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

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ABSTRACT

A water-soluble, (1→6)-branched, (1→3)-β-D-glucan (T-4-N), $[\alpha]_D^{20}$ +19° (*c* 0.1, water), was isolated from a 2% sodium carbonate extract of the fruit bodies of *Dictyophora indusiata* Fisch. T-4-N was homogeneous as judged by gel filtration, Tiselius-type electrophoresis, and ultracentrifugal analysis. By gel filtration on Sepharose CL-2B, with 0.25M sodium hydroxide as the eluant, the molecular weight of T-4-N was estimated to be $\sim 5.5 \times 10^6$. From the results of methylation analysis, periodate oxidation, Smith degradation (complete, and also mild), partial acetolysis, and enzymic degradation, it was concluded that T-4-N 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 fifth sugar residue of the main chain. In addition, the results of the enzymic hydrolysis suggested that the β-(1→6)-linked D-glucosyl side chains are mainly localized in the neighborhood of the nonreducing end of the main chain. The results of optical rotatory measurement and complex-formation with Congo Red indicated that T-4-N probably takes a triple-helical conformation.

INTRODUCTION

In earlier articles in this series[1–6] were reported the structural features of a partially *O*-acetylated (1→3)-α-D-mannan (T-2-HN)[2,3] and a (1→6)-branched (1→3)-β-D-glucan (T-5-N)[4,5] isolated from the fruit bodies of *Dictyophora indusiata* Fisch. (Japanese name: Kinugasatake). Furthermore, the biological properties of both polysaccharides, *i.e.*, antitumor activity[6] and anti-inflammatory effect[1,5], were reported. During the course of an investigation on polysaccharides of this fungus, another β-D-glucan (T-4-N) has now been isolated from a 2% sodium carbonate extract. The present article deals with the purification, characterization, and structural analysis of T-4-N.

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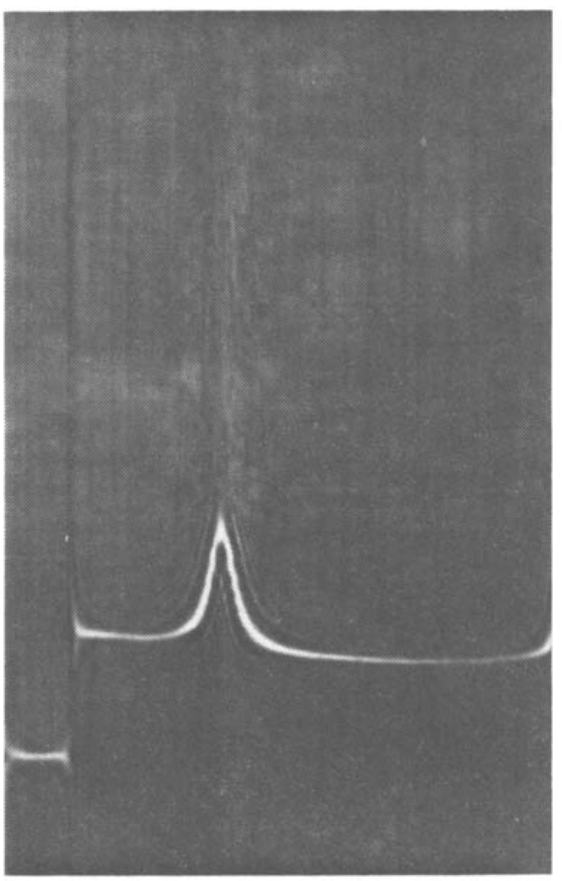


Fig. 1 Ultracentrifugal pattern of T-4-N. [T-4-N (3.5 mg/ml in 0.25M sodium hydroxide) after 50 min at 60,000 r.p.m.]

