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ENZYMIC HYDROLYSIS OF RHIZOBIAL 4-*O*-METHYL-D-GLUCURONO-L-RHAMNAN

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ABSTRACT

A *Bacillus* sp. obtained from soil-enrichment culture excretes a depolymerase that degrades rhizobial 4-*O*-methylglucuronorhamnan. The enzyme was purified to homogeneity. According to SDS gel electrophoresis, it is a polypeptide chain of M_r ~210,000. As determined by methylation analysis and n.m.r. spectroscopy, the sole product of enzymolysis is the linear tetrasaccharide 4-*O*-Me-β-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→4)-L-Rha.

INTRODUCTION

Earlier reports[1,2] described in soybean seeds a lectin that exhibits specific binding for D-glucuronic acid and its 4-methyl ether. The lectin precipitates 4-*O*-methyl-D-glucurono-L-rhamnans[3] (m.g.r.), which are secreted into the medium by a major subspecies of *Bradyrhizobium japonicum*, and also occur on the cell surfaces[1]. To define the specific sugar binding of the new lectin, enzymic and partial hydrolytic studies with acid of the m.g.r. were undertaken to determine the structure of the haptenic oligosaccharides.

This report describes (*1*) soil-enrichment growth on m.g.r. of a *Bacillus* sp. that excretes a depolymerase, (*2*) isolation of the enzyme, and (*3*) the characterization of the tetrasaccharide product, which is a repeat unit of the m.g.r.

EXPERIMENTAL*

Media. — Enrichment broth (e.b.) contained (per liter): 2.0 g of polysaccharide (PS4421), the extracellular m.g.r. from *B. japonicum* NRRL B-4421 (Nitragin 61A76), 0.5 g of $(NH_4)_2SO_4$, 0.25 g of tryptone, 0.25 g of yeast extract, 2.5 g of K_2HPO_4, 2.5 g of KH_2PO_4, 0.05 g of $CaCl_2$, 0.5 mL of Speakman salt solution B

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

(ref. 4), and 1.0 g of D-glucose, all adjusted to pH 6.8. PS4421 was sterilized separately for 12 min at 121°.

Stock agar-slant medium (p.g.r.) contained (per liter): 1.0 g of PS4421, 0.25 g of D-glucose, 1.0 g of L-rhamnose, 0.5 g of $(NH_4)_2SO_4$, 0.25 g of tryptone, 0.25 g of yeast extract, 1.8 g of K_2HPO_4, 3.2 g of KH_2PO_4, 0.05 g of $CaCl_2$, 5 mL of salt solution B, and 20 g of agar.

The test medium for enzyme production contained (per liter): 1.5 g of PS4421, 0.5 g of $(NH_4)_2SO_4$, 0.25 g of tryptone, 0.25 g of yeast extract, 1.8 g of K_2HPO_4, 3.2 g of KH_2PO_4, 0.05 g of $CaCl_2$, and 5 mL of salt solution B.

Strain isolation. — Soil samples (0.5 g) were added to 10-mL portions of e.b. medium in 50-mL Erlenmeyer flasks, and incubated for 3 to 4 weeks at 30° on a rotary shaker at 200 r.p.m. Second-stage flasks containing e.b. without D-glucose were inoculated with 0.5 mL from the first stage, and incubated under similar conditions. After inoculation of a third stage, cultures that showed good cell growth in 7 d, or less, were tested for depolymerase activity. Four of eight mixed cultures were active.

A pure culture of the best enzyme-producer was obtained by streaking the mixed culture on p.g.r. plates, and selecting isolates after incubating for one week. The Gram-positive organism, designated *Bacillus* sp. NRRL B-14089, was a sporeforming, motile rod having characteristics similar to those of a xanthanase-producting *Bacillus*[5], except that the latter strain was mucoid and grew more slowly.

Enzyme production. — Stock cultures of the enzyme-producing *Bacillus* were maintained on p.g.r. agar slants. Fresh cultures were prepared at monthly intervals by incubating the inoculated medium for 3 d at 30°; the cultures were then stored at 4°. No problems were encountered during long-term maintenance with this procedure.

Culture broths (first stage) were inoculated with a loopful of *Bacillus* cells into 10 mL of test medium in 50-mL Erlenmeyer flasks, and then incubated on a rotary shaker at 200 r.p.m. until abundant cell-growth became evident (~3–4 d). These broths were also prepared from lyophilized cultures[6] by substituting the freeze-dried pellet for fresh cells; longer incubation (3–5 d) was usually required when using this method of inoculation.

