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A BRANCHED (1→3)-β-D-GLUCAN FROM A WATER EXTRACT OF *Dictyophora indusiata* FISCH. *

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ABSTRACT

A water-soluble, (1→6)-branched, (1→3)-β-D-glucan (T-3-G). $[\alpha]_D^{27}$ +40.8° (c 0.123, water), was isolated from a hot-water extract of the fruit bodies of *Dictyophora indusiata* Fisch. T-3-G was homogeneous as judged by ultracentrifugal analysis, Tiselius-type electrophoresis, and gel filtration. By gel filtration on Sepharose CL-2B, with 0.25M sodium hydroxide as the eluant, the molecular weight ($\bar{M}_w$) of T-3-G was estimated to be ~5.1 x 10^5. From the results of methylation analysis, periodate oxidation, Smith degradation, and enzymic hydrolysis, it was concluded that T-3-G has a main chain composed of β-(1→3)-linked D-glucopyranosyl residues, and two single, β-(1→6)-linked D-glucopyranosyl groups attached as side chains to, on average, every five sugar residues of the main chain. In addition, the results of enzymic hydrolysis indicated that the branching of T-3-G occurs regularly at O-6 of the β-(1→3)-linked backbone. The results of optical rotatory measurements and complex-formation with Congo Red suggested that T-3-G probably takes a triple-helical conformation.

INTRODUCTION

Previously, we reported[2-4] the structural features of two kinds of (1→6)-branched, (1→3)-β-D-glucans [T-4-N (ref. 2) and T-5-N (refs. 3 and 4)], respectively isolated from 2% sodium carbonate and M sodium hydroxide extracts of the fruit bodies of *Dictyophora iadusiata* Fisch. Furthermore, the biological properties of the β-D-glucans, *i.e.*, their antitumor activity[5] and anti-inflammatory effect[4], were reported. During the course of an investigation on polysaccharides of this fungus, another, branched (1→3)-β-D-glucan (T-3-G) has now been isolated

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from a hot-water extract obtained prior to extraction with 2% sodium carbonate and M sodium hydroxide. We now describe the purification, characterization, and structural analysis of T-3-G.

RESULTS AND DISCUSSION

The fruit bodies of *D. indusiata*, treated with hot, 70% aqueous ethanol as previously reported[6], were thoroughly extracted with hot water, and the extract was treated with Pronase; this was followed by the Sevag procedure', and dialysis. The nondialyzable solution was mixed with ethanol (3 vol.), and the precipitate, collected by centrifugation, was dissolved in water, and the solution lyophilized. to yield a crude polysaccharide fraction (T-3) in -6.5% yield. The residue recovered after the hot-water extraction was extracted with 2% sodium carbonate and then with M sodium hydroxide, and the β-D-glucans (T-4-N and T-5-N) were isolated from each extract, as previously reported' [4].

The crude fraction T-3 contained some polysaccharides, and one of them, a β-D-glucan (T-3-G), was isolated in the following way. A solution of T-3 in water was mixed with ethanol (0.67 vol.), the resulting precipitate was dissolved in water, and the solution was subjected to the freeze-thawing procedure". The gelatinous, cold (~5°) water-insoluble materials formed were separated by centrifugation, dissolved in hot water, and then reduced with sodium borohydride. Subsequently, the product was treated, under a nitrogen atmosphere, with 0. 1M sodium hydroxide

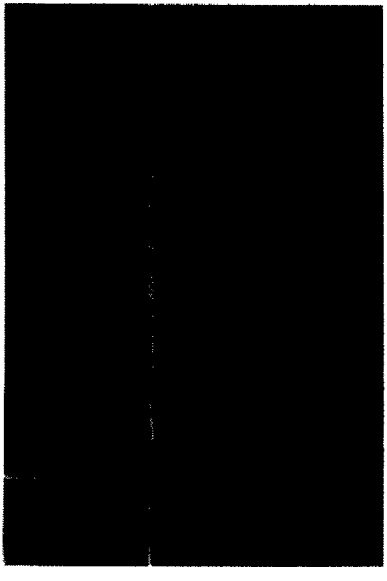


Fig. 1 Ultracentrifugal pattern of T-3-G. T-3-G (4 mg/mL in 01M sodium chloride) after 50 min at 60,000 r.p.m.

