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ACTION PATTERNS AND SUBSTRATE-BINDING REQUIREMENTS OF β-D-MANNANASE WITH MANNOSACCHARIDES AND MANNAN-TYPE POLYSACCHARIDES

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ABSTRACT

Purified (1→4)-β-D-mannanase from *Aspergillus niger* and lucerne seeds has been incubated with mannosaccharides and end-reduced (1→4)-β-D-mannosaccharides and, from the products of hydrolysis, a cyclic reaction-sequence has been proposed. From the heterosaccharides released by hydrolysis of the hot-water-soluble fraction of carob galactomannan by *A. niger* β-D-mannanase, a pattern of binding between the β-D-mannan chain and the enzyme has been deduced. The products of hydrolysis with the β-D-mannanases from *Irpex lacteus*, *Helix pomatia*, *Bacillus subtilis*, and lucerne and guar seeds have also been determined, and the differences from the action of *A. niger* β-D-mannanase related to minor differences in substrate binding. The products of hydrolysis of glucomannan are consistent with those expected from the binding pattern proposed from the hydrolysis of galactomannan.

INTRODUCTION

Such endo-enzymes as β-D-mannanase [EC 3.2.1.78] that hydrolyse unbranched homoglycans having a single linkage-type, namely, (1→4) equatorial bonds, generally can only depolymerise their substrates to oligosaccharides having a minimum degree of polymerisation (d.p.) of 2 or 3. X-Ray crystallography of lysozyme, with and without oligosaccharide inhibitors, has shown that there are interacting (binding) subsites in the enzyme for several of the monosaccharide residues that are in the active-site cleft, and which lie on either side of the catalytic site[1]. The rate of hydrolysis of oligosaccharides rises rapidly as the d.p. increases from 3 to 5 or 6, indicating the number of monomer units that are involved in optimal binding for the maximum rate of hydrolysis.

Several polysaccharides that have a regularly linked, homoglycan backbone that is substituted with single-unit branch-points, *e.g.*, galactomannan, arabino-

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xylan, and xyloglucan, can also be partly depolymerised by the relevant main-chain endo-glycanase. D-Galactomannans, with up to 34% of D-galactose, are hydrolysed to a significant degree[2], and examination of the hydrolysis products[3,4] from β-D-mannanase digestion should give information about the action pattern of the β-D-mannanase, the enzyme–substrate binding requirements, and the pattern of galactose substitution on the mannan chain. Some aspects of the specificity of β-D-mannanase with galactomannans have been discussed[5–8] and it has been pointed out that the two-fold conformation of galactomannan in aqueous solution would place neighbouring galactosyl substituents on either side of the chain, and this may be a factor in the action pattern[6].

β-D-Mannanases from different sources vary significantly in their ability to hydrolyse galactomannans, particularly when highly substituted. Fungal β-D-mannanases[2,8] readily hydrolysed highly substituted polymers, and the presence of the trisaccharide 6^1-α-D-galactosyl-(1→4)-β-D-mannobiose in the hydrolysates indicates that these enzymes can readily act at some points where single, unsubstituted mannosyl residues occur. In contrast, β-D-mannanases from *Bacillus subtilis*[8], *Trichurus spiralis*[9], and germinated legume-seeds[8] had a more limited action on highly substituted galactomannans, and only a trace of this trisaccharide was released, even on extended incubation.

D-Glucomannans are also hydrolysed by β-D-mannanase. Exhaustive hydrolysis of deacetylated glucomannan resulted[6] in nearly quantitative conversion into oligosaccharides of d.p. 2–7. Different β-D-mannanases vary in their action on glucomannan. Of two partially purified enzymes from konjac tubers[10], one had a more random pattern of hydrolysis and less activity with oligosaccharides of low d.p. The main di- and tri-saccharides found were (1→4)-β-D-mannobiose, 4-*O*-β-D-glucosyl-D-mannose, mannotriose, and 4-*O*-β-D-glucosyl-β-D-mannobiose. Cellobiose was also reported, but it has not been detected in any other study. Other oligosaccharides identified include 4-*O*-β-D-mannosyl-(1→4)-*O*-β-D-glucosyl-(1→4)-D-mannose, mannotetraose, mannopentaose, and tetra- and penta-saccharides having glucose at the non-reducing end. Native glucomannans occur acetylated, but this has not been considered in enzymic studies. In most cases, the substrate was probably de-acetylated, as ester groups would have been removed during purification *via* the alkaline copper complex[11,12], but, in some, the glucomannan may still have been acetylated[10].

β-D-Mannanases also vary in their ability to degrade mannosaccharides. *A. niger* β-D-mannanase[13] degraded[8] oligomers of d.p. ≥3, whereas *Rhizopus niveus* β-D- mannanase[14] had little action on oligosaccharides having d.p. <6. *B. subtilis* β-D-mannanase rapidly hydrolysed mannosaccharides of d.p. ≥5, and it was concluded that the third and fourth glycosidic linkages from the non-reducing terminus were preferentially cleaved[15]. Enzymes from fenugreek[16] and alfalfa (lucerne)[17] hydrolysed oligomers having d.p. ≥4, and mannotetraitol was not attacked. Incubation of fenugreek β-D-mannanase with mannotetraose gave mannobiose and mannotriose, with only traces of mannose[16]. In contrast, lucerne-seed β-D-man-

nanase afforded mannobiose plus approximately equal quantities of triose and mannose when incubated with mannotetraose[17], indicating that transglycosylation was not a significant reaction.

We now report on the pattern of transglycosylation associated with mannosaccharides and various β-D-mannanases, and the binding to, and hydrolysis of, galactomannan and glucomannan.

EXPERIMENTAL

Chromatography. — T.l.c. was performed on DC-Alufolien, Kieselgel 60 (0.2 mm), prepared plates which were developed either with *A*, 7:1:2 1-propanol–ethanol–water, or *B*, 5:2:3 1-propanol–nitromethane–water. Sugars were detected by spraying with 5% sulphuric acid in ethanol and heating to 110°. Gel-permeation chromatography was performed on a column (2.5 × 80 cm) of Bio-Gel P-2 (<400 mesh) at 60° in degassed water[18].

Preparation of polysaccharides. — Salep *(Orchis militaris)* glucomannan was prepared from ground tubers kindly provided by Professor H. Neukom (Swiss Federal Institute of Technology). The flour (20 g) was stirred in hot water (80°, 15 min), homogenised, and centrifuged (4,000*g*, 30 min). Polysaccharide in the supernatant solution was precipitated by ethanol (2 vol.), washed with 66% aqueous ethanol, and redissolved. This solution (2 L) was treated with alkaline copper, and the complex was recovered by centrifugation (4,000*g*, 20 min), suspended in ice-cold water, and dissolved by addition of 2M HCl until the solution was just acidic. After centrifugation (4,000*g*, 30 min), to remove traces of insoluble material, the supernatant solution was treated with 2M NaOH until alkaline. The copper complex re-formed, and was recovered by centrifugation and redissolved by acidification. The solution was added to 2 vol. of acidic ethanol (1% of conc. HCl in ethanol, and the precipitate was washed with acidic ethanol (3×), ethanol (2×), acetone, and ether, and dried *in vacuo*. This purification caused deacetylation of the glucomannan[11], and the polysaccharide became insoluble. For the preparation of soluble, acetylated glucomannan, the flour was extracted with hot water, and the extract was centrifuged and precipitated with alcohol. After redissolution, the polysaccharide solution was dialysed, and freeze-dried or stored in the frozen state. Galactomannans were prepared by a modification[4] of a described method[19].

Enzyme assays. — α-D-Galactosidase, β-D-mannosidase, and exo-β-D-mannanase were assayed[20] by using the appropriate *p*-nitrophenyl glycoside as substrate, and β-D-glucosidase was assayed with *p*-nitrophenyl β-D-glucopyranoside (10mM). β-D-Mannanase was routinely assayed by using RBB-carob galactomannan[21]; before use in critical experiments, it was standardised with 0.2% (w/v) carob galactomannan (total) in 0.1M acetate buffer (pH 4.5). Enzyme (0.1 mL) was incubated with carob galactomannan (0.5 mL) at 40°, the reaction was terminated with *p*-hydroxybenzohydrazide (5 mL), and the tubes were incubated for 6 min at 100°. (1→4)-β-D-Glucanase was assayed with RBB-CM-cellulose[22].

Preparation of enzymes. — α-D-Galactosidase II from germinated guar-seeds[23], β-D-mannanase from a commercial Cellulase preparation (Sigma C7502)[3,24] and from other sources[24], exo-β-D-mannanase from germinated guar-seeds[20], and β-D-mannosidase from crude gut-juice of *Helix pomatia* (Sigma G0876)[25] were prepared as described. Interfering activities were undetectable[20,23,24] or <0.01%[25].

Purification of almond emulsin β-D-glucosidase — Almond emulsin (0.5 g, Sigma G8625) in 20mM Tris buffer (100 mL, pH 8) was applied to a column (2.8 × 10 cm) of DEAE-cellulose. Protein was eluted with a linear gradient of NaCl (0→0.4M) in the same buffer. The active fractions were concentrated by $(NH_4)_2SO_4$ (80% saturation), dialysed against 20mM Tris buffer (pH 8), and applied to a column (2.5 × 88 cm) of Ultrogel AcA 44. Eluted enzyme was concentrated by dialysis against poly(ethylene glycol) 4000 and then against water. Before application to a column (1.5 × 7 cm, pre-equilibrated with 25mM imidazole buffer, pH 7.4) of Polybuffer Exchanger PBE 94, the pH was adjusted to 7.4 by the addition of buffer to a final concentration of 25mM. Proteins were eluted with Polybuffer 74-HCl (diluted 1:8 and adjusted to pH 4). The recovered enzyme had a specific activity of 1909 nkat/mg on 10mM *p*-nitrophenyl β-D-glucopyranoside (pH 4.5, 40°), and appeared as a single protein band in isoelectric focussing and SDS-gel electrophoresis.