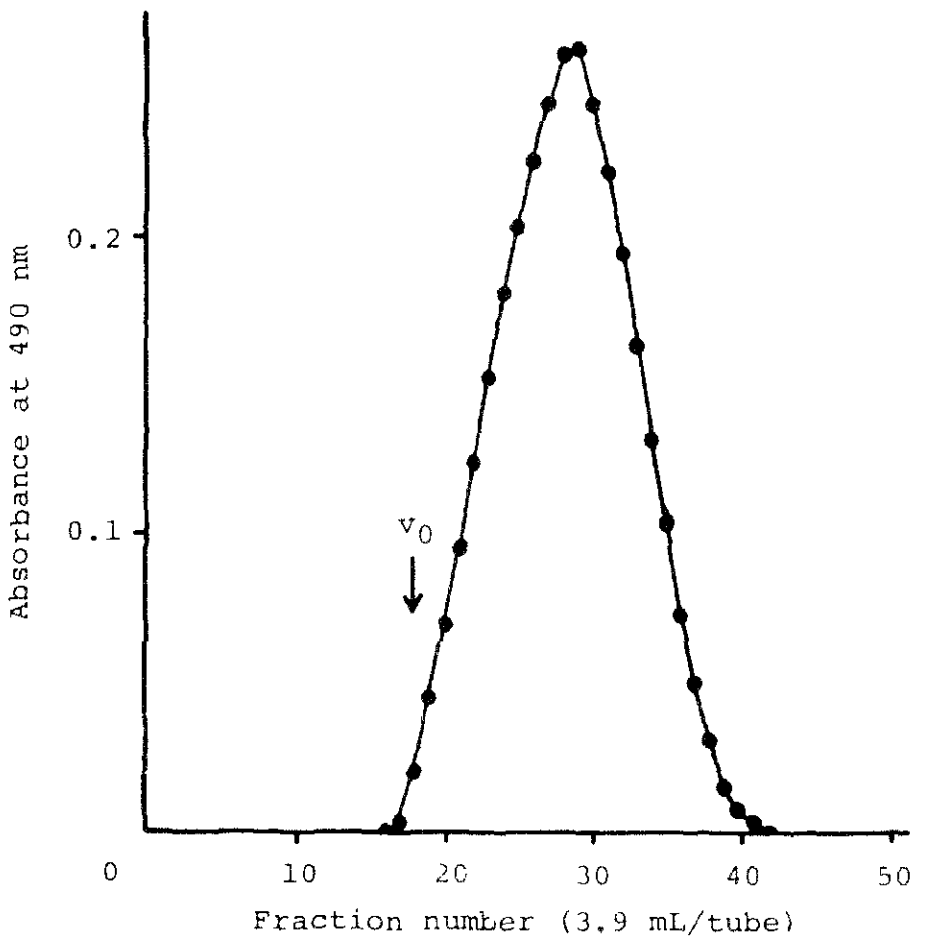


Fig. 2. Chromatogram of T-4-N on Sepharose CL-2B [The column (1.5 × 98 cm) was eluted with 0.25M sodium hydroxide.]

RESULTS AND DISCUSSION

The fruit bodies of D. indusiata, extracted with hot 70% aqueous ethanol as previously reported[2], were treated with hot water. The residue was then extracted with 2% sodium carbonate at room temperature. The alkaline extract was made neutral, and dialyzed. The nondialyzable material (T-4) was treated with Pronase, followed by the Sevag procedure[7] and dialysis. The nondialyzable solution was mixed with 1 volume of ethanol, and the precipitate obtained on centrifugation was dissolved in water, and lyophilized, to yield the polysaccharide (T-4-N) in ~0.7% yield; this slowly dissolved in water to give a highly viscous solution. T-4-N was homogeneous, as determined by ultracentrifugal analysis in 0.25M sodium hydroxide (see Fig. 1), and by Tiselius-type electrophoresis in alkaline borate buffer. T-4-N 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-4-N) 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[8] prepared from the hydrolyzate, and by the specific rotation of the hydrolyzate. T-4-N had a low, positive, specific rotation, $[\alpha]_D^{20}$ +19.0° (*c* 0.1, water), and showedcharacteristic absorbance at 890 cm^{-1} in the infrared (i.r.) spectrum, indicating the presence of the β-D configuration[9]. T-4-N contained neither nitrogen nor ash (by elementary analysis), and the total