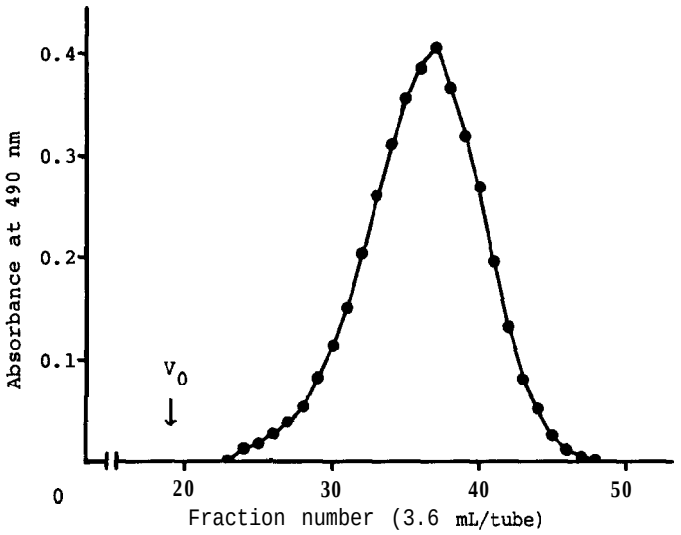


Fig. 2. Chromatogram of T-3-G on Sepharose CL-2B. The column (1.5 × 97 cm) was eluted with 0.25M sodium hydroxide.

at room temperature, the base neutralized with acid, and the mixture dialyzed. The water-insoluble polysaccharide (T-3-M, composed of D-mannose) formed in the inner solution was removed by centrifugation, and the supernatant liquor was lyophilized, to afford the polysaccharide (T-3-G) in ~0.6% yield; this slowly dissolved in water to give a highly viscous solution. Fraction T-3-G was homogeneous, as determined by ultracentrifugal analysis in 0.1M sodium chloride (see Fig. 1), and by Tiselius-type electrophoresis in alkaline borate buffer. T-3-G was also found to be pure by gel filtration on Sepharose CL-2B, with 0.25M sodium hydroxide as the eluant, as shown in Fig. 2.

The polysaccharide (T-3-G) was composed solely of D-glucosyl residues, as shown by paper chromatography (p.c.) of the hydrolyzate, by gas-liquid chromatography (g.l.c.) of the alditol acetate[9] prepared from the hydrolyzate, and by the specific rotation of the hydrolyzate. T-3-G had a positive specific rotation, $[\alpha]_D^{27} +40.8°$ (c 0.123, water), and showed characteristic absorbance at 892 cm^{-1} in the infrared (i.r.) spectrum, indicating the presence of β-D-glycosidic linkages[10]. T-3-G contained small proportions of nitrogen (0.40%) and ash (~1.9%) as found by elementary analysis, and the total sugar content was found to be 98.5% (as hexosyl residues) by the phenol-sulfuric acid method[11]. The calibration curve shown in Fig. 3 was made by gel filtration of standard dextrans on Sepharose CL-2B with 0.25M sodium hydroxide; the molecular weight ($\bar{M}_w$) of T-3-G thus estimated was ~5.1×10^5.