Enzyme-producing cultures were grown in 300-mL Erlenmeyer flasks containing 50 mL of test medium, or in 2800-mL Fernbach flasks containing 500 mL of medium. These flasks (third stage) were inoculated (2%, v/v) with a 24-h culture (second stage) seeded (5%, v/v) from the first stage. All flasks were shaken at 30°. Maximum production of the enzyme was reached in 16–20 h; enzyme activity declined with further incubation.

Enzyme recovery. — Unless otherwise indicated, all operations were carried out at 4°. Cells were removed from the fermentation broth by centrifugation for 20 min at 20,000*g*. The cell-free broth was freeze-dried, and the material was reconstituted with distilled water. The concentrate was dialyzed with daily buffer changes. Following dialysis (0.15M potassium phosphate buffer, pH 6.5, 3 d), the

concentrate was centrifuged, and again freeze-dried. This material was taken up (25 mg/mL) in 10mM Tris·HCl, pH 7.8, and 3-mL portions were chromatographed on a column (1.2 × 85 cm) of Ultrogel AcA 34 (LKB), equilibrated with the buffer. The enzyme was eluted at a flow rate of 8 mL/h; 2-mL fractions were collected. Protein was detected by the absorbance at 280 nm.

Fractions of the gel filtrate containing depolymerase activity were pooled (25 mL), and applied to a column (2 × 5 cm) of DEAE-Sephacel (Pharmacia), equilibrated with the same buffer. The column was washed with 60 mL of buffer at 15 mL/h, and the protein was eluted by means of 150 mL of a linear NaCl gradient (0 to 0.7M) in equilibration buffer with the same flow-rate.

Fractions having activity were pooled, and dialyzed against 10mM sodium phosphate, pH 7.0. The dialyzate was applied to a hydroxylapatite column (2.5 × 8 cm) that had been equilibrated with 10mM sodium phosphate, pH 7.0, at a flow rate of 20 mL/h. The column was washed with 50 mL of the buffer, and the enzyme was then eluted with a linear gradient (0.01 to 0.4M) of sodium phosphate, pH 7.0. Fractions (5 mL) were collected, and protein was detected by the absorbance at 280 nm. Fractions having enzyme activity were pooled, and dialyzed against 30mM sodium phosphate, pH 6.5, and the material was stored as a solution or freeze-dried.

Enzyme assay. — A stock substrate solution was prepared by mixing 100 mL of 0.06M potassium phosphate buffer (pH 6.5) and 1.0 mL of Speakman salt solution B with 50–100 mg of PS4421 dissolved in 99 mL of distilled water. The suspension was filtered, and the filtrate was stored at 4° under toluene.

Reaction mixtures (0.5 mL of the enzyme solution added to 2.0 mL of the stock substrate) were incubated at 40°. After elapse of a suitable time, the reaction was terminated by heating for 8 min at 100°. Enzyme activity was calculated as the amount of reducing sugar (L-rhamnose basis) liberated from PS4421.

A unit of enzyme was defined as 1 μmol of reducing sugar liberated per min at 40° and pH 6.5.

Analytical procedures. — Reducing sugars were measured by an automatic analyzer (Technicon Instruments Corp.) with potassium ferricyanide[7]. Flow times were measured at 40° with an Ostwald viscometer.

The relative molecular weight of the enzyme was determined under both native and denaturing conditions by reference to the migration of protein standards in a 7% gel. Denaturing gel electrophoresis was performed essentially as described by Laemmli[8]. Standards were myosin (200,000), β-D-galactosidase (116,250), phosphorylase b (92,500), bovine serum albumin (66,200), and ovalbumin (45,000).

The molecular weight of the native enzyme was also estimated by gel filtration on a column (1.2 × 85 cm) of Ultrogel AcA 34 equilibrated with 10mM Tris·HCl, pH 7.8. The elution volume of the enzyme was used in order to estimate the mol. wt. by reference to the elution volumes of protein standards: catalase (210,000), bovine IgG (156,000), bovine serum albumin (66,000), and ovalbumin (45,000).