Partial purification of A. niger *(1→4)-β-D-glucanase (cellulase).* — A solution of Myles Hemicellulase 100,000 (10 g) in 20mM Tris-HCl (200 mL, pH 7.5) was centrifuged (20,000*g*, 20 min). The supernatant solution was applied to a column (2.5 × 26 cm) of DEAE-Sepharose CL-6B equilibrated with 20mM Tris-HCl (pH 7.5), the column was washed with the same buffer (200 mL), and the proteins were eluted with a linear gradient of KCl (0→0.5M). Active fractions were treated with $(NH_4)_2SO_4$ (80%), and the pellet obtained on centrifugation (20,000*g*, 20 min) was dissolved in 20mM phosphate buffer (10 mL, pH 6.5) and applied to a column (2.5 × 90 cm) of Ultrogel AcA 44. Active fractions were applied to a column (1.5 × 12 cm) of DEAE-cellulose equilibrated with 20mM phosphate buffer (pH 6.5). Proteins were eluted with a linear gradient of NaCl (0→0.5M). The enzyme was concentrated by ultrafiltration (UM 10 membrane) and equilibrated with 5mM acetate buffer (pH 5). The purified enzyme was contaminated with <0.01% of β-D-mannosidase and β-D-glucosidase, and contained ~10% of β-D-mannanase on an activity basis.

Hydrolysis of galactomannan by β-D-mannanase. — To a 0.4% solution of galactomannan in 20mM acetate buffer (pH 4.5) was added β-D-mannanase (400 nkat/g of galactomannan), and the solution was incubated at 40° for 20 h. Reaction was terminated by heating at 100° for 10 min, and the solution was centrifuged (20,000*g*, 30 min), concentrated under reduced pressure (below 40°), and adjusted to 4% carbohydrate, and aliquots (2–5 mL) were fractionated on Bio-Gel P-2[4,18]. Aliquots were also removed for the determination of total carbohydrate by the anthrone procedure, reducing sugar by the Nelson–Somogyi procedure[26], and D-

galactose with α-D-galactosidase–β-D-galactose dehydrogenase[27], and the galactose–mannose ratio of the polysaccharide was determined.

Hot-water-soluble carob galactomannan (50 mL, 0.4% w/v) in 10mM acetate buffer (pH 4.5) was treated with β-D-mannanases from guar seed, lucerne seed, *B. subtilis*, *I. lacteus*, or *A. niger* culture-filtrates, or *H. pomatia* gut-solution, and concentrated as described above. Samples (10 μL) were subjected to t.l.c., and separate aliquots (1.0 mL) were fractionated by chromatography on Bio-Gel P-2 (Fig. 5). Aliquots (2 mL) were treated with guar α-D-galactosidase II (100 nkat on *p*-nitrophenyl α-D-galactopyranoside) for 20 h, stored at 4° for 20 h, and centrifuged (20,000*g*, 20 min), and aliquots (1.0 mL) were chromatographed on Bio-Gel P-2. The degree of hydrolysis of galactomannan was determined as the reducing-sugar equivalent (Nelson–Somogyi) as a percentage of total carbohydrate, and soluble carbohydrate as a percentage of total carbohydrate was determined with anthrone before and after treatment with α-D-galactosidase.

Hydrolysis of glucomannan by β-D-mannanase. — A solution of alkaline-copper purified glucomannan (2 g) in 2.5M NaOH (200 mL) was neutralised with acetic acid and dialysed for 18 h. The polysaccharide was adjusted to 0.5% in 20mM acetate buffer (pH 4.5), and aliquots (50 mL) were incubated with either *A. niger* or lucerne-seed β-D-mannanase (100 nkat) at 40° in an oscillating water-bath. The reaction was terminated at 2, 4, 8, or 26 h by heating at 100° for 5 min. The solutions were centrifuged, and total carbohydrate (phenol–sulphuric acid) and reducing sugar (Nelson–Somogyi) in the supernatants were determined. The supernatants were concentrated and redissolved in water (12 mL). An aliquot (3 mL) was fractionated on Bio-Gel P-2, and eluted carbohydrate was assayed (phenol–sulphuric acid).

Native, acetylated glucomannan (0.3 g) in water (50 mL) was treated with 2M NaOH (2.5 mL), the solution was stored for 1 h and then adjusted to pH 4.5 with 5M acetic acid, and *A. niger* β-D-mannanase (120 nkat) was added immediately. Rapid hydrolysis prevented precipitation of the deacetylated glucomannan. Also, acetylated glucomannan (0.3 g) in water was treated with 2M acetate buffer (2.5 mL, pH 4.5), and *A. niger* β-D-mannanase (120 nkat) was added. Each solution was incubated for 26 h at 40°, the reaction was terminated by heating at 100° for 5 min, the solution was centrifuged (20,000*g*, 20 min), the supernatant was concentrated, and the concentrate was redissolved in water (8 mL). An aliquot (4 mL) of this solution was chromatographed on Bio-Gel P-2. Eluted carbohydrate was monitored (phenol–sulphuric acid), and oligosaccharide fractions were concentrated and adjusted to 10 mg/mL.

Hydrolysis of salep glucomannan by A. niger *(1→4)-β-D-glucanase plus β-D-mannanase.* — Acetylated glucomannan (3 g) in water (500 mL) was treated with 2M NaOH (2.5 mL) and stored for 1 h. The solution was adjusted to pH 4.5 with 5M acetic acid, and then to 600 mL. Aliquots (150 mL) plus *A. niger* (1→4)-β-D-glucanase (12, 24, 48, or 96 nkat) were incubated at 40° for 4 h and then at 100° for 10 min. On centrifugation (20,000*g*, 20 min), aliquots were removed for the deter-

mination of total carbohydrate (phenol–sulphuric acid) and reducing sugar (Nelson–Somogyi). The degrees of hydrolysis of the four samples (calculated as reducing-sugar level as a percentage of total carbohydrate) were 10, 18, 28, and 36%, respectively. The sample that had been hydrolysed 18% was concentrated and redissolved (12 mL). Aliquots (4 mL) were fractionated on Bio-Gel P-2, and the di-, tri-, and tetra-saccharide fractions subjected to t.l.c.

Enzymic characterisation of the structures of oligosaccharides. — This was performed as described[3,4].

Preparation of β-D-mannosaccharides and reduced β-D-mannosaccharides. — β-D-Mannosaccharides were prepared either by partial hydrolysis of *Livistona australis* mannan[20] with acid, or by treatment of carob galactomannan with a particular β-D-mannanase followed by denaturation and treatment of the oligosaccharide mixture with guar α-D-galactosidase II. Oligosaccharides of d.p. 2–6 were fractionated on Bio-Gel P-2 (<400 mesh). Reduced mannosaccharides were prepared by treatment with $NaBH_4$.

Preparation of 6-α-D-galactosyl-(1→4)-β-D-mannosaccharides. — 6^1-α-D-Galactosyl-(1→4)-β-D-mannobiose (Gal^1Man_2*) and Gal^1Man_3 were prepared as previously described[3]. Gal^3Man_4 and a mixture of Gal^4Man_5 plus Gal^3Man_5 were made by reaction of hot-water-soluble carob galactomannan (250 mL, 0.4%) in 20mM acetate buffer (pH 4.5) with guar-seed β-D-mannanase (400 nkat on carob galactomannan). The solution was incubated at 40° for 20 h and then at 100°, concentrated, diluted to 25 mL, and centrifuged (20,000*g*, 30 min). Aliquots (5 mL) were chromatographed on Bio-Gel P-2. The pentasaccharide consisted exclusively of Gal^3Man_4, and the hexasaccharide fraction consisted of an approximately equal mixture of Gal^4Man_5 and Gal^3Man_5. With lucerne-seed β-D-mannanase, the pentasaccharide fraction consisted[4] of ~80% of Gal^3Man_4 and 20% of Gal^1Man_4. The latter oligosaccharide was removed by treatment with exo-β-D-mannanase from guar seeds (20 nkat on β-D-mannopentaitol/50 mg) for 6 h at 40° and pH 5.5. Products were chromatographed on Bio-Gel P-2, to give Gal^3Man_4.

Gal^3Man_3 was prepared from Gal^3Man_4 by incubation (2 mL, 10 mg/mL) in 10mM acetate buffer (pH 4.5) with *H. pomatia* β-D-mannosidase (500 nkat) at 40° for 48 h. The reaction products were separated by chromatography on Bio-Gel P-2. A mixture of Gal^4Man_4 and Gal^3Man_3 was obtained on hydrolysis of the hexasaccharide mixture Gal^4Man_5 and Gal^3Man_5 by *H. pomatia* β-D-mannosidase. The incubation conditions were the same as those in the preparation of Gal^3Man_3, and the reaction products were fractionated on Bio-Gel P-2.

Hydrolysis of β-D-mannosaccharides and reduced β-D-mannosaccharides by A. niger *β-D-mannanase.* — A solution of each oligosaccharide (0.6 mL, 10 mg/mL) in 10mM acetate buffer (pH 4.5) was incubated with *A. niger* β-D-mannanase (0.2 mL, 5.2 nkat) at 40°. Samples (15 μL) were subjected to t.l.c. after 0–240 min.

*The subscript indicates the d.p. of the mannosaccharide chain, and the superscript the position of the D-galactosyl substituent on the chain, relative to the reducing D-mannose residue.

Separate aliquots (50 μL) were heated to denature β-D-mannanase and diluted with water (0.6 mL), and aliquots (0.1–0.2 mL) were used for measurement of reducing activity (*p*-hydroxybenzohydrazide) and total carbohydrate (Fig. 1).

Transglycosylation catalysed by β-D-mannanase. — Solutions of oligosaccharides (4 mL, 10 mg/mL) in 10mM acetate buffer (pH 4.5) were incubated with *A. niger* β-D-mannanase (0.1 mL, 26 nkat) at 40° for 0 or 40 min or 20 h, and at 100° for 5 min. A sample (15 μL) was subjected to t.l.c. A separate aliquot (2.0 mL) was chromatographed on Bio-Gel P-2. The oligosaccharides of d.p. >5, produced on incubation of β-D-mannopentaose and β-D-mannopentaitol with the enzyme, were recovered after chromatography, combined, and concentrated (to 10 mg/mL). To solutions of these (50 μL, 10 mg/mL) was added 100mM acetate (10 μL, pH 4.5 or 5.5) plus *H. pomatia* β-D-mannosidase (20 μL, 14 nkat; pH 4.5) or guar-seed exo-β-D-mannanase (40 μL, 48 nkat on *p*-nitrophenyl β-D-mannopyranoside; pH 5.5), and the solutions were incubated at 40° for 20 h. Aliquots (15 μL) were subjected to t.l.c.