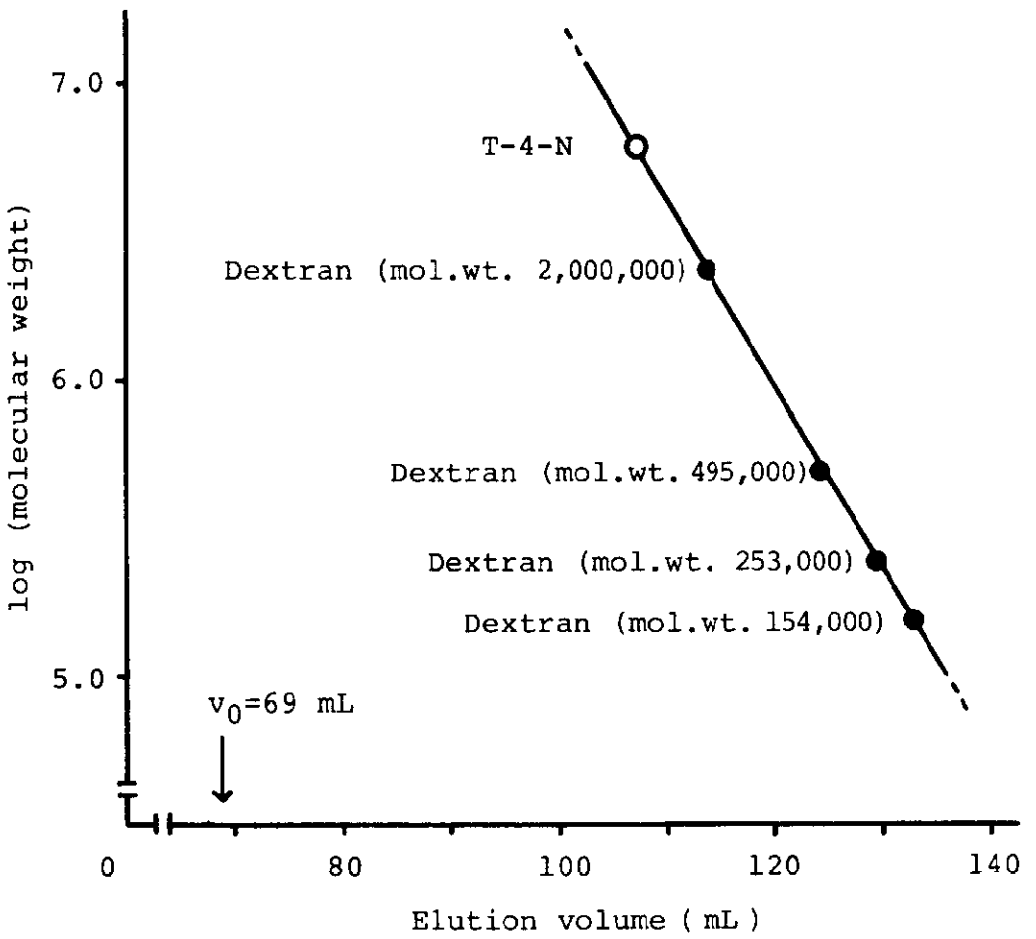


Fig. 3. Determination of molecular weight of T-4-N by gel filtration on Sepharose CL-2B. [The elution volume was plotted against the logarithm of the molecular weight of dextrans T-2,000, T-500, T-250, and T-150.]

sugar content was found to be 99.2% (as hexosyl residues) by the phenol–sulfuric acid method [10]. 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-4-N thus estimated was $\sim 5.5 \times 10^6$.