Products of enzymolysis. — PS4421 (2.4 g) was dissolved in distilled water (100 mL), and the pH of the solution was adjusted to 6.5; 50 mL of dialyzed enzyme solution was added. The mixture was incubated on a rotary shaker at 37° under a layer of toluene. The initial viscosity of 100 mPa had decreased to 5 mPa after 72 h. The mixture was filtered through a nominal 10,000 mol. wt. cut-off membrane (Amicon TCF-10) to remove protein and undigested polysaccharide. Freeze-drying of the filtrate yielded 0.48 g of the low-molecular-weight fraction (l.m.w.f.). The undigested or high-molecular-weight fraction (h.m.w.f.) was recovered by precipitation with alcohol, redissolved, and treated with fresh enzyme. After two such treatments, a total of 1.26 g of l.m.w.f. and 0.68 g of h.m.w.f. were recovered. In thin-layer chromatography (see next), all the various l.m.w.f.s migrated identically.

Hydrolysis and thin-layer chromatography (t.l.c.). — High- and low-molecular-weight fractions were hydrolyzed with 2M HCl for 1 h at 100°, and the hydrolyzates were analyzed by chromatography[9] on cellulose precoated on plastic sheets and developed with 5:5:1:3 pyridine–ethyl acetate–acetic acid–water. Carbohydrates were detected with *p*-anisidine–phthalic acid or periodate–Schiff[10] spray reagents. T.l.c. of alditols was performed with the same solvent on silica gel 60, precoated on glass. Spots were made visible by charring after spraying with 10% sulfuric acid in methanol.

Liquid chromatography (l.c.) of enzymic degradation products. — High- and low-molecular-weight hydrolytic products were chromatographed in 0.025% Brij at 0.5 mL/min on Waters E-linear and E-125 columns, respectively. The oligosaccharide was also chromatographed in 0.05M potassium phosphate at pH 3 to suppress ionization of carboxyl groups. In addition, standard sugars and the oligosaccharides were chromatographed as alditols on a Waters μBondapak carbohydrate column with 7:3 acetonitrile–H_2O at 2 mL/min. The reducing end of the oligosaccharide was converted into an alditol with $NaBD_4$, and the carboxyl group was then reduced with[11] ethylene oxide–$NaBD_4$.

Component analysis. — The oligosaccharide was methanolyzed with 3.6% HCl in MeOH for 16 h at 65°, uronic acids being converted into their methyl esters. The acid in the methanolyzate was neutralized with IR-45 anion-exchange resin, and the methyl glycosides[12] were analyzed as trimethylsilyl ethers. Gas–liquid chromatography (g.l.c.) was performed on an OV-1 column[13].

The carboxyl-reduced alditol of the oligosaccharide was hydrolyzed with 2M HCl for 1 h at 100°, and the compounds in the mixture were converted into per-*O*-acetylated aldononitriles (PAAN). G.l.c. was performed on neopentyl glycol succinate (nickel alloy, 2.25 m × 3.18 mm) at 170–210°, at 20° per min, and the sugar identification was confirmed by mass spectrometry (m.s.). Standard samples of peracetylated rhamnitol and 4-*O*-Me-glucitol were prepared by carboxyl reduction[11] of native PS4421, hydrolysis of the product, reduction of the sugars with $NaBD_4$, and peracetylation of the alditols.

Methylation analysis. — The carboxyl-reduced alditol was (trideuteriomethyl)ated by the procedure of Hakomori[14]. The permethylated oligosaccharide

was hydrolyzed for 2 h at 80° with 0.25M H_2SO_4 in acetic acid, followed by a second treatment for 2 h at 80° after 1:1 dilution with water[15]. Sulfate ions were removed with an anion-exchange resin, and the compounds in the hydrolyzate were converted into a mixture of PAAN and alditol acetate derivatives[13]. G.l.c. was performed in a Carbowax 20M, fused-silica, capillary column (25 m × 0.20 mm i.d., 0.11 μm film), with the temperature programmed from 110° (30 min) to 160° (30° per min). The identities of the methylated components were confirmed by m.s.

N.m.r. spectroscopy. — N.m.r. spectra (^1H-, 300 MHz and ^{13}C-, 75 MHz) were recorded with a Bruker WM-300 spectrometer for solutions in D_2O, with complete decoupling at ambient temperature. Chemical shifts were measured relative to sodium 2,2,3,3-tetradeuterio-4,4-dimethylsilapentanoate (t.s.p.) as the internal reference. The ^{13}C-^1H coupling-constants were determined by the heteronuclear 2D *J*-resolved method[16].