Effect of substrate concentration on the rate of hydrolysis of β-D-mannopentaitol by A. niger *β-D-mannanase.* — Solutions of β-D-mannopentaitol (3.0 mg) at final concentrations of 0.62, 1.25, 2.5, 5.0, and 10.0 mg/mL in acetate buffer (20 mM, pH 4.5) were incubated with *A. niger* β-D-mannanase (2.6 nkat) at 40°. Aliquots (0.5 mg of carbohydrate) were removed, incubated at 100° for 2 min, adjusted to a final volume of 2 mL, and assayed for reducing sugar (*p*-hydroxybenzohydrazide) and total carbohydrate.

Hydrolysis of Gal^1Man_3, β-D-mannotriose, and β-D-mannotri-itol by A. niger *β-D-mannanase.* — Solutions of oligosaccharide (0.1 mL, 15 mg/mL) in 10mM acetate buffer (pH 4.5) were incubated with *A. niger* β-D-mannanase (50 μL, 13 nkat) at 40°, and aliquots (10 μL) were removed at 0.5–20 h for t.l.c. (solvent *A*).

Hydrolysis of Gal^4Man_4, Gal^3Man_4 and β-D-mannotriose by A. niger *β-D-mannanase.* — Solutions of oligosaccharide (0.1 mL, 5 mg/mL) in 10mM acetate (pH 4.5) and *A. niger* β-D-mannanase (50 μL, 2 nkat) were incubated at 40°, and aliquots (15 μL) were removed at 15–120 min for t.l.c (solvent *B*).

Hydrolysis of α-D-galactosyl-(1→4)-β-D-mannopentaose, β-D-mannotetraose, and β-D-mannopentaose by A. niger *and lucerne-seed β-D-mannanases.* — Solutions of α-D-galactosyl-(1→4)-β-D-mannopentaose (an approx. equal mixture of Gal^3Man_5 and Gal^4Man_5), β-D-mannotetraose, and β-D-mannopentaose (0.1 mL, 15 mg/mL) in 10mM acetate buffer (pH 4.5) and *A. niger* (50 μL, 1.3 nkat) or lucerne (30 μL, 2.0 nkat) β-D-mannanases were incubated at 40°, and aliquots (10 μL) were removed at 0–120 min for t.l.c. (solvent *A*).

Hydrolysis by A. niger *β-D-mannanase and* H. pomatia *β-D-mannosidase of the tetrasaccharide and pentasaccharide fractions obtained on partial hydrolysis of salep glucomannan by* A. niger *β-D-mannanase.* — Solutions of the tetra- or pentasaccharide fractions (50 μL, 15 mg/mL) in 10mM acetate buffer (pH 4.5), and either *A. niger* β-D-mannanase (10 μL, 4.0 nkat) or *H. pomatia* β-D-mannosidase (5.9 nkat), were incubated at 40° for 2 h and then at 100° for 2 min, and aliquots

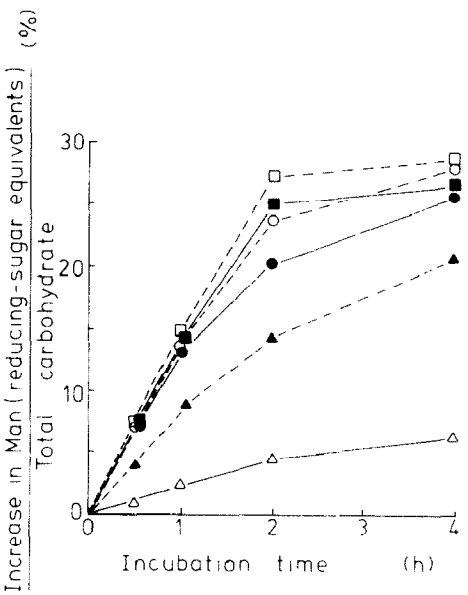


Fig. 1. Hydrolysis of β-D-mannosaccharides and reduced β-D-mannosaccharides by *A. niger* β-D-mannanase. Key: mannohexaose (■), mannohexaitol (□), mannopentaose (●), mannopentaitol (○), mannotetraose (▲), and mannotetraitol (△).

(10 μL) were removed for t.l.c. The remainder was treated with the alternative enzyme. On incubation at 40° for 2 h, aliquots (10 μL) were removed for t.l.c. (solvent *A*, 2 developments).

RESULTS

Reaction of A. niger *β-D-mannanase with mannosaccharides.* — Relative reaction rates and amounts of hydrolysis of (1→4)-β and end-reduced mannosaccharides by *Aspergillus niger* β-D-mannanase are shown in Fig. 1. The tetraose was hydrolysed more slowly than the pentaose, which reacted at an initial rate similar to that of the hexaose. Mannotriose was unreactive and the tetraitol slightly reactive. The hydrolysis of mannotetraitol was considerably less than that of mannotetraose, but the differences were less distinct with the higher oligomers (*cf.* refs. 15 and 28). The K_m value (Table I) with the hexaitol was of the same order as with the galactomannans, but V_{max} was less. Transglycosylation[29-31] was significant in the oligosaccharide reactions. Increasing the pentaitol concentration in reaction mixtures from 0.63 to 1.25 mg/ml increased the rate of release of reducing activity by 30%, but further increases in concentration resulted in a progressive decrease, until, at 10 mg/mL, the rate was 50% of that at 1.25 mg/mL. T.l.c. of the products of hydrolysis of the tetraose and pentaose in the early stages of reaction showed the transient synthesis of higher oligosaccharides. This was not observed with manno-

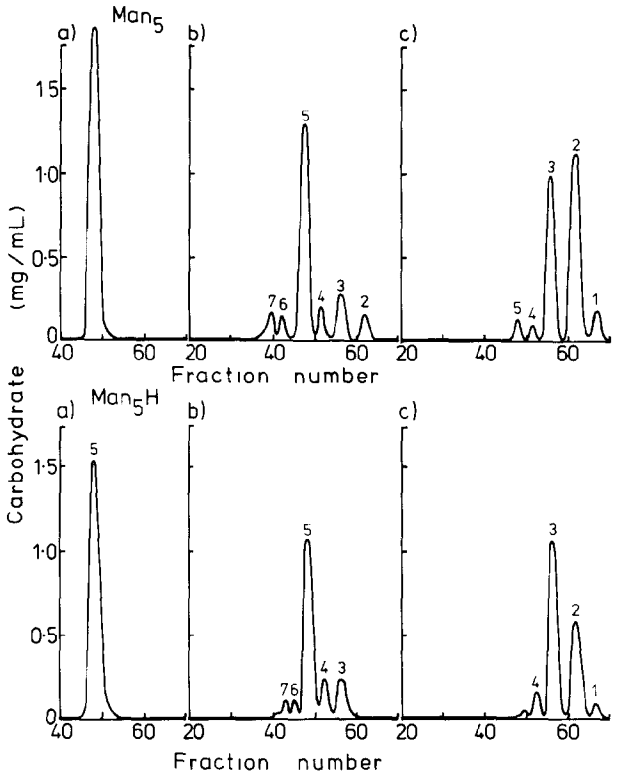


Fig. 2. Bio-Gel P-2 chromatography of the oligosaccharides produced on hydrolysis of β-D-mannopentaose and β-D-mannopentaitol by *A. niger* β-D-mannanase. A solution of each oligosaccharide was incubated with *A. niger* β-D-mannanase, and aliquots were removed at (a) 0 min, (b) 40 min, and (c) 20 h for chromatography. Numbers show the d.p. of the eluted oligosaccharides.

TABLE I

KINETIC DATA FOR *A. niger* AND LUCERNE-SEED β-D-MANNANASES[a]

Substrate	A. niger β-D-*mannanase*			*Lucerne-seed* β-D-*mannanase*		
	K_m		*Relative*[b] V_{max}	K_m		*Relative*[b] V_{max}
	mg/mL	*mM*		*mg/mL*	*mM*	
Mannotri-itol	—	—	0	—	—	0
Mannotetraitol	—	—	6	—	—	0
Mannopentaitol	0.22	0.27	79	—	—	1
Mannohexaitol	0.06	0.06	100	1.8	1.8	100
Carob galactomannan (24% Gal)	0.03	—	250	0.9	—	200
Leucaena galactomannan (40% Gal)	0.07	—	250	n.d.[c]	—	44

[a]Reduced oligosaccharide (0.5 mL, 0.5–3mM) or galactomannan (0.5 mL, 0.02–0.2%) in acetate buffer (0.1M, pH 4.5) was incubated with β-D-mannanase (0.1 mL) at 40°, and the reaction was terminated after 0, 5, 10, and 15 min. [b]Relative to 100 for mannohexaitol. [c]Not determined

TABLE II

RELATIVE INITIAL RATES OF HYDROLYSIS[a] OF CAROB AND *Leucaena* GALACTOMANNANS, MANNOPENTAITOL, AND MANNOHEXAITOL BY β-D-MANNANASES

Source of β-D-mannanase	*Substrate*			
	Carob galactomannan	Leucaena *galactomannan*	*Manno-pentaitol*	*Manno-hexaitol*
B. subtilis	100	18	1	16
Lucerne (B)	100	27	1	26
Guar (whole)	100	14	1	19
Helix pomatia	100	73	8	14
I. lacteus	100	85	14	16
A. niger	100	97	16	21

[a]Galactomannan or reduced oligosaccharide (0.5 mL, 2 mg/mL) in 0.1M acetate buffer (pH 4.5) was incubated with β-D-mannanase (0.1 mL) at 40° for 5 min.

triose. The products of extended reaction of the tetraose were mannotriose and mannobiose, with only a trace of mannose. The final products with the tetraitol were mannobiose, mannotriose, and the tri-itol; with the pentaitol, mannobiose, mannotriose, and the tri-itol and tetraitol were formed with only traces of mannose and mannobi-itol. Chromatography on Bio-Gel P-2 gave a quantitative picture of the hydrolysis and an indication of the extent of transglycosylation, and showed the intermediate production of fractions of d.p. >5 for the pentaose and pentaitol (Fig. 2).

Reaction of β-D-mannanases from other sources with mannosaccharides. — β-D-Mannanases from different sources varied in their hydrolytic capacity (Table II). The enzymes from *Irpex lacteus* and *A. niger* hydrolysed mannopentaitol and mannohexaitol at similar rates, but the β-D-mannanases from *Bacillus subtilis*, lucerne (alfalfa) seeds, and guar seeds had little action on mannopentaitol compared to mannohexaitol. The K_m values for lucerne-seed β-D-mannanase were ~30-fold those for the *A. niger* enzyme (Table I). T.l.c. of the hydrolysis products of the lucerne enzyme with manno-tetraose, -pentaose, and -pentaitol showed that transglycosylation was as extensive as with the *A. niger* enzyme.