The glucan (T-3-G) was methylated by the method of Hakomori[12], and the fully methylated polymer was hydrolyzed with acid. The partially methylated sugars were analyzed as the alditol acetate derivatives[9] by g.l.c. and g.l.c.-mass spectro-

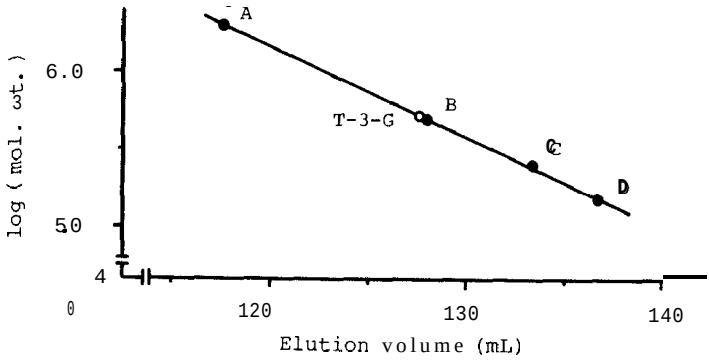


Fig. 3. Determination of molecular weight of T-3-G by gel filtration on Sepharose CL-2B. The elution volume was plotted against the logarithm of the molecular weight of dextrans T-2.000 (A: mol. wt.. 2,000,000), T-500 (B: 495,000), T-250 (C: 253.000). and T-150 (D: 154.000).

metry (g.l.c.–m.s.), and identified by comparing their retention times in g.l.c., and their mass spectra, with those of authentic samples, or with the values reported in the literature[13]. As shown in Table I, the methylation analysis indicated the presence of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucose in the molar ratios of 1.00:1.49:0.98.

T-3-G was oxidized with 2.5mM sodium metaperiodate for 19 days at 3–6°. The periodate consumption and formic acid production per hexosyl residue were 0.57 and 0.28 mol, and these values are in good agreement with those calculated from the results of the methylation analysis, namely, 0.58 and 0.29 mol (see Table I). The oxidized polysaccharide was treated with sodium borohydride, and the resulting polyalcohol was hydrolyzed with acid. The hydrolyzate (containing the Smith-degradation product[14]) was analyzed by g.l.c. as the alditol acetate derivatives", and glycerol and glucose were detected in the molar ratio of 1.00:2.58. The glycerol must have arisen from the terminal D-glucose residues, and the

TABLE I

G L C AND G L C –M S OF ALDITOL ACETATES DERIVED FROM METHYLATED T-3-G

Methylated sugar (as alditol acetate)	T[a,b]	*Main mass-fragments* (m/z)	*Molar ratio*	*Mode of linkage*
2,3,4,6-Me_4-Glc[c]	**1.00**	43, 45, 71, 87, 101.117. 129. 145, 161,205	1 00	Glc*p*-(1→
2,4,6-Me_3-Glc	1.96	43, 45, 87, 101, 117, 129, 161.233	1.49	→3)-Glc*p*-(1→
2,4-Me_2-Glc	5.03	33, X7, 117, 129. 189	0 98	→3,6)-Glc*p*-(1→

"Relative retention-time with respect to that of authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (1.00). [b]3% of ECNSS-M column at 166 °. [c]2,3,4,6-Me_4-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.*