The glucan (T-4-N) was methylated by the method of Hakomori[11]. The fully methylated glucan was successively hydrolyzed with formic acid and sulfuric acid. The hydrolyzate was analyzed as the alditol acetate derivatives[8] by g.l.c. and g.l.c.–mass spectrometry (g.l.c.–m.s.). The partially methylated alditol acetates were identified by comparing their retention times in g.l.c., and their mass spectra, with those of authentic samples, or with the values in the literature[12]. 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 1.00:1.54:0.99. The results suggested that this glucan has many nonreducing end-groups (28.3%) and a backbone of (1→3)-linked D-glucopyranosyl residues, and contains many branching points (28.1%) at O-6 of the (1→3)-linked D-glucosyl residues.

T-4-N was oxidized with 2.5mM sodium metaperiodate for 28 days at 4–7°. The periodate consumption and formic acid production per hexosyl residue were 0.55 and 0.27 mol, and the values are in good agreement with those calculated from the results of the methylation analysis, namely, 0.57 and 0.28 mol (see Table I). The oxidized polysaccharide was treated with sodium borohydride, and the resulting polyalcohol was hydrolyzed with acid. The hydrolyzate (the Smith-degradation product[13]) was analyzed by g.l.c. as the alditol acetate derivatives[8], and glycerol and glucose were detected in the molar ratio of 1.00:2.46. The glycerol must have arisen from the terminal residues, and the occurrence of glucose must be due to the presence of oxidation-resistant D-glucose, such as (1→3)-linked residues. These results are in good agreement with those expected from the methylation analysis.

The mild, acid hydrolysis of the polyalcohol just described yielded a water-insoluble product (the controlled, Smith-degradation product: T-4-NOI). As T-4-NOI was insoluble in dimethyl sulfoxide (Me_2SO), it was first dissolved in 4-

TABLE I

G.L.C. AND G.L.C.–M.S. OF ALDITOL ACETATES DERIVED FROM METHYLATED T-4-N

Methylated sugar (as alditol acetate)	T[a]		*Main mass fragments (m/z)*	*Molar ratio*	*Mode of linkage*
	Column 1[b]	*Column 2*[c]			
2,3,4,6-Me_4-Glc[d]	**1.00**	**1.00**	43,45,71,87,101,117, 129,145,161,205	**1.00**	Glc*p*-(1→
2,4,6-Me_3-Glc	1.98	1.74	43,45,87,101,117, 129,161,233	1.54	→3)-Glc*p*-(1→
2,4-Me_2-Glc	5.06	3.86	43,87,117,129,189	0.99	→3,6)-Glc*p*-(1→

[a]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 at 171°. [c]3% of Silicone OV-225 at 187°. [d]2,3,4,6-Me_4-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc.