Effect of galactose substitution on the mannan core on hydrolysis. — The rate of change of specific viscosity on incubation with *A. niger* β-D-mannanase of lucerne galactomannan showed that, even with the high level of substitution of this polysaccharide, there was still some hydrolysis (*cf.* ref. 7). The effect of galactose content on hydrolysis of galactomannans by *A. niger* β-D-mannanase is shown in Table III. Also included in Table III are the yields of polysaccharide extracted from the seeds and their limiting-viscosity numbers. There is, in general, an inverse relationship between galactose content and degree of hydrolysis, but *Leucaena leucocephala* galactomannan underwent more hydrolysis than would be predicted[2].

The reaction of hot-water-soluble carob galactomannan (Gal/Man 19:81)

TABLE III

YIELDS OF GALACTOMANNANS FROM DIFFERENT SEED SOURCES, GALACTOSE–MANNOSE RATIOS, LIMITING-VISCOSITY NUMBERS (L.V.N.) AND DEGREE OF HYDROLYSIS BY *A. niger* β-D-MANNANASE

Source of galactomannan	*Yield (%)*	*Gal·Man ratio*	*L.v.n.*[d] *(mL/g)*	*Degree of hydrolysis*[e] *by β-D-mannanase (%)*
Medicago sativa (lucerne)	8.8	48:52	1300	1
Sesbania cannabina	17.5	40:60	1440	4
Cyamopsis tetragonolobus[a] (guar)	28.0	38:62	1528	5
Leucaena leucocephala	12.0	40.60	1040	10
Hardenbergia violacea	12.0	34:66	940	15
Sesbania grandiflora	8.3	33·67	1580	16
Caesalpinia vesicaria	28.5	29:71	1330	19
Caesalpinia spinosa (tara)	—	28·72	1120	20
Crotalaria cunninghamii	14.7	29·71	1470	18
Gleditsia triacanthos (honey locust)	18.0	27:73	1380	18
Caesalpinia pulcherima	19.4	24:76	1110	22
Delonix regia	19.0	23:77	780	25
Cassia fistula	20.7	23.77	1380	21
Ceratonia siliqua (carob)[b]				
total	31	24:76	990	22
cold-water soluble	12	27:73	880	19
hot-water soluble	19	19:81	1090	26
Parkinsonia aculatea	17.8	21.79	1340	26
Sophora japonica	19.2	16:84	1570	30
α-D-Galactosidase treated	—	38·62	1410	5
guar galactomannan[c]	—	33:67	1440	14
	—	28:72	1780	20
	—	24·76	1800	23
	—	19:81	1950	29
	—	16:84	1960	30
	—	14.86	n.d.	31

[a]Var. MSSI-Type 1. [b]Var. Casuda. [c]Commercial guar-flour. [d]Determined using an Ubbelohde suspended-level viscometer. [e]Nelson–Somogyi reducing-sugar equivalents as a percentage of total carbohydrate (anthrone).

with *A. niger* β-D-mannanase gave a series of oligosaccharides that were separated by chromatography on Bio-Gel P-2. Reaction was stopped after 1, 2, 5, and 24 h, and the mixture of oligosaccharides was fractionated (Fig. 3). Hydrolysis was endo and almost complete in 5 h. After 24 h, 93% of the oligosaccharides had d.p. <9 and all had d.p. <15. The oligosaccharides of d.p. 2–9 have been identified[3,4], and their structures and yields are listed in Table IV (the reducing-end hexose is italicised; M connotes a mannosyl, and Ga a galactosyl, residue).

β-D-Mannanases from other sources (*Irpex lacteus*, lucerne and guar seed, *Bacillus subtilis*, and *Helix pomatia*) were also purified, and incubated with the

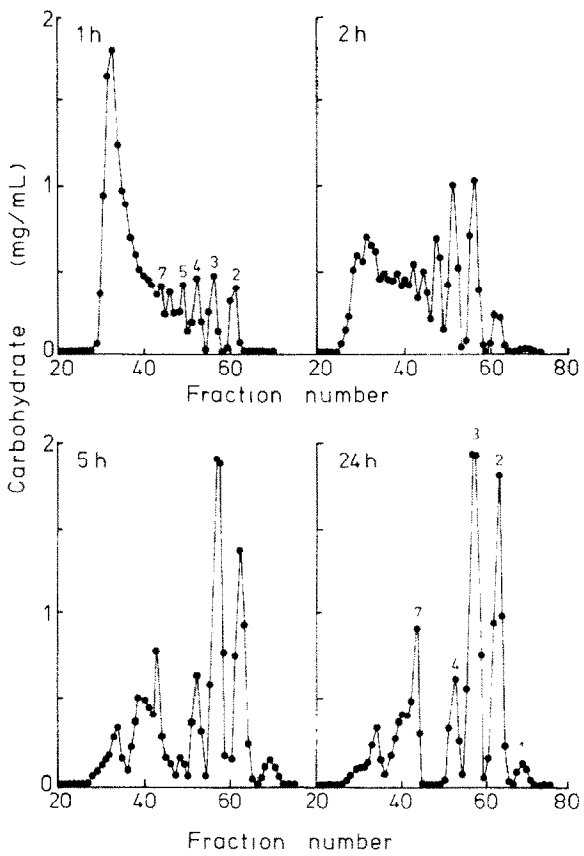


Fig. 3. Bio-Gel P-2 chromatography of the oligosaccharides produced on hydrolysis of hot-water-soluble carob galactomannan by *A. niger* β-D-mannanase. Carob galactomannan (1.0 g, 0.4%) in 20mM acetate buffer (pH 4.5) was incubated with *A. niger* β-D-mannanase (1.0 mL, 400 nkat) at 40°, aliquots (50 mL) were removed after 1, 2, 5, and 24 h, incubated at 100° for 5 min, and concentrated to dryness, each residue was then dissolved in water, and an aliquot (5 mL) was subjected to Bio-Gel P-2 chromatography.

same carob-galactomannan fraction. The extent of hydrolysis differed (Table V) and, after the hydrolysates had been treated with α-D-galactosidase, the amount of soluble mannosaccharide (d.p. <8) varied, indicating differences in the d.p. of the mannosaccharide chains forming the backbone of the heterosaccharides. The patterns produced on Bio-Gel P-2 chromatography of the final hydrolysates (24 h) are given in Fig. 4, and the amounts recovered and structures in Table V. If the hydrolysate from incubation with lucerne β-D-mannanase was treated again with the same enzyme, there was no significant change in the chromatographic pattern of heterosaccharides, but, if it was treated with *A. niger* enzyme, there was further hydrolysis and the pattern became essentially identical with that obtained by initial hydrolysis with *A. niger* β-D-mannanase (Fig. 4). Similar results were obtained

TABLE IV

OLIGOSACCHARIDES RELEASED BY β-D-MANNANASE HYDROLYSIS OF CAROB GALACTOMANNAN, AND SEGMENTS PRODUCING THESE FRAGMENTS

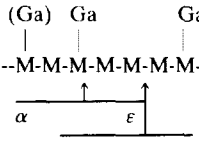
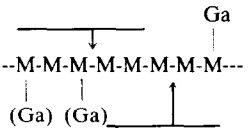
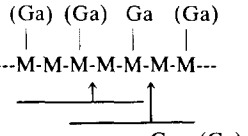
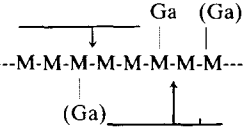
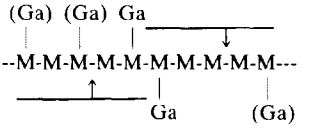
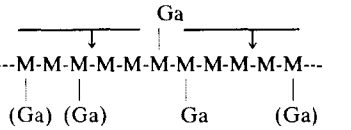
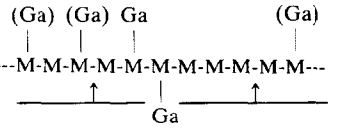
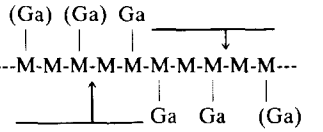
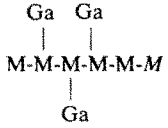
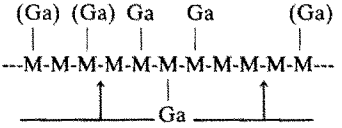
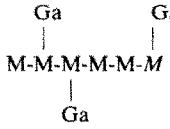
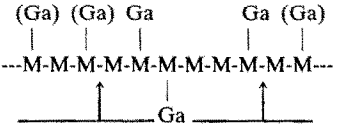
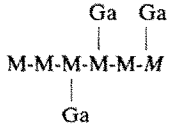
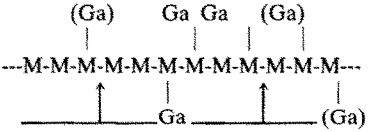
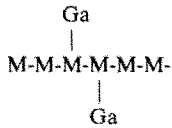
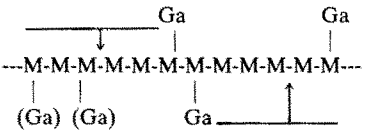
Oligosaccharide with amount recovered (wt. %)			*Polymer segment producing oligosaccharide (→ reducing end)*
Man_2	M-*M*	(23.9)	
Man_3	M-M-*M*	(20.2)	
Gal^1Man_2[a]	Ga | M-*M*	(15.9)	
Gal^1Man_3	Ga | M-M-*M*	(7.4)	
$Gal^{3,4}Man_5$	Ga | M-M-M-M-*M* | Ga	(11 1)	
$Gal^{3,4}Man_6$	Ga | M-M-M-M-M-*M* | Ga	(4.2)	
$Gal^{4,5}Man_6$	Ga | M-M-M-M-M-*M* | Ga	(1.2)	
$Gal^{1,3,4}Man_5$	Ga | M-M-M-M-*M* | Ga Ga	(0 6)	

TABLE IV *(continued)*

Oligosaccharide with amount recovered (wt. %)			*Polymer segment producing oligosaccharide (→ reducing end)*
$Gal^{3,4,5}Man_6$		(2.5)	
$Gal^{1,4,5}Man_6$		(2.3)	
$Gal^{1,3,4}Man_6$		(0.5)	
$Gal^{4,5}Man_7$		(0.7)	

[a]The subscript indicates the d.p. of the mannosaccharide chain, and the superscript the position of the galactosyl substituent on the chain, relative to the reducing D-mannose residue. (Ga) represents an optional galactosyl residue.

when the hydrolysate obtained using any of the β-D-mannanases was treated further with *A. niger* β-D-mannanase.