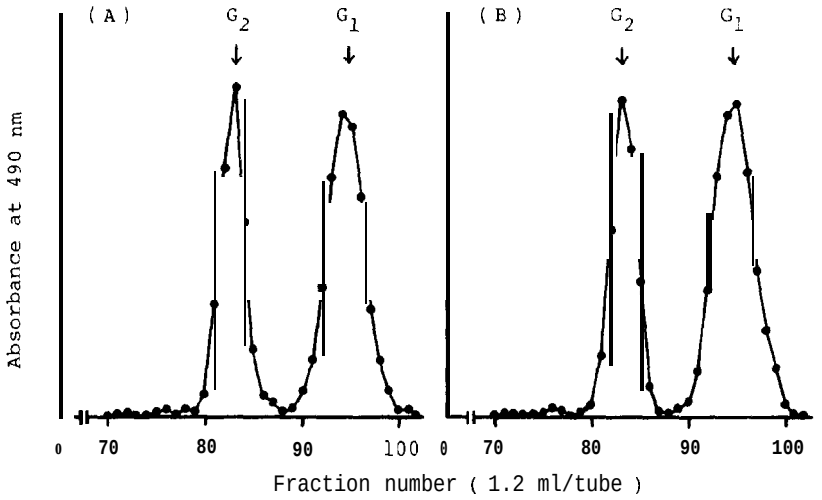
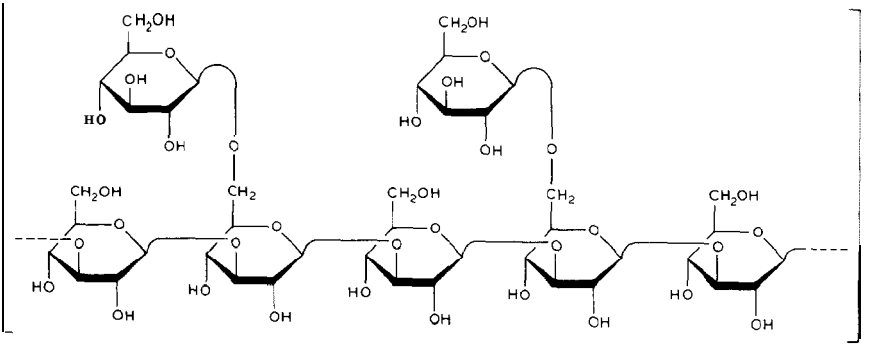


Fig. 4. Chromatograms, on Bio-Gel P-2, of enzymic-degradation product of T-3-G. The products at 4 h (A) and at 51 h (B) were applied to a column (1.5 x 98 cm) and eluted with water.

occurrence of glucose must be due to the presence of oxidation-resistant o-glucose, such as (1→3)-linked residues. These results are in good agreement with those from the methylation analysis.

The glucan was digested with exo-(1→3)-β-D-glucanase (Lysing Enzymes), from Basidiomycetes, in McIlvaine buffer (pH 4.9) for 4 and 51 h at 38". Each enzymic-degradation product was subjected to gel filtration on Bio-Gel P-2; only two peaks, corresponding to mono- and di-saccharide, were revealed (see Fig. 4). The products recovered from the eluates at 4 and 51 h were analyzed by p.c., or by g.l.c. as the trifluoroacetyl derivative of the disaccharide-alditol[15]. Consequently, in each case, glucose and gentiobiose were detected. The molar ratio of gentiobiose to glucose (Gen/Glc) was almost constant, that is, 0.70 at 4 h and 0.63 at 51 h. vs. 0.67 expected from the methylation analysis (see Table I).

The foregoing data indicate that glucan T-3-G, isolated from a hot-water extract of *D. indusiata*, has a main chain composed of (1→3)-β-D-glucopyranosyl



residues, and has as side chains two single, β-(1→6)-linked D-glucopyranosyl groups attached, on average, to every five sugar residues of the main chain. as shown in **1**. Furthermore, from the results of the enzymic hydrolysis. it was indicated that the branching of T-3-G occurs regularly at O-6 of the β-(1--+3)-linked backbone.