RESULTS AND DISCUSSION

Bacillus sp. strain NRRL B-14089 was easy to cultivate, and gave good yields of enzyme, either as a pure culture or in the presence of other micro-organisms (see

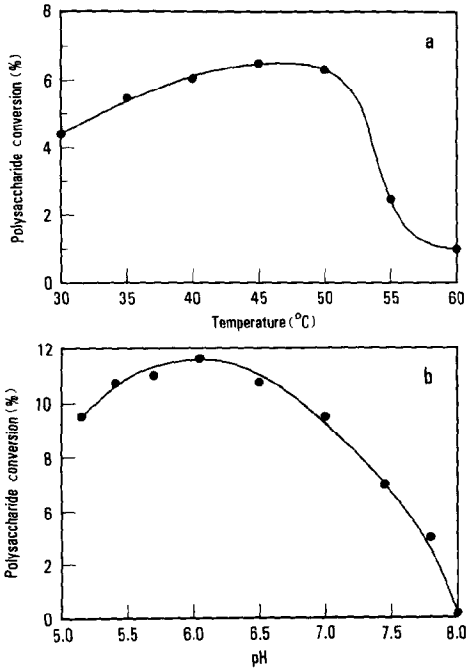


Fig. 1. (a) Effect of temperature on activity of m.g.r. depolymerase (30-min assay, pH 6.5; substrate: 50 mg of m.g.r. per 100 mL). (b) Optimum pH for m.g.r. depolymerase activity (60-min assay, 45°; substrate: 40 mg of m.g.r. per 100 mL).

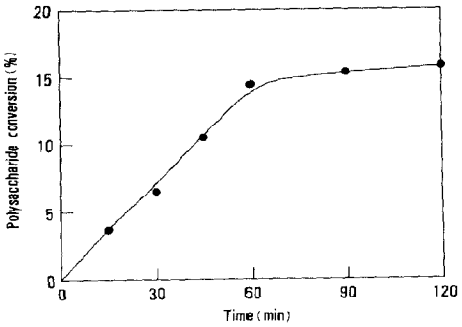


Fig. 2. Time course of depolymerase action on m.g.r. (assay at 45°, pH 6.5; substrate: 50 mg of m.g.r. per 100 mL).

ref. 5). Cultures maintained longer than three months usually required an additional broth stage before suitable yields of enzyme were obtained. Rapid inoculum build-up for production was achieved with broth cultures stored for up to two months at 4°.

No loss of enzymic activity occurred during the recovery of crude enzyme, which could be stored, either dry or in solution, for at least 6 months at 4°. Preparations were stable for 24 h at 25° at pH values between 6.6 and 8.2.

Characteristics of the enzyme. — Data on the thermal stability of the enzyme are shown in Fig. 1a. Assay mixtures (0.05M buffer, pH 6.5; 50 mg of substrate per 100 mL) were exposed for 30 min to temperatures ranging from 30 to 60°. Maximal activity was observed at 45°, with rates only slightly lower at 40 and 50°. Long incubations were routinely conducted at 35–40° in order to ensure continued activity of the enzyme.

The optimum pH of the depolymerization was determined after removing the excess of buffer by dialysis, and then adding buffers of different pH values. The results of 60-min assays at 45° are shown in Fig. 1b. Activity was optimum at pH 6.1, with less than 10% loss of activity in the pH range of 5.5 to 6.5.

The time course of PS4421 hydrolysis is given in Fig. 2. Enzyme was added to a substrate solution containing 50 mg of PS4421 in 100 mL of 0.03M potassium phosphate buffer, pH 6.5. A 3- to 4-fold diminution in viscosity was observed within 30 min. The extent of hydrolysis was measured by the increase in reducing power. After conversion of 15% of the substrate, there was no longer a direct relationship between the time and the amount of hydrolysis. Little hydrolysis occurred beyond this point, even when additional enzyme was added to the reaction mixture. Some form of inhibition by product occurs, because unaltered substrate can be recovered from the reaction mixture and then hydrolyzed further when resubmitted to enzymic action.

