Carbohydrate Research, 118 (1983) 91–109
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

CHARACTERISATION OF THE OLIGOSACCHARIDES PRODUCED ON HYDROLYSIS OF GALACTOMANNAN WITH β-D-MANNANASE

BARRY V. MCCLEARY, ELIZABETH NURTHEN,
Biological and Chemical Research Institute, N.S.W. Department of Agriculture, Rydalmere 2116 (Australia)

FRANÇOIS R. TARAVEL, AND JEAN-PAUL JOSELEAU
Centre de Recherches sur les Macromolécules Végétales (C.N.R.S.) 53X, 38041 Grenoble Cedex (France)

(Received November 8th, 1982; accepted for publication, February 2nd, 1983)

ABSTRACT

Treatment of hot-water-soluble carob galactomannan with β-D-mannanases from *A. niger* or lucerne seed affords an array of D-galactose-containing β-D-mannosaccharides as well as β-D-manno-biose, -triose, and -tetraose (lucerne-seed enzyme only). The D-galactose-containing β-D-mannosaccharides of d.p. 3–9 produced by *A. niger* β-D-mannanase have been characterised, using enzymic, n.m.r., and chemical techniques, as 6^1-α-D-galactosyl-β-D-mannobiose, 6^1-α-D-galactosyl-β-D-mannotriose, 6^3,6^4-di-α-D-galactosyl-β-D-mannopentaose (the only heptasaccharide), and 6^3,6^4-di-α-D-galactosyl-β-D-mannohexaose, 6^4,6^5-di-α-D-galactosyl-β-D-mannohexaose, and 6^1,6^3,6^4-tri-α-D-galactosyl-β-D-mannopentaose (the only octasaccharides). Four nonasaccharides have also been characterised. Penta- and hexa-saccharides were absent. Lucerne-seed β-D-mannanase produced the same branched tri-, tetra- and hepta-saccharides, and also penta- and hexa-saccharides that were characterised as 6^1-α-D-galactosyl-β-D-mannotetraose, 6^3-α-D-galactosyl-β-D-mannotetraose, 6^1,6^3-di-α-D-galactosyl-β-D-mannotetraose, 6^3-α-D-galactosyl-β-D-mannopentaose, and 6^4-α-D-galactosyl-β-D-mannopentaose. None of the oligosaccharides contained a D-galactose stub on the terminal D-mannosyl group nor were they substituted on the second D-mannosyl residue from the reducing terminal.

INTRODUCTION

Hydrolysis of galactomannans by β-D-mannanase affords an array of oligosaccharides, including the β-D-mannosaccharides of degree of polymerisation (d.p.) 2–6 and a range of mixed oligosaccharides containing both D-galactose and D-mannose[1]. The nature of the oligosaccharides produced by different β-D-mannanases varies, indicating that there are differences in the action patterns[2] of the enzymes.

0008-6215/83/$ 03.00

Initial studies[3] indicated that the D-galactosyl group of α-D-galactosyl-(1→4)-β-D-mannobiose was located exclusively on the non-reducing D-mannosyl residue, and that the D-galactosyl group of α-D-galactosyl-(1→4)-β-D-mannotriose was located either on the non-reducing terminal[3], or on the central D-mannosyl residue[4,5]. However, recent studies[6] employing n.m.r., enzymic, and chemical procedures clearly demonstrated that 6^1-α-D-galactosyl-(1→4)-β-D-mannobiose and 6^1-α-D-galactosyl-(1→4)-β-D-mannotriose were the only D-galactose-containing tri- and tetra-saccharides produced on hydrolysis of carob galactomannan by purified β-D-mannanases from culture filtrates of *A. niger*, *Irpex lacteus*, *Bacillus subtilis* TXI, the gut solution of *Helix pomatia*, and extracts of germinating seeds of lucerne and honey locust. Although these oligosaccharides were present in each hydrolysate, the relative amounts varied significantly.

D-Galactose-containing (1→4)-β-D-mannosaccharides of d.p. >4, reported to be produced on hydrolysis of galactomannan by β-D-mannanase[3,5,7,8], include a pentasaccharide (Man/Gal = 4:1), a hexasaccharide (Man/Gal = 4:2)[3,4], and a heptasaccharide (Man/Gal = 4:3)[3,4]. The location of the D-galactosyl residue(s) on the D-mannosaccharide chain was not determined[3-8]. The aim of the current study was to characterise these oligosaccharides of higher d.p. by applying the enzymic, n.m.r., and chemical procedures previously developed[6].

EXPERIMENTAL

Chromatography. — T.l.c. was performed as previously described[6,9]. The solvents employed were *A*, 7:1:2 1-propanol–ethanol–water; *B*, 5:2:3 1-propanol–nitromethane–water. Gel-permeation chromatography was performed on a column (2.5 × 80 cm) of Bio-Gel P-2 (<400 mesh) at 60° in distilled water[10].

Preparation of galactomannans. — Galactomannans were prepared by a modification of a previously described method[11]. Seeds (milled to pass a 0.7-mm mesh screen), or commercial flours, were treated with boiling, aqueous 80% ethanol for 10 min. The slurry was collected on sintered glass, and washed successively with ethanol, acetone, and ether. The flours from 10 g of seed, or commercial flours (10 g), were treated with ethanol (1–2 mL), and suspensions in cold water (200 mL) were stored at 4° for 20 h, and then equilibrated to room temperature (~22°) and homogenised by using a Waring Blendor at maximum speed for 2 min. Each slurry was centrifuged (4,000*g*, 30 min), and the pellet was re-extracted (usually twice) until no further polysaccharide was detectable in the supernatant solution. Each supernatant solution was added to ethanol (2 vol.), and the recovered polysaccharide (cold-water-soluble galactomannan) redissolved in water (to ~0.2%) and centrifuged (20,000 *g*, 1 h). The polysaccharide in the supernatant solution was precipitated with ethanol, washed with ethanol, acetone, and ether, and dried *in vacuo*. In general, further purification *via* copper complexation[12] was not necessary. The pellet remaining on extraction of cold-water-soluble galactomannan was suspended in hot water by blending, and incubated at ~100° (steam bath) for

30 min. The slurry was homogenised by using a Waring Blendor and centrifuged (4,000*g*, 30 min). The pellet was re-extracted twice and the combined supernatants were treated with ethanol (2 vol.). The precipitated polysaccharide (hot-water-soluble galactomannan) was purified as for the cold-water-soluble galactomannan fraction.

Using this extraction procedure, the yield of cold-water-soluble and hot-water-soluble galactomannan obtained on extraction of the commercial carob flour "Indal" (Industrias de Alfarrova, Portugal) was 27.7 and 41.2%, respectively. The hot-water-soluble fraction had a Gal/Man ratio of 19:81, and a limiting viscosity number (l.v.n.) of 1305 mL/g, and the cold-water-soluble fraction had a Gal/Man ratio of 26:74 and an l.v.n. of 1180 mL/g.

