-ol/pyridine/water (9:5:4, by vol.). Migration was for 48h at 20°C. The oligosaccharides were located with β -naphthylamine (Petek, 1962) and subsequently eluted with cold water. Each oligosaccharide was subjected to hydrolysis with 0.5M-H₂SO₄ at 100°C for 2h, the solution neutralized with CaCO₃ and the precipitate removed by filtration. The monosaccharides so obtained were separated by paper chromatography (Whatman no. 3) with the solvent system described above, after 24h migration at 20°C. The monosaccharides (mannose and galactose) were eluted with cold water and their relative proportions estimated by the micro-method of the Nelson-Somogyi technique (see under 'Assay of enzyme activity').

Hydrolysis of manno-oligosaccharides

The activity of β -mannanases I and II was assayed by incubating (37°C) 25 μ l of enzyme with 50 μ l of substrate (manno-biose, -triose, -tetraose,

-pentose, -hexose) and 25 μ l of McIlvaine buffer (0.03M), pH 5.4. Hydrolysis products were examined by t.l.c. on silica-gel-coated plastic sheets, with the solvent propan-1-ol/ethanol/ethyl acetate/pyridine/water (7:4:3:2:4, by vol.). Chromatograms were subsequently developed with a solution of β -naphthylamine (Petek, 1962) followed by treatment with 2% (v/v) H_2SO_4 in acetone. Products were identified by comparison with marker spots of authentic samples.

Hydrolysis products previously identified by t.l.c. were separated by paper chromatography (Whatman no. 3) with the solvent butan-1-ol/pyridine/water (9:5:4, by vol.). Each product was located with β -naphthylamine (Petek, 1962) and subsequently eluted with cold water. The ratio of products from a given manno-oligosaccharide was measured by the micro-method of the Nelson-Somogyi technique (see under 'Assay of enzyme activity'). On the basis of this measurement it was possible to calculate the percentage of total mannose (as products) that each product represented.

Gel electrophoresis

Electrophoresis was carried out on 7.5% (w/v) polyacrylamide disc gels by the technique described by Maizel (1964). Experiments were carried out at 4°C with a current of 6 mA/gel. The gels were fixed and stained with Coomassie Brilliant Blue (0.25%, w/v) in methanol/acetic acid/water (5:1:4, by vol.) for 2h, and subsequently bleached at 40°C in a solution containing 25% (v/v) methanol and 7% (v/v) acetic acid in water.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Molecular-weight determinations were carried out on 7.5% (w/v) polyacrylamide disc gels by the procedure of Weber & Osborn (1969).

Reference proteins used (with molecular weights in parentheses) were: phosphorylase *a* (94000); serum albumin monomer (68000); egg albumin (43000); pepsin (35000); trypsin (23000). Electrophoresis was carried out overnight (18h) at room temperature (20°C) with a current of 3 mA/gel (5mm diam., 110mm long). The gels were stained with Coomassie Brilliant Blue for 5h and bleached as described above.

Purification

Dry seeds of *T. repens* (200g) were soaked in water for 16h and subsequently germinated in the dark at 20–22°C for 48h. The crude extract was prepared by maceration (at 4°C) of the seeds with 3% (w/v) NaCl solution in a Waring commercial blender. All subsequent procedures were carried out at 4°C. The extract was centrifuged for 30min at

13000g and the resulting supernatant dialysed overnight against 0.2M-citrate/sodium phosphate buffer, pH 3.5. The precipitate so formed was removed by centrifugation (30min at 13000g) and $(NH_4)_2SO_4$ added to the clear solution to 80% saturation. The precipitate was collected by centrifugation (30min at 13000g), then dissolved in 80ml of water, dialysed against water (24h) and the remaining precipitate removed by centrifugation. The resulting 120–130ml of supernatant was immediately applied to a column of hydroxyapatite (column 1; see below and Table 1).