Purification of the enzyme. — Purification of the enzyme from freeze-dried culture-broths combined gel filtration, anion-exchange, and hydroxylapatite

TABLE I

PURIFICATION OF M.G.R. DEPOLYMERASE

Purification step	*Volume (mL)*	*Protein (mg/mL)*	*Total activity[a] (units)*	*Specific activity (units per mg of protein)*	*Purification (fold)*	*Yield (%)*
Crude culture broth	3	20.60	1680	27.2		100
Ultrogel AcA 34	17	0.96	938	57.1	2.0	57
DEAE-Sephacel anion exhange	47	0.14	673	104.5	4.0	40
Hydroxylapatite	21	0.01	14.1	150.0	5.5	23

[a]For definition of unit, see text.

chromatography. Gel filtration removed most of the salts and an accompanying brown pigment. The first peak eluted from the gel-filtration column contained over 50% of the enzymic activity, indicating a two-fold purification (see Table I). The enzyme fractions from gel filtration were pooled, and further purified by ion-exchange chromatography on DEAE-Sephadex (see Fig. 3). Gradient elution with NaCl produced a small peak of protein, followed by a large peak containing 25% of the initial enzyme activity. Enzyme fractions were pooled, and dialyzed against 10mM sodium phosphate buffer, pH 7.0, and the dialyzate was applied to a hydroxylapatite column. Gradient elution of the hydroxylapatite gave a single, symmetrical peak at 0.06M phosphate. This peak contained 23% of the original activity, representing an overall purification of 5.5-fold.

The purified enzyme migrated as a single band in sodium dodecyl sulfate gel-electrophoresis, and, when compared to standard samples, it exhibited a molecular weight of ~210,000, suggesting that the enzyme is a single polypeptide chain. Gel filtration of the native enzyme confirmed this result.

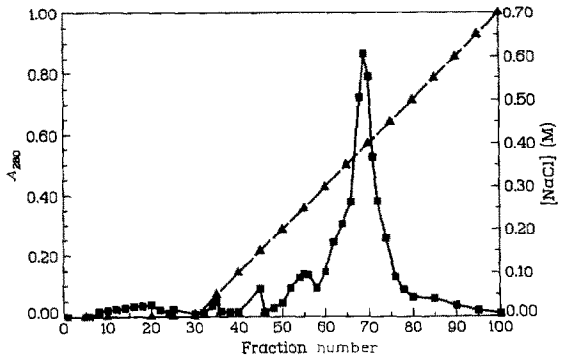


Fig. 3. DEAE-Sephacel chromatography of m.g.r. depolymerase. Key: ■—■, A_{280}; ▲—▲, NaCl concentration. Depolymerase activity coincided with fractions 60–75.

TABLE II

METHYLATION ANALYSIS OF THE CARBOXYL-REDUCED OLIGOSACCHARIDE

Methylated component	*Retention time (min)*[a]	*Percent*
1-d_1-1,2,3,5-$(CD_3)_4$-Rhamnitol	5.19	22
2,4-$(CD_3)_2$-Rhamnose	25.75	52
6,6-d_2-2,3,6-$(CD_3)_3$-4-CH_3-Glucose	27.39	26

[a]G.l.c. as PAAN or alditol acetate derivatives on a 25-m, Carbowax 20 M, fused-silica, capillary column; 30 min at 110°; 30° per min to 160°.

M.g.r. depolymerase activity was unaffected by 100mM EDTA or 10mM $HgCl_2$. The tetrasaccharide repeat-unit of m.g.r. (see later), isolated from enzymic digests, produced a slight inhibitory effect on the activity of the enzyme.

Composition of the degradation products. — T.l.c. of the l.w.m.f. gave a single spot on cellulose at R_F 0.33, and, on silica gel, at R_F 0.59. Acid hydrolyses of l.m.w. and h.m.w. fractions produced sugars that, in t.l.c., had the same R_F values as rhamnose (0.65) and 4-*O*-Me-glucuronic acid (0.39).

The l.m.w.f. migrated as a single oligosaccharide peak during l.c. on a Waters E-125 column with 0.025% Brij as the solvent. A similar result was obtained by l.c. of the carboxyl-reduced alditol of the l.m.w.f. in CH_3CN–H_2O on a Waters Carbohydrate column. Both intact B-4421 e.p.s. and the h.m.w.f. were excluded from a Waters E-linear column with 0.025% Brij as the solvent.

Structure of the oligosaccharide. — Colorimetric analysis[17] of the oligosaccharide, employing D-glucuronic acid as the standard, indicated uronic acid content of 25%; *i.e.*, a 3:1 ratio of rhamnose to 4-*O*-methylglucuronic acid. G.l.c. analysis of the per(trimethylsilyl)ated methyl glycosides after methanolysis confirmed this result.