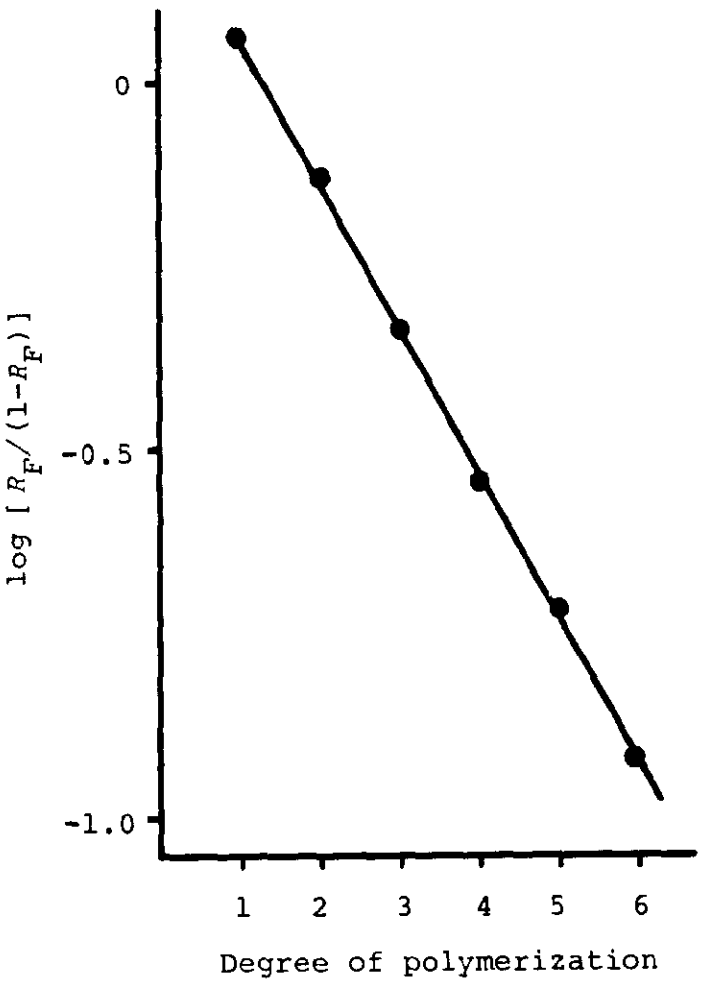


Fig. 4. Relationship between the degree of polymerization and log [R_F/(1 − R_F)] values of acetolysis products of T-4-N in p.c.

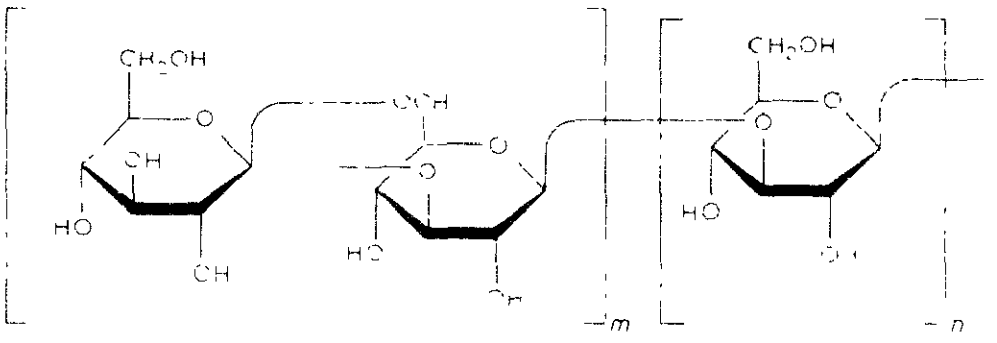
methylmorpholine *N*-oxide[14], which is a good solvent for such otherwise insoluble polysaccharides as cellulose[15], and the solution, after being diluted with Me_2SO, was treated with methylsulfinyl carbanion, and then with methyl iodide, according to the method of Hakomori[11]. The methylation analysis showed the presence of 2,4,6-tri-*O*-methyl-D-glucose (>99%), and the result indicated that T-4-NOI is essentially composed of a linear, (1→3)-linked D-glucopyranosyl chain. From these results, T-4-N has a backbone of (1→3)-linked D-glucosyl residues; some of the residues are substituted at O-6, and (1→3)-linked residues are absent from the side chains.

The partial acetolysis[16] of T-4-N gave D-glucose and a number of oligosaccharides, as shown by p.c. A linear relationship (see Fig. 4) exists between the presumed degree of polymerization of the oligosaccharides detected and their log[R_F/(1 − R_F)] values, as proposed by French and Wild[17]. Moreover, the disaccharide fraction isolated from the paper chromatograms was identified as laminarabiose by g.l.c. of the trifluoroacetyl derivative of the disaccharide-alditol[18]. These results indicated that partial acetolysis of T-4-N yielded a homologous series of β-(1→3)-linked D-gluco-oligosaccharides.