Column chromatography

Elution was by stepwise increase of buffer concentration. Column 1 (200mm $\times$ 22mm) was of hydroxyapatite equilibrated with 1mM-potassium phosphate buffer, pH 5.3. After the elimination of an inactive protein fraction with this same buffer, a second inactive peak was eluted with 5mM-potassium phosphate buffer. After washing with 5mM buffer an active fraction containing β -mannanases I and II was eluted with 50mM-potassium phosphate buffer. The average volume of this fraction was 300ml, which was dialysed for 16h before being freeze-dried. This first column permitted the complete separation of α -galactosidases (Williams *et al.*, 1977), which were retained at this pH and buffer concentration.

The freeze-dried enzyme was redissolved in 2ml of 10mM-potassium phosphate buffer, pH 6.0, and applied to a column (column 2) of acrylamide/agarose 5/4 (140cm $\times$ 2.5cm) equilibrated with the same buffer and eluted at a flow rate of 10ml/h. β -Mannanase activity was eluted from this column as a single symmetrical peak, collected in 5ml fractions, containing both β -mannanases I and II. The combined active fractions (average volume 100ml) were dialysed for 16h, freeze-dried and the resulting powder dissolved in 1.1ml of 10mM-Tris/glycine buffer, pH 8.5, to which 0.1ml of glycerol was added. This fraction was subjected to preparative polyacrylamide-gel electrophoresis (Doly & Petek, 1977).

Preparative polyacrylamide-gel electrophoresis

Polyacrylamide disc gels (15cm $\times$ 1.6cm) of 6.5% acrylamide were found to give the best resolution of separation of β -mannanases I and II. The enzyme fraction containing glycerol was layered on the gel surface; 150 μ l portions were applied to each of the eight gels. Electrophoresis was carried out at 4°C with a constant voltage of 90V for 16h. After electrophoresis the gels were removed from the glass tubes and one was stained in Coomassie Blue for 4h, to establish overall protein migration. For the remaining gels the zone containing β -mannanases I and II was sliced into 16 sections 2mm

Fig. 1. Diagram to show the different electrophoretic mobilities of β -mannanases I and II

Electrophoresis was for 16 h at 40°C with 6.5% (w/v) polyacrylamide disc gels (15 cm $\times$ 1.6 cm) with a constant voltage of 90 V. The migration is given in mm from the anode: 7.3 cm for β -mannanase I and 8.9 cm for β -mannanase II. Gels (a) and (b) are of 7.5% (w/v) polyacrylamide, and electrophoresis was carried out at 180 V and 4°C for 4 h.

thick (Fig. 1). The slices were placed in a syringe and injected into the elution chambers with electrophoresis buffer. Two elution apparatuses, each of eight chambers, were necessary for this purification step (Doly & Petek, 1977). Elution was carried out at constant voltage (300 V, 1.3 mA per chamber) for 24 h. The 16 fractions recovered after elution were dialysed against water for 24 h. Localization of the active fractions of β -mannanases I and II was by the Somogyi-Nelson method with the glucomannan of *O. intacta* as substrate (see under 'Assay of enzyme activity').

β -Mannanases I and II represented 49 and 51% respectively of the total activity recovered from this last purification step (Table 1). When examined by polyacrylamide-gel electrophoresis both enzymes I and II proved to be homogeneous protein preparations (Fig. 1). β -Mannanase I was purified approx. 1400-fold and β -mannanase II approx. 1000-fold (Table 1).

General properties

The effect of pH on the activities of enzymes I and II was determined with the glucomannan of *O. intacta* as substrate in McIlvaine buffer under standard assay conditions (Fig. 2). The pH-activity profiles for both enzymes show a maximum at pH 5.1–5.6.

Both enzymes I and II were stable to freezing (at -20°C) and thawing, and also to freeze-drying. Storage of the freeze-dried enzymes at -20°C resulted in no apparent loss of activity.