Preparation of enzymes. — β-D-Mannanases from *A. niger* (commercial Cellulase preparation, Sigma C7052) and from germinated lucerne-seed were purified as previously described[2], employing substrate-affinity chromatography on glucomannan–AH-Sepharose. Last traces of α-D-galactosidase in the *A. niger* β-D-mannanase preparation[2] were removed by chromatography of the preparation on an α-D-galactosidase affinity-support. The purified enzymes were devoid of α-D-galactosidase, β-D-mannosidase, and exo-β-D-mannanase activities (<1 p.p.m.), and gave single protein bands on isoelectric focusing. α-D-Galactosidase II from germinating guar-seed was purified by affinity chromatography[6]; it gave a single protein band on isoelectric focusing, and contamination with β-D-mannanase and exo-β-D-mannanase was <1 p.p.m. Guar seeds do not contain β-D-mannosidase. Exo-β-D-mannanase from germinating guar-seed[9] and β-D-mannosidase from crude, *Helix pomatia* gut-solution were prepared as previously described. Both enzymes were devoid of β-D-mannanase (endo-β-D-mannanase) and appeared as single protein bands on SDS-gel electrophoresis. The exo-β-D-mannanase was devoid of α-D-galactosidase, and the β-D-mannosidase was contaminated to <0.01%.

Hydrolysis of hot-water-soluble carob galactomannan by β-D-mannanase. — To solutions of hot-water-soluble carob galactomannan (1 L, 0.4%) in 10mM acetate buffer (pH 4.5) was added highly purified *A. niger* or lucerne-seed β-D-mannanase (400 nkat on carob galactomannan). The solutions were incubated at 40° for 20 h, and then at 100° for 10 min to inactivate β-D-mannanase, centrifuged (4,000*g*, 30 min), concentrated to a syrup, and adjusted to a concentration of 10% w/v. Aliquots (2–5 mL) of this solution were fractionated by chromatography on Bio-Gel P-2. A sample of the hydrolysate was diluted to 1 mg/mL, and aliquots were removed for the determination of total carbohydrate (anthrone procedure[13]), reducing sugar (*p*-hydroxybenzohydrazide method[14]), and galactose[15,16]. From these values, the degree of β-D-mannanase hydrolysis[8] and the galactose/mannose ratio of the polysaccharide were determined. The α-D-galactosidase employed here removed all of the D-galactosyl residues from each of the galactomannans studied, when used in the presence of trace levels of β-D-mannanase to prevent precipitation of mannan chains containing low levels of D-galactose[16].

Determination of galactose/mannose ratios. — Aliquots (0.1 mL) of a solution

(~1 mg/mL) of galactomannan or oligosaccharide were used for determination of total carbohydrate by the anthrone procedure[13]. Other aliquots (0.2 mL) were treated[16] with crude, guar α-D-galactosidase II (33 nkat, 20 μL) plus 0.1M acetate buffer (0.2 mL, pH 4.5) at 40° for 1 h. The pH was adjusted by adding 0.2M Tris·HCl buffer (2.5 mL, pH 8.6), and galactose was determined as previously reported[16].

Enzymic characterisation of the structures of oligosaccharides. — To solutions of the oligosaccharide (50 μL, 10 mg/mL) were added 100mM acetate buffer (10 μL, pH 4.5) plus *H. pomatia* β-D-mannosidase (20 μL, 40 nkat on *p*-nitrophenyl β-D-mannopyranoside)[17]; or guar-seed α-D-galactosidase II[15] (20 μL, 10 nkat on *p*-nitrophenyl α-D-galactopyranoside); or *A. niger* β-D-mannanase[2] (20 μL, 6 nkat on carob galactomannan); and the solutions were incubated at 40° for 20 h. Aliquots (15 μL) were used for t.l.c. (solvents *A* or *B*).

Partial hydrolysis of hepta-, octa- and nona-saccharides with α-D-galactosidase. — To solutions of the oligosaccharide (4 mL, 10 mg/mL) were added 500mM acetate buffer (80 μL, pH 4.5) and guar-seed α-D-galactosidase II[15] (140 μL, 120 nkat). The solutions were incubated at 40° for 20 min, and then at 100° for 2 min to terminate the reaction. Such treatment removed ~60% of the D-galactosyl groups. The reaction mixture was fractionated by chromatography on Bio-Gel P-2, and the recovered fractions were characterised by treatment with excess of α-D-galactosidase II[18], *H. pomatia* β-D-mannosidase[17], or *A. niger* β-D-mannanase[2] (as described above), followed by t.l.c. In some cases, the reaction products were again fractionated by chromatography on Bio-Gel P-2 and the fractions further characterised by enzymic procedures. Galactose/mannose ratios were determined as already described.

Preparation of 6^3-α-D-galactosyl-β-D-mannotetraose. — The pentasaccharide fraction produced on hydrolysis of hot-water-soluble carob galactomannan by lucerne-seed β-D-mannanase contained 6^3-α-D-galactosyl-β-D-mannotetraose and 6^1-α-D-galactosyl-β-D-mannotetraose in the ratio ~4:1. The latter oligosaccharide was removed by treating a solution of the mixture with exo-β-D-mannanase from guar seeds[6, 19] (20 nkat on β-D-mannopentaitol/50 mg of oligosaccharide) for 6 h at 40° and pH 5.5. Pure 6^3-α-D-galactosyl-β-D-mannotetraose was then obtained by chromatography on Bio-Gel P-2.

Methylation analysis[20]. — An oligosaccharide sample (5 mg) was dissolved in dry dimethyl sulphoxide (1 mL), and methylsulphinylmethanide anion (0.8 mL) was added. The mixture was stirred for 2 h under nitrogen, and methyl iodide (1 mL) was added slowly during 1 h to avoid heating of the mixture. The mixture was diluted with water, and the methylated material was extracted with chloroform and washed several times with water.

The methylated oligosaccharides were hydrolysed in 90% formic acid for 1 h at 100°, and then in 2M trifluoroacetic acid for 3 h at 100°. After evaporation of the acid, the partially methylated sugars were reduced to alditols with sodium borohydride, and the products were acetylated with pyridine–acetic anhydride for 1 h at 100° and then analysed by g.l.c.–m.s.[21].

G.l.c. was performed on a column of 3% of SP 2340 on Chromosorb W, with a temperature programme of 180° for 2 min and then 2°/min up to 220°. Quantitative analysis was performed with a Hewlett–Packard 3380A integrator. Mass spectra were recorded with an A.E.I. MS30 instrument, using a 20-m capillary column (OV 17) at 175°. The ionising potential was 70 eV and the temperature of the ionising chamber was 150°. For the permethylated oligosaccharides, the temperature of introduction was in the range of 30–80°, depending on the volatility of the samples.

N.m.r. analysis. — Samples were dissolved in D_2O (20–40 mg/mL). The deuterium resonance was used as the field-frequency lock, and acetone as the internal reference (4%). The probe temperature was 70°.