The glucan was treated with exo-(1→3)-β-D-glucanase (Lysing Enzymes; Sigma Chemical Company) from Basidiomycetes for 45 h at 38°; only glucose and gentiobiose were liberated, in the molar ratio of 1.00:0.69 in p.c. (1.00:0.65, as

evaluated from the methylation analysis). Gentiobiose isolated was independently identified by g.l.c. as the trifluoroacetyl derivative, as already described. In a similar enzymic hydrolysis, T-4-NOI liberated only glucose. The results indicated that T-4-N consists of a (1→3)-linked D-glucosyl backbone, partially branched at O-6 of the D-glucosyl residues of the backbone, and that each side chain is composed of only one D-glucosyl group.

The foregoing data indicate that glucan T-4-N, isolated from a 2% sodium carbonate extract of *D. indusiata*, has a main chain composed of (1→3)-β-D-glucopyranosyl residues, and has many side chains of two single, β-(1→6)-linked D-glucopyranosyl groups attached, on average, to every fifth sugar residue of the main chain, as shown in **1**. The ratio of the side chains to the main chain is very

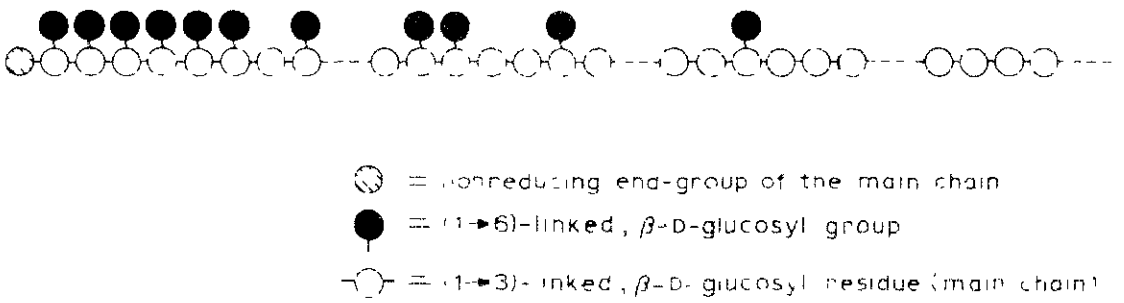


(*m*, *n* = 2, 3)

1

similar to that observed for lentinan from *Lentinus edodes*[19] and for a β-D-glucan from *Botrytis cinerea*[20].

On the other hand, the enzymic degradation products of T-4-N formed with elapse of time were examined by gel filtration on Bio-Gel P-2. In the earlier stage (at 5 h) of enzymic hydrolysis with exo-(1→3)-β-D-glucanase, gentiobiose was mainly liberated, the molar ratio of gentiobiose to glucose (Gen/Glc) being ~5.5. The ratio gradually decreased, and reached a constant value (0.8) at 33 and 46 h (see Fig. 5). In addition, a small proportion of the undigested polysaccharide fraction (T-4-R22) recovered from the reaction mixture at 22 h was again treated with the enzyme for 42 h; glucose, together with a negligible proportion of gentiobiose, was liberated. These findings indicate that the branching of T-4-N occurs irregularly, and that the β-(1→6)-linked D-glucosyl groups are mainly localized in the neighborhood of the nonreducing end of the main chain. Accordingly, a possible structure of T-4-N may be represented as in **2**.