The conformational behavior of linear, and branched, (1→3)-β-D-glucans, including our β-D-glucans (T-4-N and T-5-N), has been discussed in regard to changes in specific rotation and in the visible absorption spectra of the complexes formed with Congo Red, at various concentrations of alkali[2,4,16]. The values of specific rotation of T-3-G ($[\alpha]_D^{27}$ +35.8 to +41.0°) at concentrations of sodium hydroxide lying between 0 and 0.15M decrease abruptly (down to $[\alpha]_D^{27}$ +8.7°) at concentrations of alkali in the range of 0.15–0.25M, as shown in Fig. 5. The change in the specific rotation reverted almost to the initial value ($[\alpha]_D^{27}$ +35.5°) when a solution of T-3-G in M sodium hydroxide was made neutral (pH 7.3) with acid. In addition, the value of the visible absorption maximum (A_{max}) of Congo Red in 0.1M sodium hydroxide was largely shifted to a longer wavelength (508 nm) by the presence of T-3-G (A_{max} of Congo Red only: 486 nm). These observations suggested that the reversible, conformational transition of T-3-G occurs at concentrations of sodium hydroxide in the range of 0.15–0.25M, and that T-3-G, as well as T-4-N and T-5-N, also has an ordered, triple-helical structure in neutral or weakly alkaline solution (<0.15M NaOH), as previously reported[2,4].

As described in the earlier and present articles in this series[2–4,6,17], the fruit bodies of ***D. indusiata*** were successively extracted with solvents; that is, at first, with hot, 70% aqueous ethanol; secondly with hot water; thirdly with 2% sodium

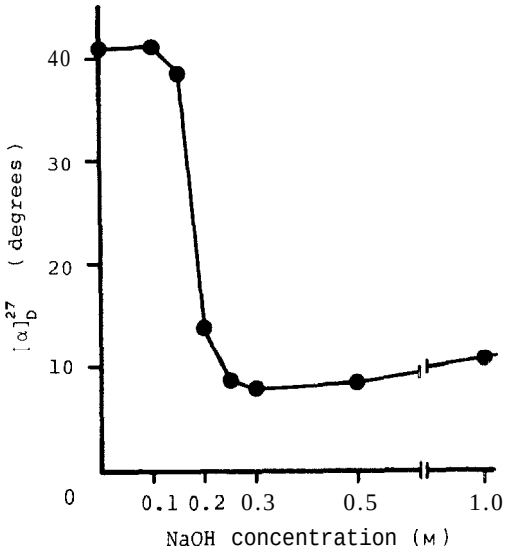


Fig. 5. Dependence of specific rotation, at 589 nm. of T-3-G on the concentration of sodium hydroxide.

carbonate; and lastly with M sodium hydroxide, and consequently, three kinds of homogeneous, β-(1→6)-branched, (1→3)-β-D-glucans, namely, the present glucan T-3-G (obtained from the hot-water extract), T-4-N (from the 2% sodium carbonate extract), and T-5-N (from the M sodium hydroxide extract) were isolated. These β-D-glucans have essentially a main chain composed of β-(1→3)-linked D-glucopyranosyl residues, and many side chains consisting of a single, β-(1→6)-linked D-glucopyranosyl group. However, the (1+3)+-D-glucans differ from each other in specific rotation ($[\alpha]_D^{27}$ +40.8° for T-3-G; $[\alpha]_D^{20}$ +19.0° for T-4-N; and $[\alpha]_D^{18}$ +28.7° for T-5-N, in water), and in molecular weight (5.1 X 10^5 for T-3-G; 5.5 x 10^6 for T-4-N; and 3.3 x 10^5 for T-5-N) determined by gel filtration on Sepharose CL-2B with 0.25M sodium hydroxide as the eluant. The glucans can also be distinguishable in the degree of branching; two side-chains out of five residues of the main chain for T-3-G and T-4-N, and two out of seven for T-5-N. More interestingly, the branching of T-3-G and T-5-N occurs regularly at O-6 of the β-(1→3)-linked backbone. On the other hand, the side chains in T-4-N are mainly localized in the neighborhood of the nonreducing end of the main chain, as already reported[2]. Furthermore, T-3-G and T-4-N do not contain such other types of glycosidic linkage as internal, (1→6) linkages, and branching points at O-2 of the β-(1→3)-linked backbone, found[3] in T-5-N.