^{13}C-Spectra (62.8 MHz) were recorded with a Bruker WM250 spectrometer equipped with an Aspect 2000 computer. Free induction decays were accumulated with 16k data block-size and a spectral width of 6000 Hz. The pulse width was 15 μs and the acquisition time 1.36 s (resolution, 0.735 Hz per point). Chemical shifts were expressed in p.p.m. downfield from internal acetone (fixed at 31.07 p.p.m. from D.S.S.)

RESULTS

Fractionation of oligosaccharides. — The Bio-Gel P-2 chromatographic-patterns obtained on hydrolysis of hot-water-soluble carob galactomannan by *A. niger* and lucerne-seed β-D-mannanases are shown in Fig. 1. *A. niger* β-D-mannanase catalysed more extensive hydrolysis than did lucerne-seed β-D-mannanase, and this difference was reflected in the products. The major products of hydrolysis by *A. niger* β-D-mannanase were di-, tri-, tetra-, hepta-, octa-, and nona-saccharides with minor quantities of D-mannose and oligosaccharides having higher d.p. Penta- and hexa-saccharides were absent (Fig. 1). The lucerne-seed β-D-mannanase hydrolysate contained significant amounts of penta- and hexa-saccharides, and appreciably more material of d.p. >7.

Enzymic analysis of oligosaccharides. — (*a*) *Mono-, di-, tri- and tetra-saccharide fractions.* The only monosaccharide present was chromatographically identical with D-mannose, and the single disaccharide chromatographed as (1→4)-β-D-mannobiose and gave only D-mannose on acid hydrolysis or treatment with *Helix pomatia* β-D-mannosidase[17]. The disaccharide epimelibiose (6-α-D-galactosyl-D-mannose), reported[7] to be a product of β-D-mannanase hydrolysis of galactomannan, was not present in these hydrolysates. Two trisaccharides (β-D-mannotriose and 6^1-α-D-galactosyl-β-D-mannobiose) and a single tetrasaccharide (6^1-α-D-galactosyl-β-D-mannotriose) were present, and were characterised by using purified α-D-galactosidase and β-D-mannosidase, as previously described[6].

(*b*) *Pentasaccharide fraction.* — The pentasaccharide fraction produced by lucerne-seed β-D-mannanase (Fig. 1) migrated as a single spot in t.l.c., with a mobility similar to that of β-D-mannopentaose. However, α-D-galactosidase quantita-

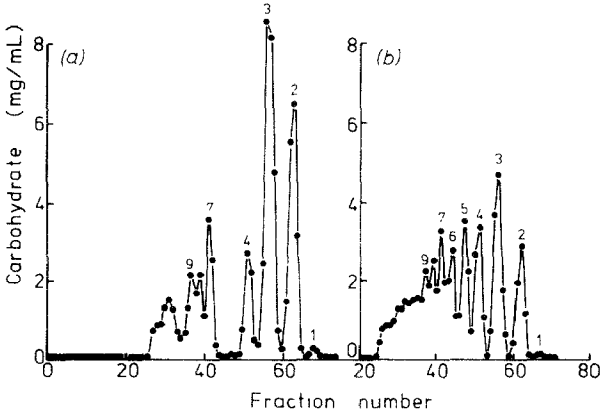


Fig. 1 Chromatography, on a column (2.5 × 80 cm) of Bio-Gel P-2 (<400 mesh) with distilled water at 60°, of the oligosaccharides produced on hydrolysis of hot-water-soluble carob galactomannan by (*a*) *A niger* and (*b*) lucerne-seed β-D-mannanases. Fraction volume, 5.4 mL.

tively hydrolysed this fraction to D-galactose and an oligosaccharide chromatographically indistinguishable from β-D-mannotetraose. This result demonstrated that β-D-mannopentaose was absent, and was consistent with the determined galactose–mannose ratio of 19:81. Exhaustive treatment with snail β-D-mannosidase effected complete hydrolysis to D-mannose plus a galactose-containing tetra- and di-saccharide. Since snail β-D-mannosidase sequentially removes[6] single D-mannosyl groups from the non-reducing terminal of (1→4)-linked β-D-mannosaccharides, but is unable to cleave beyond a D-mannosyl residue substituted by D-galactose, it is concluded that the pentasaccharide fraction contains two oligosaccharides in which the D-galactosyl groups are located on either the reducing D-mannose or the third D-mannosyl residue from the reducing end, *i.e.*, that the oligosaccharides are 6^1-α-D-galactosyl-β-D-mannotetraose and 6^3-α-D-galactosyl-β-D-mannotetraose. This mixture, on treatment with *A. niger* β-D-mannanase, quantitatively yielded β-D-mannobiose and 6^1-α-D-galactosyl-β-D-mannobiose, consistent with the proposed structures. The susceptibility of the pentasaccharide fraction to hydrolysis by *A. niger* β-D-mannanase explains its absence from the oligosaccharide mixtures produced on hydrolysis of hot-water-soluble carob galactomannan by *A. niger* β-D-mannanase (Fig. 1).

(*c*) *Hexasaccharide fraction.* The hexasaccharide fraction present in the lucerne-seed β-D-mannanase hydrolysate had a Gal/Man ratio of 16:84. Treatment with α-D-galactosidase effected complete hydrolysis to D-galactose plus β-D-mannopentaose and a small proportion of β-D-mannotetraose, indicating that the major hexasaccharide was β-D-mannopentaose substituted by a single D-galactosyl group, and that the minor hexasaccharide was β-D-mannotetraose substituted by two D-galactosyl groups. Treatment of the hexasaccharide fraction with snail β-D-mannosidase gave D-mannose, two major components having chromatographic

mobilities similar to those of α-D-galactosyl-β-D-mannotriose[6] and α-D-galactosyl-β-D-mannotetraose (the pentasaccharide fraction), and a minor component having a mobility slightly lower than that of the pentasaccharide fraction. Heat-treatment of this mixture followed by treatment with α-D-galactosidase gave β-D-mannotriose and β-D-mannotetraose as the only oligosaccharide products. Incubation of the original hexasaccharide with *A. niger* β-D-mannanase effected complete hydrolysis to β-D-mannobiose, β-D-mannotriose, 6^1-α-D-galactosyl-β-D-mannobiose, and 6^1-α-D-galactosyl-β-D-mannotriose. These results conclusively demonstrate that the two major hexasaccharides are 6^3-α-D-galactosyl-β-D-mannopentaose and 6^4-α-D-galactosyl-β-D-mannopentaose. The minor hexasaccharide-fraction contains two D-galactosyl groups attached to a β-D-mannotetraose backbone. Since treatment with β-D-mannosidase removed only a single D-mannosyl group, it was concluded that one of the D-galactosyl groups is located on the second D-mannosyl residue from the non-reducing terminal. Knowing the products of hydrolysis of the whole hexasaccharide-fraction by *A. niger* β-D-mannanase (see above) and the fact that β-D-mannanase is unable to cleave the glycosidic linkage between two D-mannosyl residues both substituted by D-galactose [see (*d*)], it was concluded that the second D-galactosyl group is located on the reducing D-mannose residue. Further support for this conclusion comes from the observation that none of the oligosaccharides characterised had D-galactosyl substitution on the penultimate D-mannosyl residue at the reducing end. Thus, the structure proposed for this oligosaccharide is 6^1,6^3-di-α-D-galactosyl-β-D-mannotetraose.