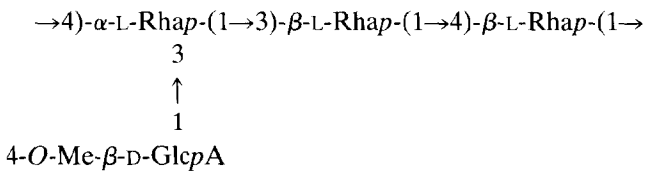
TABLE III

N.M.R. DATA FOR TETRASACCHARIDE

^{1}H-N.m.r.			*^{13}C-N.m.r.*		
δ[a]	*Integral peak size*	*Assignment*	*δ*[a]	$J_{C,H}$ *(Hz)*	*Assignment*
5.14	0.6	H-1 of α-Rha-OH	178.6		CO_2H of 4-*O*-Me-GlcA
5.05	1.1	H-1 of α-Rha	106.3	162	C-1 of 4-*O*-Me-β-D-GlcA
4.90	0.3	H-1 of β-Rha-OH	104.9	170	C-1 of α-L-Rha
4.70	1	H-1 of β-Rha	103.0	162	C-1 of β-L-Rha
4.67	1	H-1 of 4-*O*-Me-β-GlcA	96.4	170	C-1 of α-Rha-OH
3.51	3	OCH_3 of 4-*O*-Me-GlcA	96.2	162	C-1 of β-Rha-OH
1.40–1.30	9	CH_3 of three Rha	85.5–69.5		Remaining C-2, -3, -4, -5
			62.5		OCH_3 of 4-*O*-Me-GlcA
			19.5		CH_3 of three Rha

[a]Chemical shifts relative to sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP).

Methylation analysis of the carboxyl-reduced oligosaccharide (see Table II) revealed a linear tetrasaccharide having 4-*O*-methyl-D-glucuronic acid as the non-reducing end-group. A likely structure corresponds to a sequence that is present in the native polysaccharide[3], namely, 4-*O*-Me-β-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→4)-L-Rha. The product appears to be a repeating unit derived through cleavage of the β-(1→4) link to the rhamnosyl branch point in the original polysaccharide[3]:



The n.m.r. spectra. — The oligosaccharide structure proposed was supported by the n.m.r. data (see Table III).

The ^{1}H-n.m.r. spectrum of the tetrasaccharide showed five peaks, at 5.14, 5.05, 4.90, 4.70, and 4.67 p.p.m., corresponding to the protons of four anomeric carbon atoms. The two peaks beyond 5 p.p.m. correspond to protons of α-anomeric carbon atoms, and the three other peaks correspond to protons of β-anomeric carbon atoms. Peaks at 5.14 and 4.90 p.p.m. integrated to 0.6 and 0.3 proton, respectively, and were assigned to the α and β forms of the (reducing) rhamnopyranose residue. The three other peaks (5.05, 4.70, and 4.67 p.p.m., integrating for one proton each) represent sugars in glycosidic linkage. The peak at 5.05 p.p.m. was assigned the α-rhamnosyl residue. Signals at 4.70 and 4.67 p.p.m. must represent a β-rhamnosyl residue and a 4-*O*-Me-β-D-glucopyranosyluronic acid group, and were assigned thus: that at 4.70 p.p.m. to β-rhamnosyl, and that at 4.67 p.p.m. (J 7.7 Hz; all other coupling constants are <2 Hz) to the β-D-glucosyluronic group. These spectral and peak assignments agree with the ^{1}H-n.m.r. spectra recorded by Dudman[3] for the intact polysaccharide (4.99, 4.70, 4.67, and 4.53 p.p.m.), except for Dudman's assignment of the peak at 4.67 p.p.m. to one of the β-rhamnosyl residues, and of that at 4.53 p.p.m. to the β-4-*O*-Me-glucopyranosyluronic acid group. We consider that these assignments should probably be reversed, because the spectrum of the tetrasaccharide lacked a signal at 4.53 p.p.m. that was present in that of the polysaccharide. This result indicates that the signal at 4.53 p.p.m. represents the proton attached to C-1 of the β-rhamnosyl residue involved in the (1→4) linkage that is cleaved by the enzyme. Dudman[3] assigned the signal at 4.70 p.p.m. as being due to the other β-rhamnosyl residue (1,3-di-*O*-substituted), on the basis of the n.m.r. spectrum of a periodate-oxidation product, which agrees with our assignment.