Table 1. Purification of β -mannanases I and II

The substrate used was the glucomannan of *O. intacta* (final concn. 1%, w/v) under standard assay conditions. Liberation of reducing sugars was measured by the Nelson-Somogyi technique, by the standard or the micro-method as appropriate. β -Mannanases I and II represented 49.1 and 50.9% of the total β -mannanase activity recovered after the final purification step (step 6). Subsequently, calculation of the recovery and purification of each enzyme was based on the proportion of the total activity that each enzyme represented.

Step	Volume (ml)	Total activity (μmol of man-nose equiv./min)	Total protein (mg)	Specific activity (units/mg of protein)	Recovery (%)	Purification (-fold)
(1) Crude extract	970	556.1	8555	0.065	100	
(2) After acid dialysis at pH 3.5	940	479.4	3948	0.121	86.2	1.86
(3) After 80%-satd.-(NH_4) $_2\text{SO}_4$ precipitation and dialysis	108	456.2	927.2	0.49	82.0	7.57
(4) Column 1 (hydroxyapatite)	285	264.8	58.7	4.51	47.6	69.4
(5) Column 2 (acrylamide/agarose)	95	145.1	17.6	8.26	26.1	127.1
(6) Preparative electrophoresis						
β -Mannanase I	12	27.0	0.593	45.61	4.86	1429.8
β -Mannanase II	12	27.99	0.852	32.86	5.03	992.75

Fig. 2. Effect of pH on the activities of β -mannanases I and II

McIlvaine buffer with a pH range of 2.5–8 was used. The incubation mixture contained 25 μ l of buffer, 25 μ l of enzyme and 50 μ l of glucomannan (1%). Incubation was for 10 min at 37°C and the reducing sugars liberated were measured by a micro-technique adapted from the Nelson–Somogyi method (see under 'Assay of enzyme activity'). Symbols: ●, β -mannanase I; ○, β -mannanase II.

Molecular-weight determinations

Electrophoretically pure samples of β -mannanases I and II were examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; a single protein band was obtained in each case. Molecular weights were calculated to be 43000 and 38000 for β -mannanases I and II respectively. These estimations were confirmed by the electrophoretic method of Hedrick & Smith (1968).

Inactivation of hydrolytic activity as a function of temperature and time

Samples of β -mannanases I and II (0.1 mg/ml) of approximately the same protein concentration were incubated at 37, 40, 45 and 50°C (Fig. 3). At the indicated times, 50 μ l portions were withdrawn and residual hydrolytic activity was assayed by the Somogyi–Nelson technique under standard assay conditions (see under 'Assay of enzyme activity' for details). Whereas at 37°C both enzymes were stable for up to 60 min, at 60°C inactivation was complete for β -mannanase I in 60 min, and for enzyme II it was complete in 40 min.

Energy of activation

To study the effect of temperature on the initial

Fig. 3. Inactivation of hydrolytic activity as a function of temperature and time

Samples of β -mannanases I and II of approximately the same protein concentration were incubated at 37, 40, 45 and 50°C. At the indicated times 25 μ l samples were withdrawn and residual hydrolytic activity was assayed with glucomannan as substrate (final concn. 0.5%, w/v) under standard assay conditions. Liberation of reducing sugars was measured by a micro-technique adapted from the Nelson–Somogyi method (see under 'Assay of enzyme activity'). The ordinate represents percentage residual activity. Symbols: ○, 37°C; ●, 40°C; ▲, 45°C; △, 50°C. —, β -Mannanase I; ----, β -mannanase II.

Fig. 4. Energy of activation

The reaction was carried out in McIlvaine buffer, pH 5.4 (0.03 M), at 20, 25, 30, 37 and 40°C for 10 min with glucomannan as substrate. The assay medium consisted of 25 μ l of buffer, 50 μ l of glucomannan (1%, w/v) and 25 μ l of enzyme. Liberation of reducing sugars was measured by a micro-technique adapted from the Nelson–Somogyi method (see under 'Assay of enzyme activity'). Symbols: ●, β -mannanase I; ○, β -mannanase II.

velocity for enzymes I and II, incubations were carried out (at pH 5.4) at 20, 25, 30, 35, 37 and 40°C for 10 min with glucomannan as substrate (see under 'Assay of enzyme activity'). The energy of activation was calculated from the Arrhenius equation to be 41.66 kJ/mol for β -mannanase I and 31.71 kJ/mol for β -mannanase II (Fig. 4).