Thus, some significant differences between these three kinds of branched, (1→3)-β-D-glucans (T-3-G, T-4-N, and T-5-N) were observed, although the chemical and high-order structures appear to be similar to each other. These findings are of considerable interest in connection with the structure of branched, (1→3)-β-D-glucans and their physiological role in the fruit bodies of this fungus. T-3-G, together with other polysaccharides, e.g., a partially 0-acetylated (1→3)-α-D-mannan (T-2-HN) isolated [6,17] from a 70% aqueous ethanol extract, may occur as one of the cell-surface glycans of this fungus, whereas T-4-N and T-5-N, which were respectively extracted with dilute and concentrated alkaline solutions, might arise from intracellular components, or be present as components of the cell wall[18].

EXPERIMENTAL

Materials. -The dried fruit-bodies of *D. indusiata* are commercially available in Hong Kong. Pronase (45,000 p.u.k./g), and Lysing Enzymes were respectively purchased from Kaken Chemical Ind., Tokyo, and Sigma Chemical Company. Sepharose CL-2B and standard dextrans were purchased from Pharmacia Fine Chemicals. Bio-Gel P-2 was purchased from Bio-Rad Laboratories.

General. — All evaporations were conducted under diminished pressure at bath temperatures not exceeding 40". Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. I.r. spectra were recorded with a JASCO A-102 spectrometer. Ultracentrifugal analysis was conducted with a MOM 3170/b analytical ultracentrifuge at 20". P.c. was performed by the double-ascending method, using Toyo Roshi No. 51A filter-paper and the following solvent systems

(v/v): *(A)* 6:4:3 1-butanol-pyridine-water, (B) 10:4:3 ethyl acetate–pyridine–water, and (C) 6:1:3 1-propanol-ethyl acetate-water. Sugars were detected with an alkaline silver nitrate reagent'". G.l.c. was performed in a Shimadzu GC-4CM apparatus equipped with a flame-ionization detector. Glass columns (0.3 x 200 cm) were used, with nitrogen as the carrier gas at a flow rate of 4.5 mL/min. The columns used were (*1*) 3% of ECNSS-M on Gaschrom Q (100-120 mesh) at 166°, and (*2*) 2% of GE-XF 1105 on Chromosorb P (SO-100 mesh) at 200°. Peak areas were measured with a Shimadzu E1A Chromatopac. G.l.c.–m.s. was conducted with a JEOL JMS-D 300 apparatus equipped with a glass column (0.2 x 100 cm) packed with 3% of ECNSS-M, at 185°, at a pressure of helium of 127.5 kPa. The mass spectra were recorded under the conditions previously reported".

Isolation of the polysaccharide. — After being extracted with hot, 70% aqueous ethanol as already reported[6], the fruit bodies (100 g) were 4 times extracted, with stirring, with water (1.5 L) for 5 h in a boiling-water bath. The extracts. obtained on centrifugation for 30 min at 4,000 r.p.m., were concentrated, and dialyzed against distilled water for 4 days. Proteins in the nondialyzable fraction were removed by Pronase treatment followed by the Sevag procedure' and dialysis, as previously reported'. Ethanol (3 vol.) was then added to the nondialyzable solution (1 L). and the resulting precipitate was separated by centrifugation, dissolved in water, and the solution lyophilized, to afford a crude. polysaccharide fraction (T-3); yield. 6.5 g.

A portion of T-3 (5.5 g) was dissolved in water (800 mL), and ethanol (0.67 vol.) was added to the solution. The resulting precipitate (3.5 g) was again dissolved in water (800 mL), and the solution was frozen and allowed to thaw[8] at ~5°. Cold-water-insoluble, gelatinous materials were formed in the solution. The materials collected by centrifugation for 40 min at 8,000 r.p.m. were dissolved in hot water (-70, 150 mL), and reduced with sodium borohydride (2.5 g), with stirring, for 20 h at room temperature. The mixture was treated, under a nitrogen atmosphere. with 0.6M sodium hydroxide (30 mL) for 5 h at room temperature, made neutral with acetic acid, and then dialyzed. The water-insoluble materials (T-3-M) formed in the inner solution were centrifuged off, and the supernatant liquor thus obtained was concentrated, and lyophilized, to afford the purified polysaccharide (T-3-G) as colorless flakes; yield, 58.5 mg.