An examination of the oligosaccharides produced on hydrolysis of hot-water-soluble carob galactomannan by the different β-D-mannanases showed that oligosaccharides, when present, had common structures (Table V), consistent with the same type of linkages being cleaved by all of the enzymes. However, some linkages cleaved by *A. niger* β-D-mannanase were cleaved to a lesser degree, or not at all, by the other enzymes. Oligosaccharides present in guar-seed and lucerne-seed β-D-mannanase hydrolysates of carob galactomannan, but not present in the *A. niger* β-D-mannanase hydrolysate, were further hydrolysed by the *A. niger* enzyme. Gal^3Man_4 from a guar β-D-mannanase hydrolysate, and a mixture of this with Gal^1Man_4 from lucerne β-D-mannanase hydrolysate, were hydrolysed by *A. niger* β-D-mannanase more rapidly than was mannotetraose. There was transient production of oligosaccharides of higher d.p. The hexasaccharides Gal^3Man_5 and Gal^4Man_5 were both hydrolysed, the former more readily, as shown by the rapid production of Gal^1Man_3 with more limited amounts of Gal^1Man_2. Similar results were obtained on hydrolysis of this hexasaccharide mixture by excess amounts of lucerne-seed β-D-mannanase (Fig. 5). $Gal^{1,3}Man_4$ from a lucerne-seed β-D-man-

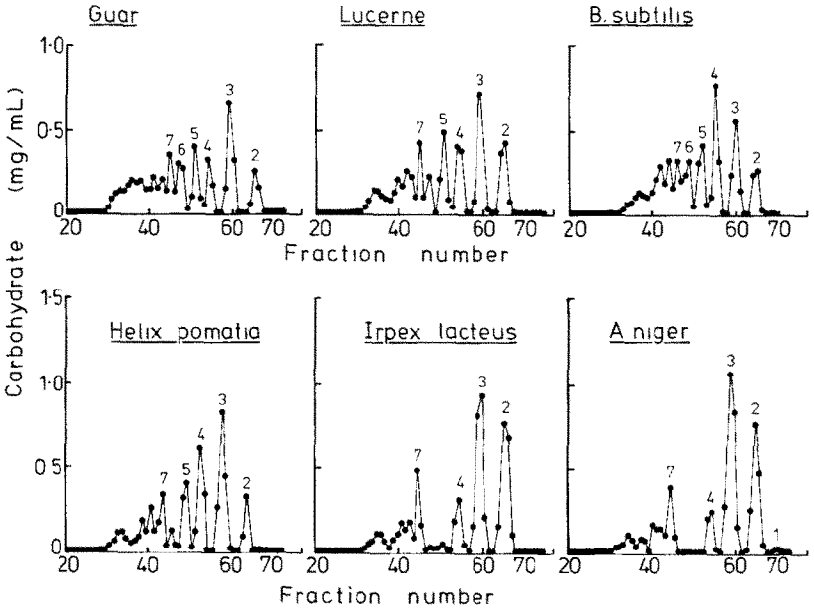


Fig. 4. Bio-Gel P-2 chromatographic patterns of oligosaccharides produced on hydrolysis of hot-water-soluble carob galactomannan by β-D-mannanases from guar seed, lucerne seed, *B. subtilis*, *H. pomatia*, *I. lacteus*, and *A niger*.

nanase hydrolysate was also attacked by the *A. niger* enzyme. The pentasaccharide Gal^4Man_4, prepared by β-D-mannosidase hydrolysis of Gal^4Man_5, was hydrolysed by *A. niger* β-D-mannanase at about a tenth the rate of Gal^3Man_4 and ten times the rate of Man_3. The products were mainly Gal^3Man_3 and mannose, whereas Gal^1Man_4 and Gal^3Man_4 gave only Man_2 and Gal^1Man_2.

Some heterosaccharides from the *A. niger* hydrolysate were hydrolysed further at much higher concentrations (30-fold) of enzyme, as previously found with *I. lacteus* β-D-mannanase[3]. Gal^1Man_3 gave Gal^1Man_2 plus Man_2 and a small proportion of Man. These products arise from transglycosylation. Under the reaction conditions, the rate of degradation of Gal^1Man_3 paralleled that of mannotriose. $Gal^{3,4}Man_5$ and $Gal^{1,3,4}Man_5$ were resistant even at higher concentrations, although traces of material of higher d.p. (immobile on t.l.c.) were observed with the former compound. $Gal^{4,5}Man_6$ and $Gal^{3,4}Man_6$ were hydrolysed to $Gal^{3,4}Man_5$, $Gal^{1,4,5}Man_6$ gave $Gal^{3,4}Man_5$ plus GalMan, and $Gal^{4,5}Man_7$ produced $Gal^{3,4}Man_5$, Man, and traces of Man_2. $Gal^{3,4,5}Man_6$ was not attacked.

Hydrolysis of glucomannan. — Glucomannans occur in Nature with some sugar residues acetylated, and these may have an effect on hydrolysis and lead to a much larger number of hydrolytic products than when the de-esterified polymer is used. When native (acetylated) salep mannan and the deacetylated polysaccharide were incubated with *A. niger* β-D-mannanase, the extents of hydrolysis were 23% and 31%, respectively, and the patterns of products on Bio-Gel P-2

TABLE V

RELATIVE AMOUNTS OF OLIGOSACCHARIDES PRODUCED ON HYDROLYSIS OF HOT-WATER-SOLUBLE CAROB GALACTOMANNAN BY β-D-MANNANASES

	Source of β-D-mannanase					
	A. niger	L. lacteus	H. pomatia	B. subtilis	*Lucerne*	*Guar*
Degree of hydrolysis (%)[a]	30	29	22	18	22	19
Soluble carbohydrate on α-D-galactosidase treatment (%)	96	95	95	92	93	87
Oligosaccharides produced (wt. %)						
Man	1.8	0.3	—	—	tr[b]	—
Man_2	23.9	26.6	14.3	8.7	13.3	7.3
Man_3	20.2	19.4	21.5	13.6	19.3	16.8
Gal^1Man_2	15.9	12.9	2.4	0.7	1.0	—
Man_4	—	tr	8.4	15.0	8.0	8.9
Gal^1Man_3	7.4	9.1	8.4	3.7	4.2	—
Man_5	—	—	—	1.8	—	tr
Gal^1Man_4	—	1.7	2.3	3.0	2.0	—
Gal^3Man_4	—		9.3	7.1	9.0	9.5
Gal^3Man_5	—	—	3.0	3.8	3.3	4.6
Gal^4Man_5	—	—		3.8	3.3	4.4
$Gal^{1,3}Man_4$	—	—	tr	1.4	0.4	—
$Gal^{3,4}Man_5$	11.1	11.4	8.0	10.0	9.2	9.0
Gal^4Man_6	—	—	—	—	—	2.2
Gal^3Man_6	—	—	—	—	—	0.4
Oligomers of higher d.p.	19.7	18.6	22.6	27.4	26.9	37.5

[a]Nelson–Somogyi reducing-sugar equivalents as a percentage of total carbohydrate (anthrone). [b]Trace.

chromatography were quite distinct (Fig. 6). The acetylated mannan gave more of the fractions of higher molecular weight. The deacetylated material gave a small peak at the void volume but, as this was absent from the hydrolysate of the polysaccharide purified as the copper complex (Fig. 6), it was probably a contaminating polysaccharide. The even larger peak at the void volume of the native-glucomannan hydrolysate suggests that some substrate of higher molecular weight may have been left.

The major products on hydrolysis of insoluble, deacetylated, salep glucomannan with *A. niger* β-D-mannanase were di- and tri-saccharides with lesser amounts of tetra- and penta-saccharides (Fig. 6). The ratio of di- to tri-saccharide was different when soluble deacetylated polymer was hydrolysed (Fig. 6), which may have been a consequence of the different physical properties of substrates.

When lucerne β-D-mannanase was incubated with purified salep-glucoman-

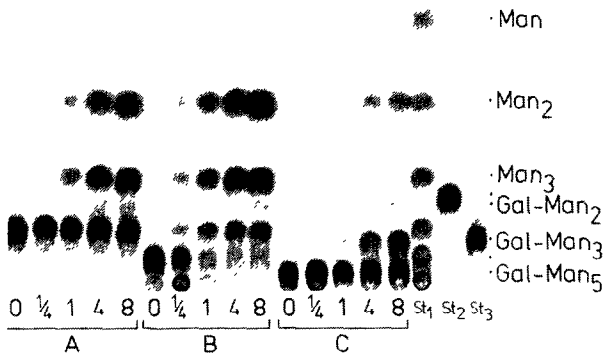


Fig. 5. T.l.c. of the oligosaccharides produced on limited hydrolysis of mannotetraose (A), mannopentaose (B), or α-D-galactosyl-β-D-mannopentaose (C) by lucerne-seed β-D-mannanase. St_1, mannose–mannohexaose; St_2, Gal^1Man_2, St_3, Gal^1Man_3

nan and the results were compared with those for the *A. niger* enzyme reaction, the initial rates of hydrolysis and solubilisation were similar, but the final amounts of solubilisation and the proportions of oligosaccharide fragments were different. *A. niger* β-D-mannanase solubilised all of the glucomannan, but lucerne β-D-mannanase gave only 85% conversion into soluble products and there was much more tetra- and penta-saccharide. T.l.c. of the hydrolysate fractions from Bio-Gel P-2 chromatography (Fig. 7) showed that, up to a d.p. of 4, both enzymes produced the same oligosaccharides. Each hydrolysate contained two disaccharides, two trisaccharides, and four tetrasaccharides in more than trace amounts. The di- and tri-saccharide fractions were isolated by thick-paper chromatography, and the sugar sequence was characterised by enzymic and chemical procedures. The more slowly migrating disaccharide was identified as (1→4)-β-D-mannobiose, since it gave only mannose on acid hydrolysis, was quantitatively converted into mannose by *Helix pomatia* β-D-mannosidase, and co-chromatographed with an authentic sample. The second disaccharide was identified as β-Glc-(1→4)-Man, because acid hydrolysis gave equimolar amounts of glucose and mannose, mannitol and glucose were the only sugars detected after borohydride reduction and hydrolysis, and the chromatographic mobility was consistent with published values[32]. *H. pomatia* β-D-mannosidase gave no reaction. Unusual behaviour was observed on incubation with almond β-D-glucosidase. There was insignificant hydrolysis with a preparation that readily hydrolysed cellobiose at one tenth the enzyme–substrate ratio used. The lack of glycon specificity of this enzyme has been documented[33], but this aglycon effect suggests that the enzyme should be used with care in oligosaccharide