Substrate specificity: polysaccharides

Since the molecular weights of the polysaccharides used as substrates (glucomannan of *O. intacta*; galactomannans of *T. repens*: T₁, T₂, T₃, T₄) are not known, stock solutions were made up on the basis of percentage weight/volume. Consequently values of the Michaelis constant are expressed as percentages (Table 2).

Examination of the kinetic data presented in Table 2 shows that the affinity of both β -mannanases I and II for the glucomannan is greater than for any of the galactomannans of *T. repens*. The affinity of enzyme I for the galactomannan of *T. repens* increases as the percentage of galactose removed increases. Thus the value of K_m decreases by a factor of 2, from 0.38% for T₁ to 0.16% for T₄.

β -Mannanase II also shows an increasing affinity for the galactomannan of *T. repens* as the degree of galactose substitution decreases. The value of K_m decreases by a factor of 6, from 1.23% for galactomannan T₁ to 0.2% for T₄. Unlike enzyme I, for β -mannanase II the value of V_{max} decreases at the same time by a factor of 2.

The time course of hydrolysis for the galactomannans of *T. repens* (T₀, T₁ and T₂) was determined for both enzymes (Fig. 5). Initial substrate concentrations were identical in all experiments. The results show that both β -mannanases release increasing amounts of reducing sugars as the galac-

Fig. 5. Hydrolysis with time of the galactomannan of *T. repens*

The galactomannans used were as follows [percentage galactose removed (w/w) in parentheses]: ○, T₀ (0%); ●, T₁ (40%); △, T₂ (62%). The reaction was carried out at 37°C and pH 5.4 in a volume of 100 μ l containing 50 μ l of galactomannan (1%, w/v), 25 μ l of McIlvaine buffer and 25 μ l of enzyme. Liberation of reducing sugars was measured by a micro-technique adapted from the Nelson-Somogyi method. —, β -Mannanase I; ----, β -mannanase II.

Table 2. Specificity of β -mannanases I and II in McIlvaine buffer pH 5.4, at 37°C

Hydrolysis of the glucomannan of *O. intacta* and of the galactomannans of *T. repens*: T₁, T₂, T₃ and T₄ was assayed by incubating 50 μ l of substrate, 25 μ l of McIlvaine buffer, pH 5.4 (0.03 M) and 25 μ l of enzyme. Incubation was at 37°C for 10 min and liberation of reducing sugars estimated by a micro-technique adapted from the Nelson-Somogyi method (see under 'Assay of enzyme activity').

Substrate	β -Mannanase I		β -Mannanase II	
	K_m (g/100 ml)	V_{max} . (μ mol of mannose equivalent liberated/ min per mg of protein)	K_m (g/100 ml)	V_{max} . (μ mol of mannose equivalent liberated/ min per mg of protein)
Glucomannan of <i>O. intacta</i>	0.06	58.8	0.08	45.9
Galactomannan of <i>T. repens</i> T ₁	0.38	71.4	1.23	117.6
Galactomannan of <i>T. repens</i> T ₂	0.19	68.9	0.47	76.9
Galactomannan of <i>T. repens</i> T ₃	0.17	74.1	0.25	58.8
Galactomannan of <i>T. repens</i> T ₄	0.16	95.2	0.20	66.7