Five peaks appear in the anomeric-carbon region (96.2–106.3 p.p.m.) of the ^{13}C-n.m.r. spectrum of the tetrasaccharide (see Table III). The peaks at 96.4 and 96.2 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 170 and 162 Hz) represent the α- and β-anomeric carbon

atoms, respectively, of the (reducing) rhamnose end-group[16,18]. Three peaks having $^1J_{C\text{-}1,H\text{-}1}$ 162 Hz represent β-anomeric carbon atoms; two with $^1J_{C\text{-}1,H\text{-}1}$ 170 Hz represent α-anomeric carbon atoms[19]. The peak at 106.3 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 162 Hz) was assigned to the β-anomeric carbon atom of the 4-*O*-methyl-D-glucopyranosyluronic acid group. Joseleau *et al.*[20] found a chemical shift of 104.80 p.p.m. and $^1J_{C\text{-}1,H\text{-}1}$ 160 Hz for the (1→4)-linked β-D-glucopyranosyluronic acid of *Klebsiella* serotype K41 polysaccharide. The peak at 104.9 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 170 Hz) represents the 3-*O*-linked α-L-rhamnosyl residue. Joseleau *et al.*[20] recorded 104.5 p.p.m. and *J* 171 Hz for that residue in the K41 polysaccharide. The signal at 103.0 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 162 Hz) represents the β-L-rhamnosyl residue that is also 3-*O*-linked; Hall and Morris[16] obtained values of 101.1 p.p.m. ($^1J_{C\text{-}1,H\text{-}1}$ 162 Hz) for that residue in *Klebsiella* serotype K32 polysaccharide. The shift values reported here are ~2 p.p.m. downfield from those previously reported[16,20], as sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP) was used as the reference standard, instead of (indirectly) tetramethylsilane.

REFERENCES

1 M. A. Dombrink-Kurtzman, W. E. Dick, Jr., K. A. Burton, M. C. Cadmus, and M. E. Slodki, *Biochem. Biophys. Res. Commun.*, 111 (1983) 798–803.
2 W. M. Rutherford, W. E. Dick, Jr., J. F. Cavins, M. A. Dombrink-Kurtzman, and M. E. Slodki, *Biochemistry*, 25 (1986) 952–958.
3 W. F. Dudman, *Carbohydr. Res.*, 66 (1978) 9–23.
4 E. E. Snell and F. M. Strong, *Ind. Eng. Chem., Anal. Ed.*, 11 (1939) 346–350.
5 M. C. Cadmus, L. K. Jackson, K. A. Burton, R. D. Plattner, and M. E. Slodki, *Appl. Environ. Microbiol.*, 44 (1982) 5–11.
6 W. C. Haynes, L. J. Wickerham, and C. W. Hesseltine, *Appl. Environ. Microbiol.*, 3 (1955) 361–368.
7 W. S. Hoffman, *J. Biol. Chem.*, 120 (1937) 51–55.
8 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–695.
9 L. K. Jackson, M. E. Slodki, M. C. Cadmus, K. A. Burton, and R. D. Plattner, *Carbohydr. Res.*, 82 (1980) 154–157.
10 J. Baddiley, J. G. Buchanan, R. E. Handschumacher, and J. F. Prescott, *J. Chem. Soc.*, (1956) 2818–2823.
11 I. W. Sutherland, *Biochemistry*, 9 (1970) 2180–2185.
12 C. C. Sweeley, R. Bentley, M. Makita, and W. W. Wells, *J. Am. Chem. Soc.*, 85 (1963) 2497–2507.
13 L. K. Jackson, M. E. Slodki, R. D. Plattner, K. A. Burton, and M. C. Cadmus, *Carbohydr. Res.*, 110 (1982) 267–276.
14 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
15 K. Stellner, H. Saito, and S. Hakomori, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
16 L. D. Hall and G. A. Morris, *Carbohydr. Res.*, 82 (1980) 175–184.
17 N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
18 V. Pozsgay, P. Nánási, and A. Neszmélyi, *Carbohydr. Res.*, 90 (1981) 215–231.
19 K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293–297.
20 J.-P. Joseleau, M. Lapeyre, M. Vignon, and G. G. S. Dutton, *Carbohydr. Res.*, 67 (1978) 197–212.