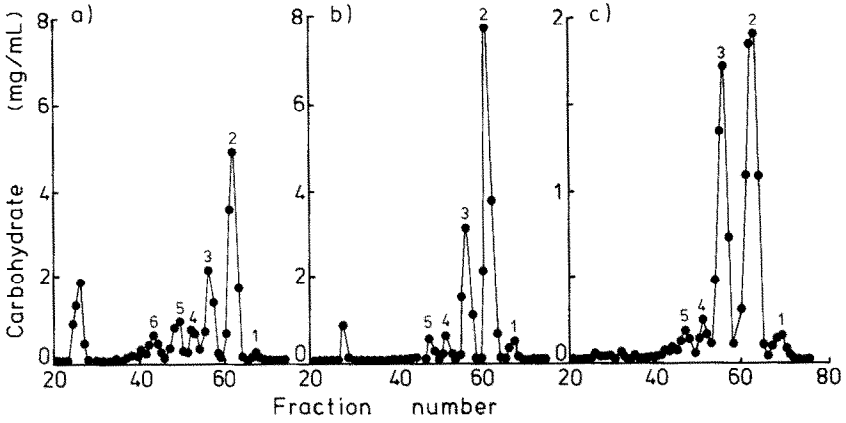


Fig. 6. Bio-Gel P-2 chromatography of the oligosaccharides produced on hydrolysis of (a) soluble native (acetylated), (b) soluble deacetylated, and (c) insoluble deacetylated (copper-purified) salep-glucomannan by *A. niger* β-D-mannanase.

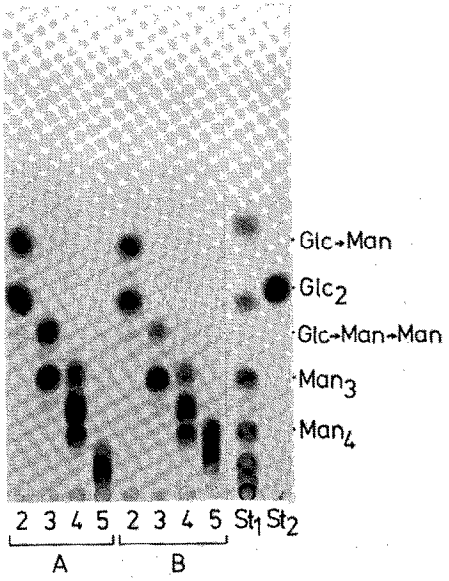


Fig. 7. T.l.c. (solvent *A*, 2 developments) of the di-, tri-, tetra-, and penta-saccharide fractions obtained on Bio-Gel P-2 chromatography (Fig. 6) of the oligosaccharide mixture produced on treatment of insoluble salep-glucomannan with *A. niger* (A) or lucerne-seed (B) β-D-mannanase for 2 h: St_1, mannose–mannohexaose; St_2, cellobiose.

sequencing. The preparation hydrolysed *p*-nitrophenyl β-D-mannopyranoside at 0.03% of the rate for *p*-nitrophenyl β-D-glucopyranoside and, on incubation with mannobiose, transglycosylation slowly occurred, giving mannotriose and mannose at a rate higher than the hydrolysis of β-Glc-(1→4)-Man.

The more slowly migrating trisaccharide was (1→4)-β-D-mannotriose. It co-chromatographed with an authentic sample, gave only mannose on acid hydrolysis, and was quantitatively converted into mannose by *H. pomatia* β-D-mannosidase. The sequence of the other trisaccharide was Glc→Man→Man. Acid hydrolysis gave glucose and mannose in the ratio of 1:2, but, after borohydride reduction and hydrolysis, these sugars were obtained in equimolar amounts. No monosaccharide was released with *H. pomatia* β-D-mannosidase. (This enzyme rapidly hydrolysed β-Man-(1→4)-Glc, prepared by (1→4)-β-D-glucanase hydrolysis of salep glucomannan, showing that β-D-mannosidase is still active if mannose is replaced by glucose at the penultimate sugar residue from the non-reducing end.) This trisaccharide has been identified previously in a β-D-mannanase hydrolysate of glucomannan[6,10].

The structures of the components of the tetrasaccharide fraction were indicated by their behaviour on incubation with *H. pomatia* β-D-mannosidase and then treatment with more *A. niger* β-D-mannanase (Fig. 8) to be a mixture of mannotetraose (*D*), Man→Man→Glc→Man (*C*), and the probable structures Glc→Man→Man→Man (*B*), and Glc→Man→Glc→Man (*A*). These conclusions

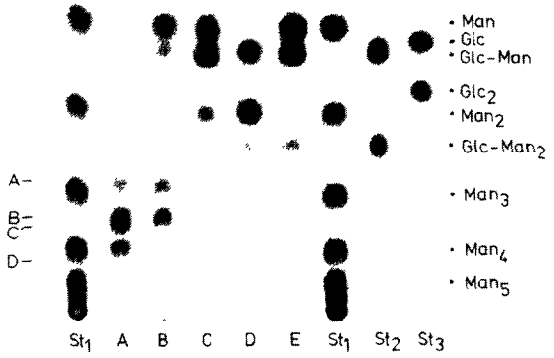


Fig. 8. T.l.c. of the products of hydrolysis by *A. niger* β-D-mannanase and *H. pomatia* β-D-mannosidase of the tetrasaccharide fraction produced on partial hydrolysis of insoluble salep-glucomannan by *A. niger* β-D-mannanase: A, tetrasaccharide; B, A plus β-D-mannosidase; C, A plus β-D-mannosidase, heat inactivation, and then β-D-mannanase; D, A plus β-D-mannanase; E, A plus β-D-mannanase, heat inactivation, and then β-D-mannosidase; St_1, mannose–mannohexaose; St_2, (1→4)-β-D-glucosyl-D-mannose plus (1→4)-β-D-glucosyl-(1→4)-β-D-mannobiose; St_3, glucose plus cellobiose.

are based on the observations that *D* co-chromatographed with (1→4)-β-D-mannotetraose and was hydrolysed by β-D-mannosidase. *A* and *B* were not affected by β-D-mannosidase, and *C* was incompletely hydrolysed. The products from incubation of the mixture were unchanged *A* and *B*, mannose, and β-Glc-(1→4)-Man, indicating that *C* was Man→Man→Glc→Man. If the β-D-mannosidase in the hydrolysate was then denatured and β-D-mannanase added, then mannobiose and more β-Glc-(1→4)-Man were produced, but no other disaccharides, consistent with structures of Glc→Man→Glc→Man and Glc→Man→Man→Man. Since glucose-containing oligosaccharides are more mobile than mannose-containing oligosaccharides, *A* is probably the former.

When the whole tetrasaccharide-fraction was treated with β-D-mannanase, a minor product was a trisaccharide fraction that co-chromatographed with β-Glc-(1→4)-β-Man-(1→4)-Man and which was resistant to β-D-mannosidase. Its production from the total mixture, but not on hydrolysis of tetrasaccharides *A* and *B*, can be explained in terms of transglycosylation and the subsite-binding requirements of β-D-mannanase (see Discussion).

The pentasaccharide fraction was completely hydrolysed by β-D-mannanase to only (1→4)-β-D-mannobiose, β-Glc-(1→4)-Man, and β-Glc-(1→4)-β-Man-(1→4)-Man, with no cellobiose, mannotriose, or trisaccharides containing more than one glucose residue. β-D-Mannosidase gave limited hydrolysis, indicating that these oligosaccharides mainly had a glucosyl group at the non-reducing end.

The β-D-mannanases from all sources gave only the same oligosaccharides of d.p. up to 4 that were produced by the *A. niger* enzyme. Man→Glc→Man, Glc→Glc→Man, and cellobiose were not detected in any hydrolysate, although these have been previously reported from hydrolyses of konjac glucomannan with konjac β-D-mannanase[10].

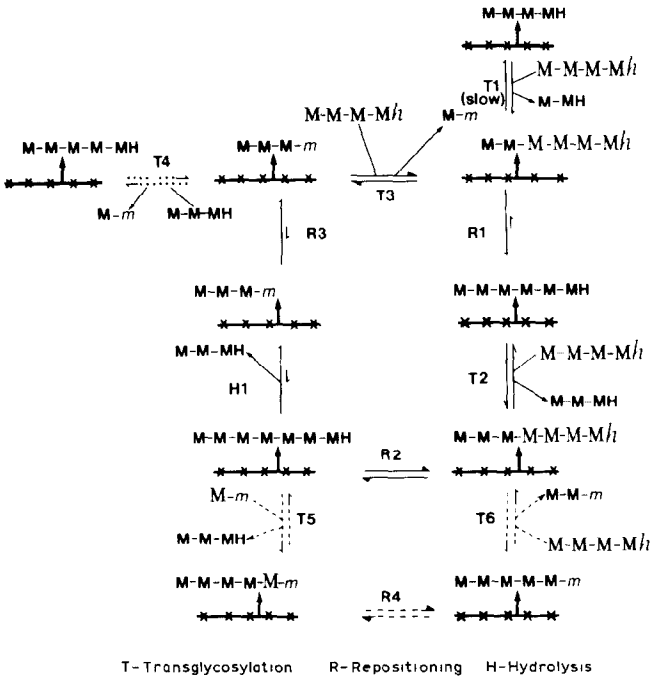
DISCUSSION

The relative rates of hydrolysis of the tri-, tetra-, penta- and hexa-oses (Fig. 1 and Table I) indicate that a chain of at least four mannose residues is required for a significant hydrolysis rate; with five subsites (pentaose), the initial rate is as high as with the hexaose, suggesting five significant binding-subsites. These can be designated as α–ε on the enzyme, with ε toward the reducing end of the substrate; the five neighbouring pyranose rings that bind in the substrates are designated as A–E. Since the products of hydrolysis contain only traces of mannose, the catalytic site is between C and D, or B and C. The hydrolytic behaviour of galactomannosaccharides indicates that hydrolysis occurs between pyranose rings C and D (see later).