tose/mannose ratio decreases. The products of hydrolysis of similar experiments were examined by t.l.c. on silica-gel-coated plastic sheets. The solvent system was propan-1-ol/ethanol/ethyl acetate/pyridine/water (7:4:3:2:4, by vol.). Samples (2 μ l) were removed after 1, 2, 4, 6 and 24h of incubation at 37°C. Hydrolysis of the initial galactomannan of *T. repens* T₀ was extremely limited for both β -mannanases I and II. After 24h of incubation at 37°C with β -mannanase I we observed by t.l.c. the formation of the cores, with R_F values approximating to those of mannotriose, mannotetraose and mannohexaose. After separation by paper chromatography, analysis of these products showed them to be: a trisaccharide composed of 2 molecules of mannose for 1 of galactose (Table 3; core 1), a tetrasaccharide composed of 3 molecules of mannose for 1 of galactose (core 2) and a hexasaccharide composed of 4 molecules of mannose for 2 of galactose (core 3). The possible structures of these

oligosaccharides are shown in Table 3. β -Mannanase II, after 24h incubation with galactomannan from *T. repens* T₀, gave mannose, mannobiose and two oligosaccharides of R_F values (by t.l.c.) approximating to those of mannotriose and mannotetraose. After separation by paper chromatography, analysis of these cores showed them to be: a trisaccharide composed of 2 molecules of mannose for 1 of galactose (Table 3; core 1) and a tetrasaccharide composed of 3 molecules of mannose for 1 of galactose (core 2).

With the galactomannans of *T. repens* T₁, T₂, T₃ and T₄ β -mannanase I liberated initially a hexasaccharide and oligosaccharides of DP (degree of polymerization) greater than 6, which by t.l.c. did not migrate with the solvent used. After 24h we observed the liberation of di-, tri-, tetra- and penta-saccharides. β -Mannanase II, however, gave after 1h, di-, tri-, tetra- and penta-saccharides and after 24h the same oligosaccharides were present in

Table 3. Oligosaccharides of mixed composition produced by hydrolysis of the galactomannan of *T. repens* by β -mannanases I and II

Purification was by paper chromatography (Whatman no. 3) with the solvent butan-1-ol/pyridine/water (9:5:4, by vol.). After elution with cold water each core was hydrolysed (0.5M-H₂SO₄ at 100°C for 2h), neutralized (CaCO₃), filtered and monosaccharides obtained were separated by paper chromatography (as above). The mannose/galactose ratios were measured by the Nelson-Somogyi micro-method. The composition of products was also verified by attack with α -galactosidase II and β -mannanase II; this latter hydrolysed core 3 to give uniquely core 1.

Fig. 6. Action of β -mannanases on manno-oligosaccharides

Mannobiose and mannotriose are not hydrolysed by either β -mannanase I or II. The preferential points of attack ($\uparrow$) secondary point of hydrolysis ($\uparrow$) and very light hydrolytic attack ($\uparrow$) are indicated. ⁰ represents the reducing end of the molecule.

increased concentrations. The results of the analysis of these products are shown in Table 3. The series of products given by β -mannanases I and II were found to be identical and to consist of mannobiose, core 1 and core 2 (as with galactomannan of *T. repens* T₀) as well as mannotriose, mannotetraose, epimelibiose (Galpa1→6Man) and core 4.

The composition of the products shown in Table 3 was further verified by attack with α -galactosidase II (Williams *et al.*, 1978): with core 1 we obtained mannobiose and galactose, with core 2 mannotriose and galactose, with core 3 mannotetraose and galactose and with core 4 mannotetraose and galactose. The relative proportions of mannose/galactose previously established were confirmed by the Nelson-Somogyi micro-method after separation of products by paper chromatography.

Neither enzyme was capable of hydrolysing the galactomannan of *M. sativa* (galactose/mannose ratio, 1:1).