Electrophoresis. — Tiselius-type electrophoresis of T-3-G was performed under the conditions already reported?. Electrophoretic mobility (u) = 0.58 x 10^{-4} $cm^2/V \cdot sec$.

Gel filtration, and estimation of molecular weight. — Gel filtration of T-3-G and standard dextrans on a column of Sepharose CL-2B, with 0.25M sodium hydroxide as the eluant, was performed as previously reported[2]. A calibration curve, constructed by use of standard dextrans, is shown in Fig. 3. and therefrom, the molecular weight was estimated.

Analysis of component sugars. — The polysaccharide (T-3-G) was hydrolyzed

with 90% formic acid for 3 h at loo", and then with 0.25M sulfuric acid for 15 h thereat. The specific rotation of the hydrolyzate was $[\alpha]_D^{14}$ +48.2° (c 0.085, 0.05M sulfuric acid). The hydrolyzate was made neutral with barium carbonate in the usual way, and analyzed by p.c. (solvents ***A*** and B), and by g.l.c. (condition I) as the corresponding alditol acetates'.

Methylation ***analysis. -T-3-G*** (5 mg) was methylated twice by the Hakomori procedure[12], and the fully methylated polysaccharide was hydrolyzed with 90% formic acid and 0.25M sulfuric acid, as previously reported". After removal of the acid with Amberlite CG-400 (CO_3^{2-}) ion-exchange resin, the partially methylated sugars were converted into the alditol acetates[9], and analyzed by g.l.c. (condition 1) and g.l.c.-m.s.

Periodate oxidation, and Smith degradation. — The polysaccharide (26 mg) was subjected to periodate oxidation with 2.5mM sodium metaperiodate (230 mL), with stirring, at 3–6° in the dark. The periodate consumption was measured by the spectrophotometric method used by Ikenaka[20], and the formic acid produced was determined by titration with 5mM sodium hydroxide[21]. The oxidation was complete after 19 days. A portion (3 mg) of the polyalcohol derivative (yield, 21 mg), prepared from the periodate-oxidized polysaccharide with sodium borohydride, was successively treated with 90% formic acid and 0.25M sulfuric acid, and the hydrolyzate was analyzed by g.l.c. as the alditol acetates, as already reported[2].

Enzymic hydrolysis. — T-3-G (4.2 mg) was treated with exo-(1+3)-P-D-glucanase (Lysing Enzymes; 2.1 mg) in 17mM McIlvaine buffer, pH 4.9 (10 mL) at 38"; half of the reaction mixture was taken at 4 h, and the residual portion was allowed to react for 51 h. Each enzymic degradation product, prepared according to the procedure outlined[2], was subjected to gel filtration on a column of Bio-Gel P-2, and two peaks were revealed (see Fig. 4). The products recovered were analyzed by p.c. (solvent *C*), and two spots, corresponding to glucose and gentiobiose (R_{Glc} 0.72), were detected. The gentiobiose was further identified by g.l.c. (condition 2) as the trifluoroacetate of the disaccharide-alditol[15], as previously reported[2]. The molar ratio of the sugars was calculated from the peak areas in gel filtration on Bio-Gel P-2.

Specific ***rotations in aqueous sodium hydroxide.*** — Specific rotations of T-3-G (0.98 mg/mL) were measured at 27" at various concentrations of sodium hydroxide in the range of 0–1.0M, as previously reported[2,4].

Interaction with Congo Red in aqueous sodium hydroxide. — T-3-G (1 mg/mL) was dissolved[2,4] in 0.1M sodium hydroxide containing Congo Red (46μM), and the visible absorption spectra were recorded.

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