(*d*) *Heptasaccharide fraction.* A heptasaccharide fraction was produced by both *A. niger* and lucerne-seed β-D-mannanase. The heptasaccharide fraction produced by *A. niger* β-D-mannanase was completely hydrolysed to D-galactose and β-D-mannopentaose by α-D-galactosidase, was resistant to hydrolysis by *A. niger* β-D-mannanase even at enzyme concentrations 30-fold higher than those used in the initial hydrolysis, and was quantitatively hydrolysed to D-mannose and a hexasaccharide fraction (Gal/Man = 2:4) by β-D-mannosidase (Fig. 2). These results indicated that the oligosaccharide was β-D-mannopentaose substituted by two D-galactosyl groups, one of which was located on the second D-mannosyl residue from the non-reducing end of the β-D-manno-oligomer backbone. The second D-galactosyl group was located as shown in Fig. 2. Treatment of the heptasaccharide with α-D-galactosidase to remove ~60% of the D-galactosyl groups, and subsequent chromatography on Bio-Gel P-2 yielded D-galactose plus hepta-, hexa-, and pentasaccharide fractions. The heptasaccharide was unhydrolysed material, and the pentasaccharide was β-D-mannopentaose. The hexasaccharide fraction was a mixture of two oligosaccharides, each of which contained a single D-galactosyl group. Treatment of the hexasaccharide fraction with β-D-mannosidase gave two oligosaccharide products which had chromatographic mobilities similar to those of α-D-galactosyl-β-D-mannotriose and α-D-galactosyl-β-D-mannotetraose, indicating that the D-galactosyl groups are located on the third and the fourth D-mannosyl residues, respectively, from the reducing end of β-D-mannopentaose. Furthermore,

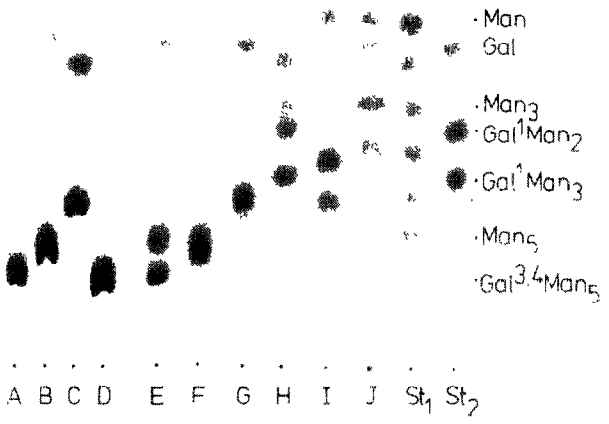
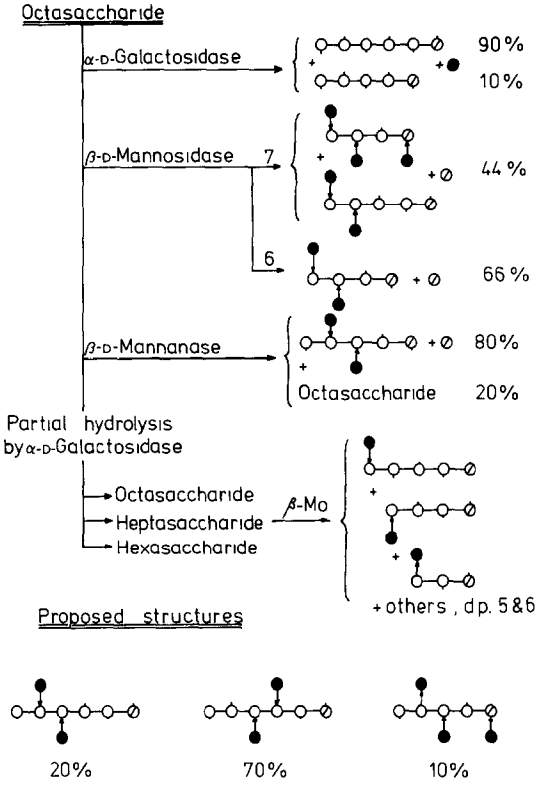


Fig. 2. Enzymic characterisation of the heptasaccharide produced on hydrolysis of hot-water-soluble carob galactomannan by *A. niger* β-D-mannanase. Untreated heptasaccharide (A) or enzyme-treated heptamer was subjected to t l c (solvent *B*). Heptasaccharide was treated with excess of β-D-mannosidase (B), α-D-galactosidase (C) or β-D-mannanase (D); or partially hydrolysed by α-D-galactosidase (E) with purification of the hexasaccharide fraction (F) by chromatography on Bio-Gel P-2. This fraction was then treated with excess of α-D-galactosidase (G), β-D-mannanase (H) or β-D-mannosidase (I). The β-D-mannosidase digest was heated to inactivate the enzyme and then treated with excess of α-D-galactosidase (J). St_1, mannose–mannohexaose, St_2, galactose, 6^1-α-D-galactosyl-β-D-mannobiose, and 6^1-α-D-galactosyl-β-D-mannotriose.

treatment of this mixture with α-D-galactosidase, following inactivation of the β-D-mannosidase, gave β-D-mannotriose and β-D-mannotetraose as the only oligomeric reaction-products (Fig. 2). Consistent with these results, treatment of the hexasaccharide fraction with *A. niger* β-D-mannanasegave β-D-mannobiose, β-D-mannotriose, 6^1-α-D-galactosyl-β-D-mannobiose, and 6^1-α-D-galactosyl-β-D-mannotriose. Thus, the heptasaccharide fraction is 6^3,6^4-di-α-D-galactosyl-β-D-mannopentaose. Similar studies of the heptasaccharide produced on hydrolysis of galactomannan by lucerne-seed β-D-mannanase showed that the major fraction was identical to that produced by *A. niger* β-D-mannanase, and that other, very minor components were also present.

(*e*) *Octasaccharide fraction.* Characterisation of the octa-and nona-saccharide fractions was complicated by the fact that each contained more than one oligosaccharide, and each oligosaccharide contained at least two D-galactosyl branch-units. The current studies were limited to the characterisation of the octa- and nona-saccharides produced by *A. niger* β-D-mannanase.