Specificity of hydrolysis of manno-oligosaccharides

Under the assay conditions used (2 and 24 h at 37°C), neither β -mannanase I nor II was able to hydrolyse mannobiose or mannotriose. β -Mannanase I showed no hydrolysis of mannotetraose, whereas β -mannanase II hydrolysed mannotetraose completely after 24 h to give mannotriose (10%), mannobiose (80%) and mannose (10%). The most apparent difference between the two enzymes was observed with mannopentaose as substrate (Fig. 6). Whereas after 24 h incubation β -mannanase I had liberated

only minor quantities of mannobiose and mannotriose, β -mannanase II had achieved an almost total hydrolysis of mannopentaose to give mannobiose, mannotriose and mannose (trace). Hydrolysis of mannohexaose by β -mannanase I is almost complete after 2 h; hydrolysis by β -mannanase II is much slower and complete degradation was only achieved after 24 h (Fig. 6).

Discussion

The β -mannanase of *Bacillus subtilis* (Emi *et al.*, 1972) is the only bacterial enzyme of this type that has been purified and studied in any great detail. This enzyme was shown to be a typical endo-enzyme and to degrade various D-galacto- and D-gluco-mannans to mannose, manno-oligosaccharides of DP 2-8 and oligosaccharides of mixed composition, containing either glucose or galactose residues as well as mannose. The mode of action of this enzyme on D-manno-oligosaccharides is of a preferential attack on the D-mannose chain at the third and fourth β 1→4 mannopyranosyl linkages from the non-reducing end of the molecule. Mannobiose and mannotriose are not attacked, whereas mannotetraose was slowly degraded to mannobiose and mannotriose with D-mannose appearing after 24 h of hydrolysis.

The action, by two purified β -mannanases from *Amorphophallus konjak* on the glucomannan from the same tuber (Shimahara *et al.*, 1975) and on manno- and gluco-manno-oligosaccharides of DP

greater than 2, is similar. Hydrolysis of the substrate is by a typical random or endo-mechanism, and three consecutive mannose residues were concluded to be necessary for the binding of substrates to the active centre of either enzyme.

In a previous paper (Villarroya & Petek, 1976) we showed that the β -mannanase from *M. sativa* (mol.wt.40000) did not hydrolyse mannobiose or mannotriose and hydrolysed mannotetraose relatively slowly (30% after 2h). Mannopentaose and mannohexaose were almost completely hydrolysed after 2h. The galactomannan of *M. sativa* was not attacked, whereas the α -galactosidase-degraded galactomannan was hydrolysed.

The present paper describes a study of the kinetic properties of β -mannanases I and II from the germinated seeds of *T. repens*. These enzymes have similar molecular weights; 43000 (I) and 38000 (II). Neither enzyme I nor II attacks the galactomannan of *M. sativa*, but both slowly hydrolyse the galactomannan of *T. repens* T₀. β -Mannanase I hydrolyses the latter to give only three oligosaccharides, namely cores 1, 2 and 3, whereas β -mannanase II gives mannose and mannobiose as well as two oligosaccharides, namely cores 1 and 2. Attack by either β -mannanase I or II on the galactomannan of *T. repens* (T₁, T₂, T₃ and T₄) results in the formation of an additional series of oligosaccharides: epimelibiase, mannotriose, mannotetraose and core 4 (Table 3). Moreover, as the degree of substitution by galactose decreases, both enzymes show an increased affinity for the galactomannan of *T. repens* and an increased rate of hydrolysis.

The study of the hydrolysis of a series of manno-oligosaccharides suggests certain structural specificities for the binding sites of β -mannanases I and II. Enzyme I seems to require a chain length of at least five consecutive mannopyranose residues for its activity, whereas enzyme II seems to require at least four. Furthermore, β -mannanase I shows a preferential attack on the D-mannose chain at the fourth β 1 $\rightarrow$ 4 mannopyranosyl linkage from the non-reducing end of the molecule; for β -mannanase II this attack is at the second linkage from the non-reducing end (Fig. 6).

The results presented in this paper suggest that β -mannanases I and II are distinct multiple forms of β -mannanase, of complementary hydrolytic function in the degradation of the galactomannan of this seed, and also that the degradation of the galacto-

mannan *in vivo* during germination could proceed by a simultaneous attack by β -mannanase and α -galactosidase.

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