Transglycosylation is significant during the hydrolysis of mannosaccharides. This has previously been described for preparations from fenugreek seeds[16]. With the tetraose, the enzyme gave mannotriose and mannobiose with only traces of mannose, and it was proposed that this resulted from transfer of initially produced

mannobiose to the tetraose, giving the hexaose, which was then hydrolysed to mannotriose, in addition to direct hydrolysis of the tetraose to two molecules of mannobiose. Mannohexaose was not detected. The lucerne-seed enzyme was reported[14] as catalysing only a hydrolytic mechanism. In the present study, reaction with the lucerne enzyme resembled the activity from fenugreek, effecting transglycosylation. Chromatographic evidence was obtained for the transient production of higher oligosaccharides.

Considering the products from the reaction of *A. niger* β-D-mannanase with reduced mannosaccharides of d.p. 4 and 5, any reaction sequence must explain the transient production of significant amounts of oligosaccharides having d.p. one and two higher than the starting material, and the production of mannobiose, but only traces of mannobi-itol, from mannotetraitol. A cyclic sequence for mannotetraitol that requires the initial production of only a trace of mannobi-itol from a slow transglycosylation reaction (T1), to give these transient and final products, is shown in Scheme 1. The sequence can be applied generally to any mannosaccharide substrate having fewer than the optimal number of binding sites, *e.g.*, to mannopentaitol and mannotetraose, and may also occur with such similar enzymes as cellulase and xylanase. In the rearrangements designated R1 and R3, the negative free-energy change resulting from the increased number of binding subsites favours the reaction direction shown (clockwise). In the transglycosylation reac-



Scheme 1. Reaction of mannotetraitol with *A. niger* β-D-mannanase.

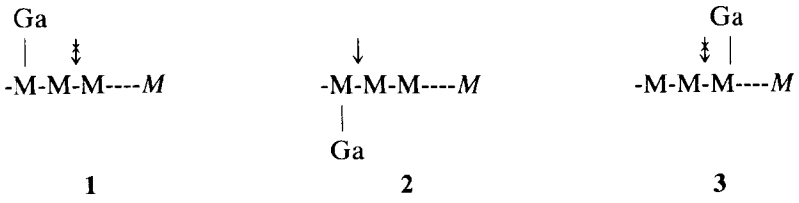
tions, T1 is very slow, T4 is a "dead-end" reaction since the product cannot reposition, and for T5 to occur, sufficient mannobiose must first be produced. The cycle with the broken arrows is designed to explain the appearance of mannotriose. This bicyclic system shows how the degradation of mannotetraitol can proceed without the production of significant levels of mannobi-itol, but with the production of the oligomers indicated by chromatography. All of the reactions within the cycle are much more rapid than the first transglycosylation (T1). The production of a significant amount of fractions of d.p. 4 and 7 during reaction of mannopentaose with β-D-mannanase shows that transglycosylation followed by repositioning also occurs with larger substrates.

Using [^{3}H]mannosaccharides, it was concluded[34] that *Streptomyces* β-D-mannanase transferred single mannosyl groups, whereas fenugreek enzyme transferred oligosaccharides. Transfer of a single monosaccharide is inconsistent with present knowledge of the pattern of binding and hydrolysis by endo-hydrolases[1,29,30,35,36], *e.g.*, lysozyme, dextranase, alpha-amylase, cellulase, and xylanase. X-Ray crystallography with lysozyme shows a catalytic site that fits the half-chair conformation[37] at the carboxonium ion, and this model is supported by inhibition studies with tetra-*N*-acetylchitotetraonolactone[38]. The transfer of a single repeating-unit by testicular hyaluronidase has been discussed[30], but the repeating unit is a disaccharide and the enzyme has specificity for the uronic acid residue whose glycosidic bond is not hydrolysed.

Although the K_m for mannohexaitol is of the same order as those for *Leucaena* and carob galactomannans, the relative V_{max} values of the galactomannans are 2.5 times that of the oligosaccharide (Table I). This probably reflects the extent of transglycosylation occurring with the smaller substrate, even when initial reaction-rates are being measured, and the capacity for multiple attack on a single polysaccharide chain.

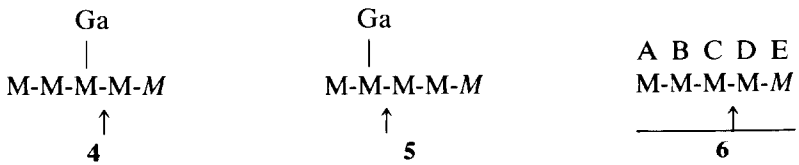
Substitution of the mannose residues in mannan with galactose interferes with hydrolysis. Generally, as the galactose content increases, the amount of hydrolysis by β-D-mannanase decreases, the V_{max} decreases, and the K_m increases[2,5,6,9,39,40]. If D-galactosyl residues are partly removed from a highly substituted galactomannan, there is an increase in the rate of hydrolysis. This relationship holds for *A. niger* β-D-mannanase (Table III). However, hetero-oligosaccharides having a d.p. as low as 3 are released in significant amounts, showing that β-D-mannanase can still hydrolyse the mannan chain, even if it does not have a sequence of five neighbouring, unsubstituted mannosyl residues. That is, substituting galactosyl groups do not interfere completely. Information on the action pattern and binding between glycan and enzyme can be obtained from the presence and absence of heterosaccharides released on hydrolysis (Table IV). 6-α-D-Galactosylmannose, Gal^2Man_2, Gal^2Man_3, and Gal^3Man_3 (Tables IV and V) are not released, and the smallest oligosaccharide having two adjacent D-galactosyl residues is $Gal^{3,4}Man_5$. Galactosyl residues are absent from the non-reducing, terminal mannosyl groups in all structures. The penultimate mannosyl residue from the reducing end is always unsubstituted. Therefore, hydrolysis of the glycosydic linkage

of a mannosyl residue cannot occur if there is galactosyl substitution on the hydroxymethyl group of the next mannosyl residue towards the non-reducing end, as in **1.** On the other hand, substitution of the mannosyl residue whose glycosidic bond is cleaved has little effect on hydrolysis, as in **2**. If the hydroxymethyl group on the mannosyl residue on the reducing side of the hydrolysed bond is substituted, as in **3**, then hydrolysis is blocked.



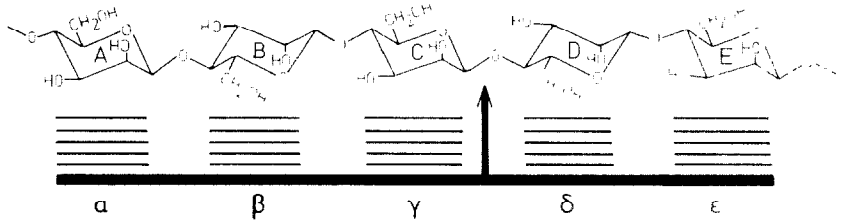
1 **2** **3**

The position of the catalytic site, relative to the five binding-subsites, was established from reaction with an equimolar mixture of the two hexasaccharides **4** and **5**, when the only hydrolysis products were mannobiose, mannotriose, Gal^1Man_2, and Gal^1Man_3 (Fig. 5). These would result from hydrolysis at the positions shown by the arrows. In one of the oligosaccharides, the position of hydrolysis would allow only four binding-subsites. Since the tetrasaccharide Gal^1Man_3 was released more rapidly than the trisaccharide Gal^1Man_2, the catalytic site lies between pyranose rings C and D, as in **6**.



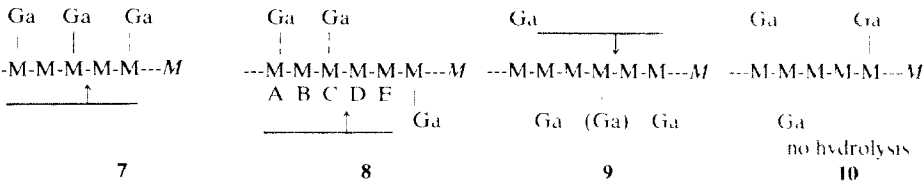
4 **5** **6**

The hydrolysis products of carob galactomannan by *A. niger* β-D-mannanase can then be explained, if the enzyme primarily binds to alternate edges of the five pyranose rings. Rings B and D bind through the hydroxymethyl groups (and possibly the ring oxygen), and rings A, C, and E through the other edge (*i.e.*, through HO-2 and/or HO-3). The favoured conformation of the (1→4)-β-D-linked mannan chain is a flat ribbon with a two-fold axis, which places neighbouring hydroxymethyl groups on opposite sites of the ribbon[41–43], so that, in galactomannan, galactosyl substituents separated by zero or an even number of mannosyl residues lie on opposite sides of the chain, and those separated by an odd number, on the same side. Then the enzyme binds to one edge of this ribbon, as in Scheme 2. Galactosyl substitution on either ring B or D would block binding; on rings A, C, or E, it would not, thereby allowing hydrolysis to proceed between rings C and D.



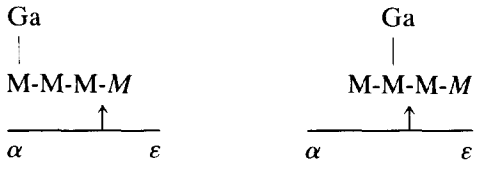
Scheme 2 Schematic representation of subsite binding between *A. niger* β-D-mannanase and the (1→4)-β-D-mannan chain

Structural requirements for *A. niger* β-D-mannanase hydrolysis of galactomannan chains cannot be defined only by the number of unsubstituted mannosyl residues between galactosyl-substituted units. The glycosidic linkage of a substituted mannosyl residue can be hydrolysed, if there are unsubstituted mannosyl residues on both sides of it, as in **7**. Where there are two adjacent, unsubstituted residues on the reducing side of a substituted mannosyl residue and at least one unsubstituted on the non-reducing side, as in **8**, then the only susceptible bond is between pyranose rings C and D. If two or more neighbouring mannosyl residues are substituted, then, towards the reducing end of the molecule, three unsubstituted residues or a sequence of unsubstituted-substituted-unsubstituted residues can be split (**9**), but not a sequence of two unsubstituted residues, as in **10**. (Ga) represents an optional substitution.



The oligosaccharide products of hydrolysis of carob galactomannan with *A. niger* β-D-mannanase came from the segments of polymer shown in Table IV.