Treatment of the octasaccharide fraction with α-D-galactosidase gave D-galactose plus β-D-mannopentaose and β-D-mannohexaose in the ratio 1:9 (estab-



Scheme 1. Enzymic characterisation of the octasaccharides produced on hydrolysis of hot-water-soluble carob galactomannan by *A. niger* β-D-mannanase: ●, (1→6)-linked α-D-galactosyl group; ○, (1→4)-linked β-D-mannosyl residue; ∅, reducing, terminal D-mannose; β-Mo, β-D-mannosidase.

lished by chromatography on Bio-Gel P-2). Incubation with β-D-mannosidase produced hexa- and hepta-saccharide fractions. The relative proportions and structures proposed for these fragments are shown in Scheme 1. The location of the D-galactosyl stubs was determined as for the heptasaccharide fraction, *i.e.*, by partial hydrolysis with α-D-galactosidase followed by fractionation of the products and treatment with β-D-mannosidase (Scheme 1). Partial hydrolysis of the octasaccharide fraction by α-D-galactosidase (~60% removal of D-galactose), followed by fractionation on Bio-Gel P-2 gave hexa-, hepta-, and octa-saccharide fractions, together with traces of pentasaccharide. Since the bulk of the original octasaccharide-fraction consisted of β-D-mannohexaose substituted by two D-galactosyl groups, it follows that the bulk of the heptasaccharide(s) produced on partial hydrolysis with α-D-galactosidase would contain a single D-galactosyl branch-unit. Consequently, the nature of the major oligosaccharides produced on treatment of this fraction with β-D-mannosidase should give direct information on the location of the D-galactosyl branch-units in octasaccharides having a β-D-mannohexaose backbone.

Experimental results showed that oligosaccharides which co-chromatographed with α-D-galactosyl-β-D-mannotriose, α-D-galactosyl-β-D-mannotetraose, and α-D-galactosyl-β-D-mannopentaose (lesser amounts) were produced, but that di- and tri-saccharides containing a single D-galactosyl branch-unit were absent. Since the tetra-, penta-, and hexa-saccharide fractions were resistant to further attack by β-D-mannosidase, it was concluded that the D-galactosyl group was located on the non-reducing D-mannosyl residue and consequently that, in the original octasaccharide based on a β-D-mannohexaose backbone, the D-galactosyl stubs were located on the third, fourth, and fifth D-mannosyl residues from the reducing end. These results indicate that there are two octasaccharides having a β-D-mannohexaose backbone, namely, 6^4,6^5-di-α-D-galactosyl-β-D-mannohexaose (**1**) and 6^3,6^4-di-α-D-galactosyl-β-D-mannohexaose (**2**). 6^3,6^5-Di-α-D-galactosyl-β-D-mannohexaose is not a possible structure, as it would be rapidly cleaved by *A. niger* β-D-mannanase, as is 6^1,6^3-di-α-D-galactosyl-β-D-mannotetraose (produced on hydrolysis of galactomannan by lucerne-seed β-D-mannanase). On treatment of the heptasaccharide (derived from partial hydrolysis of the octasaccharide fraction with α-D-galactosidase) with snail β-D-mannosidase, two oligosaccharides, other than the three already mentioned were produced in lesser quantities. These most probably originate from the octasaccharide(s) based on a β-D-mannopentaose backbone, and their chromatographic mobilities indicated that they had d.p. values of 5 and 6, respectively, suggesting that they were di-α-D-galactosyl-β-D-mannotriose and di-α-D-galactosyl-β-D-mannotetraose.

To obtain information on the location of the D-galactosyl branch-units in the octasaccharide having a β-D-mannopentaose backbone, the hexasaccharide fraction produced on partial hydrolysis of the octasaccharide with α-D-galactosidase was studied. Treatment of this hexasaccharide fraction with β-D-mannosidase gave a large proportion of D-mannose, together with small proportions of α-D-galactosyl-D-mannose, 6^3-α-D-galactosyl-β-D-mannotriose, and 6^4-α-D-galactosyl-β-D-mannotetraose. Thus, this hexasaccharide fraction consists mostly of β-D-mannohexaose, together with a small proportion of β-D-mannopentaose substituted by D-galactose on either the reducing D-mannose or on the third or fourth D-mannosyl residue from the reducing end. Thus, the octasaccharide based on β-D-mannopentaose is 6^1,6^3,6^4-tri-α-D-galactosyl-β-D-mannopentaose (**3**).

Treatment of the octasaccharide fraction at high concentrations of substrate (10 mg/mL) with high concentrations of *A. niger* β-D-mannanase (30 times that used in the initial incubation) caused further hydrolysis. The major reaction-products were the heptasaccharide, 6^3,6^4-di-α-D-galactosyl-β-D-mannopentaose, and resistant octasaccharide in the ratio 4:1, together with traces of D-mannose. Heptasaccharide production is consistent with removal of a single D-mannose residue from the reducing end of octasaccharide **1** and from the non-reducing end of octasaccharide **2**. The resistant octasaccharide-fraction has a D-galactose content of ~37%, consistent with its being octasaccharide **3**.

The relative proportions of the octasaccharides **1**–**3** were calculated as

~2:7:1 from the amounts of oligosaccharides produced on treatment of the octasaccharide with each of the three enzymes (Scheme 1).

(*f*) *Nonasaccharide fraction.* Characterisation was achieved by techniques similar to those used for the octasaccharides and heptasaccharide. The α-D-galactosidase treatment gave β-D-mannohexaose and β-D-mannoheptaose in the ratio 86:14, and treatment with β-D-mannosidase effected complete hydrolysis to octasaccharide and heptasaccharide (4:1). The location of the D-galactosyl branch-units was determined by partial hydrolysis experiments employing α-D-galactosidase followed by fractionation of the products on Bio-Gel P-2 and treatment with β-D-mannosidase. Treatment of the heptasaccharide fraction with β-D-mannosidase gave D-mannose, α-D-galactosyl-D-mannose, 6^3-α-D-galactosyl-β-D-mannotriose, 6^4-α-D-galactosyl-β-D-mannotetraose, and 6^5-α-D-galactosyl-β-D-mannopentaose as the only reaction products. The last two oligosaccharides were present in approximately three times the amount of 6^3-α-D-galactosyl-β-D-mannotriose; 6^2-α-D-galactosyl-β-D-mannobiose was absent. This indicates that, in the nonasaccharide based on a β-D-mannohexaose backbone, the D-galactosyl groups are located on the reducing D-mannose or on the third fourth, or fifth D-mannosyl residue from the reducing end. Three structures consistent with these data are 6^3,6^4,6^5-tri-α-D-galactosyl-β-D-mannohexaose (**4**), 6^1,6^4,6^5-tri-α-D-galactosyl-β-D-mannohexaose (**5**), and 6^1,6^3,6^4-tri-α-D-galactosyl-β-D-mannohexaose (**6**).