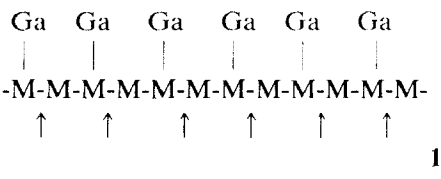
The relative significance of sites α and ε can be established from a comparison of the hydrolysis of oligosaccharides **11** and **12**. The maximum subsite-binding for each occurs as shown. Gal^3Man_4 (**12**) was hydrolysed at a rate similar to mannotetraose, which was ten times the rate for Gal^4Man_4 (**11**). Oligosaccharide **11** produced almost exclusively Gal^3Man_3 and mannose, and was hydrolysed at only ~10 times the rate for mannotriose. Oligosaccharide **12** produced only Gal^1Man_2 and mannobiose. The rates indicate that binding at ε is more significant in hydrolysis than at α. This is supported by the observation that Gal^3Man_5 (**4**) is hydrolysed much more rapidly than Gal^4Man_5 (**5**) (*cf.* Fig. 5).



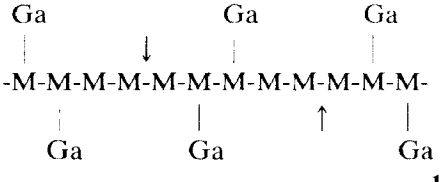
11 **12**

The nature and amounts of oligosaccharides produced by different β-D-mannanases indicate (Table V) that similar rules apply to all β-D-mannanases, but that not all linkages split by the *A. niger* enzyme can be hydrolysed as readily by the others. Some of the heterosaccharides not attacked by guar, lucerne, *B. subtilis*, and *H. pomatia* enzymes reflect a limited ability to cleave mannosaccharides having d.p. <5. However, the presence of such compounds as Gal^3Man_5 and Gal^4Man_5, combined with the absence of mannopentaose, indicates that a galactosyl substituent on ring C has an effect on binding and hydrolysis by these enzymes. The ratio of galactosylmannotetraoses produced by the enzymes is consistent with this conclusion. There is always much more 6^3 substituted than 6^1. The effect becomes more pronounced on moving from *I. lacteus* to guar β-D-mannanases. The absence of any 6^1-α-D-galactosyl-substituted oligosaccharides in guar-enzyme hydrolysates shows that substitution of the C ring stops hydrolysis completely with this enzyme. However, galactosyl substituents on ring C and E do not affect hydrolysis by *A. niger* β-D-mannanase. A 4:1 mixture of Gal^3Man_4 and Gal^1Man_4 had an initial rate of hydrolysis slightly higher than that for mannotetraose. $Gal^{1,3}Man_4$ is present in hydrolysates of lucerne, *B. subtilis*, and *H. pomatia*, but not in those of *A. niger*, *I. lacteus*, and guar. Its non-appearance with guar β-D-mannanase is caused by the inability of this enzyme to hydrolyse the glycosidic linkage of a substituted mannosyl residue; with *A. niger* and *I. lacteus* β-D-mannanases, the reason is further hydrolysis to Gal^1Man_2.

β-D-Mannanase from *I. lacteus* hydrolyses *L. leucocephala* galactomannan rapidly[3], releasing Gal^1Man_2 as the major product of low d.p. A comparison of the ability of a particular β-D-mannanase to depolymerise this polysaccharide, relative to carob galactomannan, should include the pattern of substitution across five binding-subsites. Glycan segment **13**, which has a galactose:mannose ratio of 1:2, has six potential hydrolysis-sites with *A. niger* and *I. lacteus* β-D-mannanases, indicated by the arrows. Segment **14**, with the same galactose:mannose ratio, has only two scission-sites. The high degree of hydrolysis of *Leucaena leucocephala* galactomannan, despite its high galactose-to-mannose ratio (Table III), has been attributed[2] to a high content of such sequences as **13**.



13 reducing end →

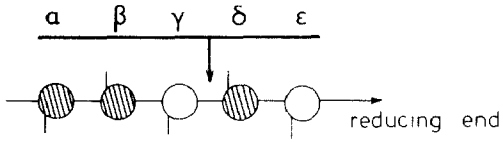


14 reducing end →

On hydrolysis of deacetylated glucomannan by *A. niger* β-D-mannanase, not all of the theoretically possible oligosaccharides of glucose and mannose were produced. The products can be predicted from the model shown in Scheme 2. Pyranose rings B and D present the hydroxymethyl edge of the ring; therefore, glucose or mannose can bind because the stereochemistry of both molecules on that side of the ring is similar. Rings C and E present the HO-2,3 edge of the ring and, as these differ in the stereochemistry of HO-2 in glucose and mannose, only mannose residues will bind. The isolation of Glc→Man→Man indicates that the stereochemical requirement for binding at the fifth (α) site is not as rigid, so that binding can occur to both glucose and mannose. Perhaps only HO-3 is involved. Alternatively, four binding sites may be sufficient for hydrolysis. Hydrolysis of galactosylmannotetraoses indicated the greater significance of the ε site over the α.

Binding therefore occurs as shown in Scheme 3. The heterosaccharide products arise as shown in Table VI; the oligosaccharides not produced are shown in Table VII together with the binding subsites where binding cannot occur.

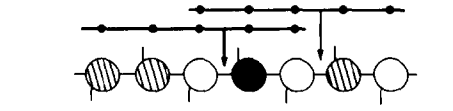
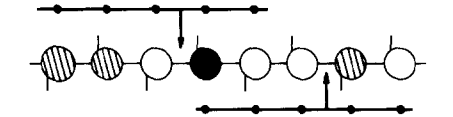
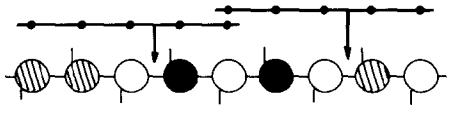
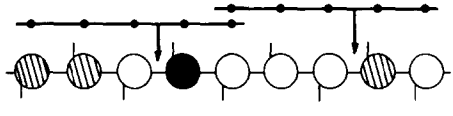
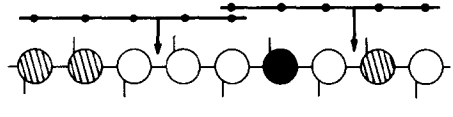
The production of the trisaccharide Glc→Man→Man on β-D-mannanase treatment of the whole tetrasaccharide fraction from hydrolysis of glucomannan by β-D-mannanase, and its absence when only the mixture of Glc→Man→Man→Man and Glc→Man→Glc→Man was incubated, is also a consequence of the binding-subsite requirements. If Glc→Man→Man→Man or Glc→Man→Glc→Man undergoes transglycosylation with mannotetraose, the product is



Scheme 3 Subsite binding between *A. niger* β-D-mannanase and glucomannan
Key: ○, glucose or mannose; ●, mannose; the projection indicates the orientation of the [illegible]

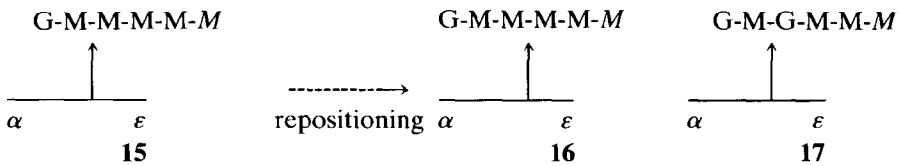
TABLE VI

BINDING AND HYDROLYSIS THAT PRODUCES THE HETERO-OLIGOSACCHARIDES RELEASED ON REACTION OF *A. niger* β-D-MANNANASE AND GLUCOMANNAN

Polymer section[a] (reducing end ⟶)	Oligosaccharide products
	Glc→Man
	Glc→Man→Man
	Glc→Man→Glc→Man
	Glc→Man→Man→Man
	Man→Man→Glc→Man

[a] Key ◎, glucose or mannose, ○, mannose, ●, glucose
The projection indicates the orientation of the $-CH_2OH$ group

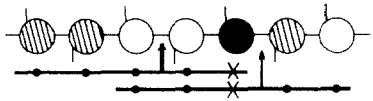
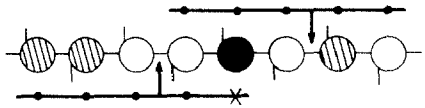
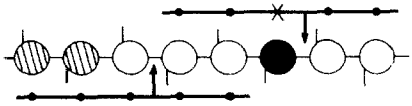
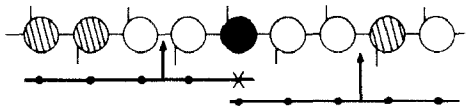
Glc⟶Man⟶Man⟶Man⟶Man⟶Man (**15**). On repositioning on the active site (**16**), this hydrolyses to Glc⟶Man⟶Man and Man⟶Man⟶Man. If any other tetrasaccharide containing glucose at the non-reducing terminal were involved in transglycosylation as the second substrate, the product **17** could not reposition, as this would require glucose to be bound to the γ binding-subsite and this cannot occur.



The results obtained using glucomannan as a substrate complement those with galactomannan, and both are consistent with the model illustrated in Scheme 2, where subsites β and δ bind to the hydroxymethyl edge of a pyranose ring

TABLE VII

SOME HETERO-OLIGOSACCHARIDES NOT PRODUCED ON REACTION OF *A. niger* β-D-MANNANASE AND GLUCOMANNAN, AND THE BINDING SUBSITES THAT CANNOT FORM

Oligosaccharide	*Required binding "x" shows a subsite that cannot occur*[a] *(reducing end ⟶)*
Man→*Glc*	
Man→Glc→*Man*	
Man→Man→*Glc*	
Man→Glc→Man→*Man*	

[a] Key ○, glucose or mannose; ◐, mannose; ●, glucose. The projection indicates the orientation of the CH_2OH group.

(equatorial CH_2OH) and α, γ, and ϵ bind to the HO-2,3 edge. Binding at γ and ϵ requires an axial HO-2 and an equatorial HO-3; at the α position, HO-2 can probably be axial or equatorial. This pattern applies generally to all of the β-D-mannanases examined, but the presence of a galactosyl substituent on mannose residue C (whose glycosidic bond is split) has a variable effect with the enzymes from various sources. At one extreme, *A. niger* β-D-mannanase, there is little or no effect; at the other, the guar-seed enzyme, reaction is blocked completely.

The results are significant with respect to the relationship between the degree of hydrolysis and the amount of galactosyl substitution as well as the distribution of galactosyl substituents along the (1→4)-β-D-mannan chain.

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