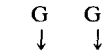
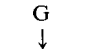
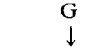
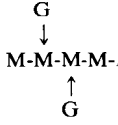
Treatment of the nonasaccharide fraction with excess of *A. niger* β-D-mannanase gave the heptasaccharide, 6^3,6^4-di-α-D-galactosyl-β-D-mannopentaose plus α-D-galactosyl-D-mannose, and resistant nonasaccharide as the major products, together with a small proportion of octasaccharide. The ratios of the hepta-, octa-, and nona-saccharide fractions were 45:10:45. These results indicate that nonasaccharides **4** and **5** (Table I) preponderate. The heptasaccharide is produced mainly from **5** by removal of the D-mannosyl residue substituted by D-galactose, at the reducing terminal. Borohydride reduction of the nonasaccharide alters its susceptibility to hydrolysis by β-D-mannanase. Reduced **5** is resistant to hydrolysis by β-D-mannanase, as shown by the absence of heptasaccharide in the reaction mixture. By analogy with results obtained on hydrolysis of octasaccharide **2** by *A. niger* β-D-mannanase, it would be expected that nonasaccharide **6** (Table I), when treated with excess of β-D-mannanase, would yield an octasaccharide product plus D-mannose by cleavage of the terminal D-mannosyl group. This reaction does occur, but the production of only traces of octasaccharide indicates that **6** is only a minor component. The production of octasaccharide from nonasaccharide by β-D-mannanase is not affected by prior reduction of the nonasaccharide with borohydride, providing further evidence for the proposed structure for **6**.

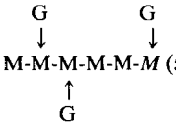
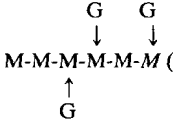
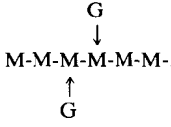
The location of the D-galactosyl branch-units in the nonasaccharide(s) based on a β-D-mannoheptaose backbone was determined by β-D-mannosidase treatment of the octasaccharide fraction obtained on partial hydrolysis of the nonasaccharide with α-D-galactosidase. Among the reaction products were oligosaccharides that co-chromatographed with 6^3-α-D-galactosyl-β-D-mannotriose and 6^4-α-D-galac-

TABLE I

OLIGOSACCHARIDES PRODUCED ON HYDROLYSIS OF HOT WATER-SOLUBLE CAROB GALACTOMANNAN BY *A. niger* AND LUCERNE-SEED β-D-MANNANASES

Oligosaccharide			*Amount (%)*		*Oligosaccharide*			*Amount (%)*	
D.p.	*Compound*	*Structure*	A. niger	*Lucerne seed*	*D.p.*	*Compound*	*Structure*	A. niger	*Lucerne seed*
1	Man	*M*	1.8	Trace			G ↓ M-M-M-M-*M* (**3**) ↑ ↑ G G		
2	Man_2	M-*M*	23.9	13.3	8	$Gal^{1,3,4}Man_5$		0.6	26.9[b]
3	Man_3	M-M-*M*	20.2	19.3					
3	Gal^1Man_2[a]	G ↓ M-*M*	15.9	1.0	8	$Gal^{4,5}Man_6$	G ↓ M-M-M-M-M-*M* (**1**) ↑ G	1.2	26.9[b]
4	Gal^1Man_3	G ↓ M-M-*M*	7.4	4.2					
4	Man_4	M-M-M-*M*		8.0	8	$Gal^{3,4}Man_6$	G ↓ M-M-M-M-M-*M* (**2**) ↑ G	4.2	26.9[b]
5	Gal^3Man_4	G ↓ M-M-M-*M*		9.0					
5	Gal^1Man_4	G ↓ M-M-M-*M*		2.0	9	$Gal^{3,4,5}Man_6$	G G ↓ ↓ M-M-M-M-M-*M* (**4**) ↑ G	2.5	26.9[b]

6	$Gal^{1,3}Man_4$			0.4
6	$Gal^{4}Man_5$			3.3
6	$Gal^{3}Man_5$			3.3
7	$Gal^{3,4}Man_5$		11.1	9.2

9	$Gal^{1,4,5}Man_6$	 M-M-M-M-M-*M* (**5**)	2.3	26.9[b]
9	$Gal^{1,3,4}Man_6$	 M-M-M-M-M-*M* (**6**)	0.5	26.9[b]
9	$Gal^{4,5}Man_7$	 M-M-M-M-M-M-*M* (**7**)	0.7	26.9[b]
>9		—	7.7	26.9[b]

[a]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. [b]The structures of oligosaccharides of d.p. >7 produced by lucerne-seed β-D-mannanase were not characterised. These oligosaccharides represented 26.9% of the total hydrolysate.

tosyl-β-D-mannotetraose as well as oligosaccharides of higher d.p. having chromatographic mobilities similar to those of β-D-mannoheptaose and β-D-manno-octaose. Oligosaccharides having the chromatographic mobilities of α-D-galactosyl-D-mannose, 6^2-α-D-galactosyl-β-D-mannobiose, or 6^5-α-D-galactosyl-β-D-mannopentaose were absent, indicating that the only nonasaccharide having a β-D-mannoheptaose backbone was 6^3,6^4-di-α-D-galactosyl-β-D-mannoheptaose. The oligosaccharides in the hydrolysate with chromatographic mobilities similar to those of β-D-mannoheptaose and β-D-manno-octaose were derived from nonasaccharides having a β-D-mannohexaose backbone.

Methylation studies. — Data for the methylation analysis of the tetra-, penta-, and hepta-saccharides produced on hydrolysis of hot-water-soluble carob galactomannan by *A. niger* or lucerne-seed β-D-mannanase are recorded in Table II. The permethylated oligosaccharides were subjected to mass spectrometry, and the alditol acetates derived from partial hydrolysates were analysed conventionally by g.l.c.–m.s. The results in Table II show that, in each oligomer, D-galactose occupies a terminal position as a single-unit side-chain. When the reduced end-unit was unsubstituted, a 1,2,3,5,6-penta-*O*-methylmannitol derivative was obtained, identified by its low T value and characteristic fragment-ions at *m/z* 45, 89, 133, 157, and 205. When the terminal mannitol was substituted at O-6 by a D-galactosyl group, a 1,2,3,5-tetra-*O*-methylmannitol derivative was obtained with characteristic fragment-ions at *m/z* 45, 89, 117, 133, and 233. In this way, it became clear that α-D-galactosyl-β-D-mannotriose was a β-D-mannotriose having a D-galactosyl group at O-6 of its reducing residue. Comparison of the methylated derivatives given by α-D-galactosyl-β-D-mannotetraose and by borohydride-reduced α-D-galactosyl-β-D-mannotetraose of the mixed-pentasaccharide fraction showed that the D-galactosyl group in this oligosaccharide is attached to an internal D-mannosyl residue. Examination of the mixture of two D-galactose-containing pentasaccharides (Table II), before and after borohydride reduction, confirmed that they are present in the ratio ~4:1, as judged by the relative proportions of 1,2,3,5-tetra-*O*-methylmannitol and of 2,3-di-*O*-methylmannose derivatives. Also, the formation of 1,2,3,5-tetra-*O*-methylmannitol from the reduced mixture indicates that the minor pentasaccharide carried a D-galactosyl group at O-6 of its reducing end, as in 6^1-α-D-galactosyl-β-D-mannotriose. The D-galactosyl group in the major α-D-galactosyl-β-D-mannotetraose oligosaccharide could not be located unequivocally by methylation analysis, although it was shown to be attached to one of the two internal D-mannosyl residues. This result is consistent with enzymic studies that identified this oligosaccharide as 6^3-α-D-galactosyl-β-D-mannotetraose. Likewise, methylation analysis indicated that the two D-galactosyl groups in the heptasaccharide di-α-D-galactosyl-β-D-mannopentaose were attached to two of the internal mannosyl residues, but did not identify these. However, the results were consistent with the structure of the heptasaccharide determined by enzymic procedures.

Mass spectrometry of the permethylated oligosaccharides. — Ions at *m/z* 88 represented the base peak in the mass spectra of the permethylated oligosac-

TABLE II

METHYLATION ANALYSIS OF OLIGOSACCHARIDES PRODUCED ON β-D-MANNANASE HYDROLYSIS OF D-GALACTO-D-MANNANS[a]

Oligosaccharides	*1,2,3,5,6-Me$_5$-Mannitol*	*1,2,3,5-Me$_4$-Mannitol*	*2,3,4,6-Me$_4$-Mannose*	*2,3,4,6-Me$_4$-Galactose*	*2,3,6-Me$_3$-Mannose*	*2,3-Me$_2$-Mannose*
Gal^{1}Man$_3$			1	1	1	0.8
Gal^{1}Man$_3$H		0.7	1	0.9	0.9	
Gal^{3}Man$_4$			1	1	1.8	0.9
GalMan$_4$H MIX[b]	1.5[c]	0.25	1	1	1.3	0.9
Gal^{3,4}Man$_5$			1	1.8	1.8	1.5
Gal^{3,4}Man$_5$H	1.1		1	1.7	0.8	1.4

[a]Results expressed in molar proportions. [b]A mixture of two reduced pentasaccharides. [c]In excess, because of the presence of overlapping impurities.

charides before and after reduction, as is to be expected[22,23] for oligosaccharides having (1→4) and (1→6) linkages. In all spectra, the high intensity of the ions of the aA1 and aA2 series[24] at *m/z* 219, 187, and 155, arising from non-reducing terminal units, accords with the branched structure of the oligosaccharides.

Further structural information on borohydride-reduced α-D-galactosyl-β-D-mannotriose is provided by the absence of the ion at *m/z* 235 characteristic of an unsubstituted alditol end-group. This is further confirmed by the presence of the ion at *m/z* 439, which arises from the mannitol end-group substituted at O-6 by a D-galactosyl group. The peak at *m/z* 423 (baA1), a fragment that corresponds to the two residues at the non-reducing end, is present in the mass spectrum of reduced α-D-galactosyl-β-D-mannotriose, but not in that of 6^3,6^4-di-α-D-galactosyl-β-D-mannopentaose. This is consistent with the second D-mannosyl residue from the non-reducing end of 6^3,6^4-di-α-D-galactosyl-β-D-mannopentaose carrying a substituent at O-6. The formation of the ion at *m/z* 391 from this heptasaccharide could thus arise from the D-galactosyl-D-mannose unit[25].

Although some structural features of the oligomers were confirmed by their mass spectra, the assignment of the relative position of the branch points in di-α-D-galactosyl-β-D-mannopentaose, for example, requires ions at higher mass-numbers.

N.m.r.-studies. — The proton-decoupled, ^{13}C-n.m.r. spectra of 6^1-α-D-galactosyl-β-D-mannobiose and 6^2-α-D-galactosyl-β-D-mannobiose have been described[6]. Table III contains the chemical shifts of the resonances observed at 70°.

(*a*) *6^1-α-D-Galactosyl-β-D-mannotriose.* The spectrum of Gal^{1}Man$_3$ has the same characteristics as that of 6^1-α-D-galactosyl-β-D-mannobiose. The resonances associated with the reducing D-mannose residue can be easily assigned, as their intensities reflect the α:β ratio. Comparison of the resonances observed for these compounds leads to a ready assignment of the other lines. Thus, the spectral region of the anomeric carbons (94–101 p.p.m.) shows four signals, three of them being sensitive to the anomeric configurations. This corresponds to a structural arrange-

TABLE III

^{13}C-N.M.R. CHEMICAL SHIFTS[a] FOR OLIGOSACCHARIDES PRODUCED ON HYDROLYSIS OF D-GALACTO-D-MANNANS

Compound	*Unit*		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Gal^{1}Man$_2$	Gal	α	99.8	69.4	70.5	70.25	72.05	62.05
		β	99.75					
	Reducing Man	α	94.7	71.25	69.8	77.95	70.55	67.8
		β	94.65	71.55	72.55	77.6	74.25	67.65
	Man	α	100.85	71.5	73.95	67.75	77.35	62.0
		β	100.9					
Gal^{2}Man$_2$	Gal		99.4	69.5	70.45	70.3	71.85	62.1
	Reducing Man	α	94.8	71.25	69.95	78.15	71.85	61.6
		β	94.65	71.55	72.65	77.85	75.7	
	Man		101.2	71.45	73.95	67.75	75.6	67.5
Gal^{1}Man$_3$	Gal	α	99.8	69.4	70.5	70.25	72.1	62.05
		β	99.75					
	Reducing Man	α	94.75	71.25	69.8	77.95	70.6	67.75
		β	94.70	71.6	72.5	77.6	74.3	67.65
	Man′	α	100.8	71.0	72.5	77.4	76.05	61.55
		β	100.9					
	Man″		101.1	71.45	73.9	67.75	77.3	62.0
Gal^{3}Man$_4$	Gal		99.8	69.45	70.45	70.25	72.15	62.1
	Reducing Man	α	94.75	71.35	69.90	77.75[e]	72.0	61.6[f]
		β	94.6	71.7	72.6		75.75	
	Man′		100.95[b]	70.95[c]	72.5[d]	77.7[e]	75.95	61.55[f]
	Man″		101.05[b]	70.9[c]	72.35[d]	77.65[e]	74.40	67.6
	Man‴		101.1[b]	71.45	73.90	67.75	77.40	62.0
Gal^{3,4}Man$_5$	Gal[g]		99.8	69.4	70.4	70.25	72.1	62.1
			99.75					
	Reducing Man	α	94.75	71.35	69.9	77.75[e]	71.95	61.6[f]
		β	94.6	71.65	72.6		75.75	
	Man′		100.9[b]	70.95[c]	72.45[d]	77.70[e]	75.9	61.55[f]
	Man″		100.95[b]	70.90[c]	72.35[d]	77.60[e]	74.4	67.55
	Man‴		101.0[b]	70.8[c]	72.30[d]	77.85	74.3	67.5
	Man⁗		101.1[b]	71.45	73.9	67.7	77.40	61.95

[a]Chemical shifts (p.p.m.) are expressed downfield from D.S.S., with acetone as internal standard (31.07 p.p.m. from D.S.S.), at 70°. Man′ connotes the D-mannosyl residue adjacent to the reducing D-mannose residue, *etc.* [b–f]Within a column, ^{13}C assignments for different D-mannosyl residues may be exchanged [g]Two C-1 signals relative to two D-galactosyl groups are distinguished in the case of Gal^{3,4}Man$_5$

ment in which the α-D-galactopyranosyl group is linked to the reducing D-mannose, as in 6^1-α-D-galactosyl-β-D-mannobiose[6]. Examination of the C-6 region (60–62 p.p.m.) reveals three signals corresponding to three carbons. The fourth signal is found at lower field (67.75 and 67.65 p.p.m., as it is also sensitive to the anomeric configuration of the reducing D-mannose residue), in agreement with a downfield shift of 6 p.p.m. which is commonly experienced for a carbon attached to a

glycosidic linkage in this series[26]. The resonances (at 70.6 and 74.3 p.p.m., respectively) associated with C-5α and C-5β correspond to signals shifted by 1.5 p.p.m. upfield, in agreement with what occurs to a carbon adjacent to a carbon attached to a glycosidic linkage (β-effect) and with the corresponding resonances of β-D-mannobiose and 6^2-α-D-galactosyl-β-D-mannobiose. Other characteristics are given by the resonances at 76.05 and 61.55 p.p.m. (C-5 and C-6, respectively, of an internal mannosyl residue not substituted by a D-galactosyl group), and at 77.3 and 101.1 p.p.m. (C-5 and C-1, respectively, of an unbranched, terminal mannosyl group).

These observations provide conclusive evidence that the tetrasaccharide is 6^1-α-D-galactosyl-β-D-mannotriose.

(*b*) 6^3-α-D-*Galactosyl*-β-D-*mannotetraose.* Assignment of the resonances observed is made possibly by comparisons[6] with the resonances shown by Gal^1Man_2, Gal^2Man_2, and Gal^1Man_3. Also, the lines associated with the reducing D-mannose residue (sensitive to the anomeric configuration) are easily recognised. The signals in the spectral region of the anomeric carbons (94–101 p.p.m.) accord with the structure of the compound. Six resonances are observed, corresponding to C-1α and C-1β of the reducing D-mannose residue, C-1 of galactose, and C-1 of the three non-reducing D-mannosyl residues.

Four theoretical structures for $GalMan_4$ can be envisaged, as the D-galactosyl substituent may be attached to O-6 of any of the four mannose residues of the mannotetraose backbone. However, the resonances of C-5α and C-5β, easily identifiable at 75.75 and 72.0 p.p.m., respectively, clearly show that C-5 belongs to an unbranched, reducing D-mannose residue (if branched, an upfield shift of 1.5–2.0 p.p.m. would be observed). Furthermore, the C-6 signal at 67.6 p.p.m., relative to a carbon attached to a glycosidic linkage, is present as only one line (as the corresponding carbon is not sensitive to the anomeric configuration). These features eliminate 6^1-α-D-galactosyl-β-D-mannotetraose as a possible structure. Substitution on the non-reducing terminal D-mannosyl residue is also excluded, as this would involve three 4-*O*-substituted mannose residues, each giving a C-6 signal[27] at 61.5–61.6 p.p.m.; only two were observed. Also, four C-5 signals (including C-5β) would be observed at 75.6–75.05 p.p.m., but only two were found. A comparison of the chemical-shift data for Gal^2Man_2 and those for 6^2-α-D-galactosyl-β-D-mannotetraose allows the latter to be excluded as the structure of the pentasaccharide. Such a pentasaccharide would be expected to show the signals from C-1 of the galactosyl group at 99.4 p.p.m. (instead of 99.8), from C-5 of the same unit at 71.85 p.p.m. (instead of 72.15), and from C-4 of the reducing D-mannose residue at 78.15 p.p.m. (instead of 77.75).

Thus, the structure for the pentasaccharide is 6^3-α-D-galactosyl-β-D-mannotetraose, which accords with the results of the enzymic and chemical studies.

(*c*) 6^3,6^4-*Di*-α-D-*galactosyl*-β-D-*mannopentaose.* Similar reasoning to that used in the characterisation of Gal^3Man_4 leads to the conclusion that there is a close relationship between Gal^3Man_4 and the heptasaccharide. The data indicate

that the latter must include a common part, in the form of a 6^3-α-D-galactosyl-β-D-mannotriose moiety, and that there are only two possible locations for the second D-galactosyl group, namely, attached to O-6 of either the fourth D-mannosyl residue (from the reducing residue) or the non-reducing terminal residue. The ^{13}C-n.m.r. spectrum of the compound corresponding to this last hypothesis would show three C-6 signals at 61.5–61.6 p.p.m. for three 4-*O*-substituted D-mannose residues[27], but only two were seen. Furthermore, such a compound would be expected to show four C-5 signals (including C-5β) at 75.6–75.9 p.p.m., because of the different chemical-shifts for C-5 of D-mannose residues either 4-*O*-substituted, 6-*O*-substituted, or both. However, only two C-5 signals were observed in this region. Thus, the heptasaccharide must be 6^3,6^4-di-α-D-galactosyl-β-D-mannopentaose.

DISCUSSION

The structures of the oligosaccharides produced on hydrolysis of hot-water-soluble carob galactomannan by *A. niger* and lucerne-seed β-D-mannanase have been determined by using enzymic, n.m.r., and chemical procedures. There was excellent agreement between the results obtained with the different procedures, and it is evident that only a limited number of the theoretically possible branched-oligosaccharides were produced. Oligosaccharides of d.p. 2–9 produced on hydrolysis by *A. niger* β-D-mannanase are shown in Table I. Lucerne-seed β-D-mannanase produced the same di-, tri-, tetra-, and hepta-saccharides, but the relative proportions were different. Also, this enzyme has a limited ability to hydrolyse β-D-mannotetraose, so that this oligosaccharide, and other oligosaccharides having a β-D-mannotetraose backbone, also accumulated. These oligosaccharides included the pentasaccharides 6^3-α-D-galactosyl-β-D-mannotetraose and 6^1-α-D-galactosyl-β-D-mannotetraose, and the hexasaccharide 6^1,6^3-di-α-D-galactosyl-β-D-mannotetraose. Significant quantities of hexasaccharide having a β-D-mannopentaose backbone were also produced, namely, 6^3-α-D-galactosyl-β-D-mannopentaose and 6^4-α-D-galactosyl-β-D-mannopentaose. The presence of these oligosaccharides in the hydrolysate, even though β-D-mannopentaose was absent, indicates that the presence of a D-galactosyl substituent on the β-D-manno-oligomer backbone reduces the susceptibility of the oligosaccharide to attack by the lucerne-seed β-D-mannanase. However, these oligosaccharides were rapidly attacked by *A. niger* β-D-mannanase, as shown by their absence from hydrolysates where this enzyme was employed.

A detailed knowledge of the structures of the oligosaccharides produced on hydrolysis of D-galactomannans and D-glucomannans by β-D-mannanase, together with kinetic data on the hydrolysis of β-D-manno-oligosaccharides and D-galactose-substituted β-D-manno-oligosaccharides, provides information on both the substrate-binding requirements of these enzymes and on the "fine-structures" of the polysaccharides. These points will be discussed in a subsequent paper[28].

ACKNOWLEDGMENTS

The authors thank Mrs. Andrée Lefebvre for skilled technical assistance, and Mr. M. Hill for photography.

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