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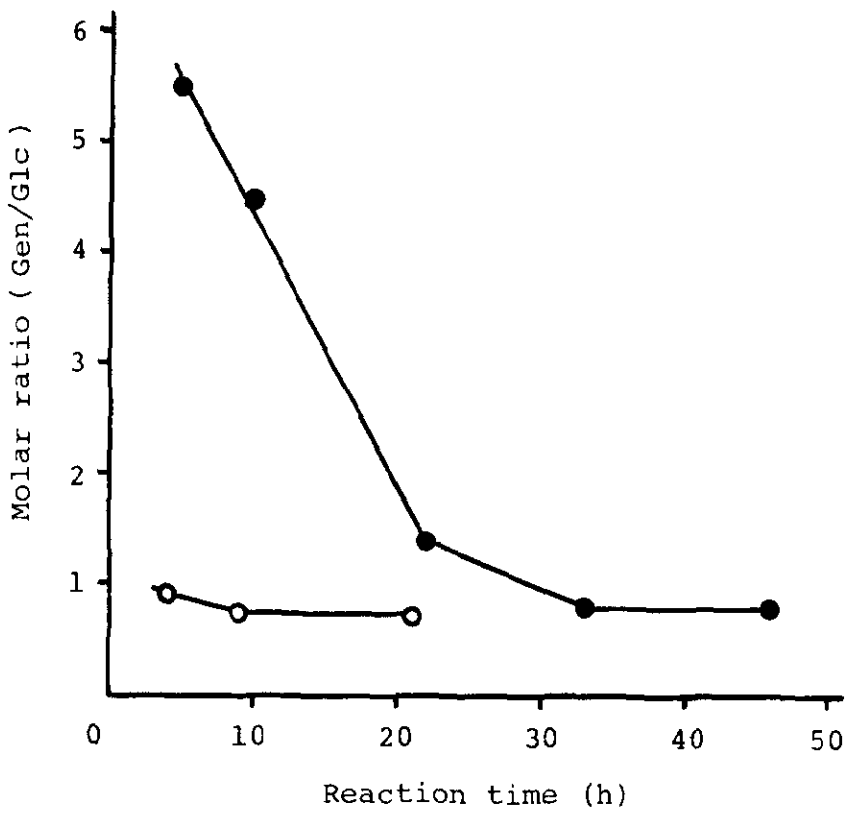

Fig. 5. Molar ratio of gentiobiose to glucose in enzymic hydrolyzates of T-4-N and T-5-N. [The ratio was calculated on the basis of the peak-area in gel filtration on Bio-Gel P-2. Key: —●—, T-4-N; —○—, T-5-N.]

Branched (1→3)-β-D-glucans having side chains of single, D-glucosyl groups at O-6, such as sclerotan[21], schizophyllan[22], and others[4,23], have been isolated, and these have been subjected to enzymic hydrolysis with exo-(1→3)-β-D-glucanase, but not examined in regard to the relative value of Gen/Glc with elapse of time. For the enzymic hydrolysis of a branched (1→3)-β-D-glucan (AP) from *Grifora umbellata*[24], it was reported that the molar ratio Gen/Glc was constant (0.5) throughout the hydrolysis. Similarly, in the case of enzymic hydrolysis of the β-D-glucan[4] T-5-N which was isolated from a M sodium hydroxide extract of *D. indusiata*, the ratio Gen/Glc was also almost constant, that is, 0.9 at 4 h and 0.7 at 9 and 21 h (see Fig. 5). These results, in contrast to those for T-4-N, suggest that the branching of these β-D-glucans (AP and T-5-N) occurs regularly at O-6 of the (1→3)-β-linked backbone. Thus, the first evidence for the existence of heterogeneity in the branching of the (1→6)-branched (1→3)-β-D-glucan (T-4-N) was provided.

The conformational behavior of linear, and branched, (1→3)-β-D-glucans, including the β-D-glucan 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[5,22,25]. The values of specific rotation of T-4-N ($[\alpha]_D^{26}$ +20.0 to +22.1°) at concentrations of sodium hydroxide lying between 0 and 0.15M decrease abruptly (down to $[\alpha]_D^{26}$ −2.5°) at concentrations of alkali in the range of 0.15–0.25M, as shown in Fig. 6. The change in the specific rotation reverted almost to the initial value ($[\alpha]_D^{26}$ +18.0°), when a solution of T-4-N in M NaOH was made neutral (pH 6.8) with acid. In addition, the values of the visible

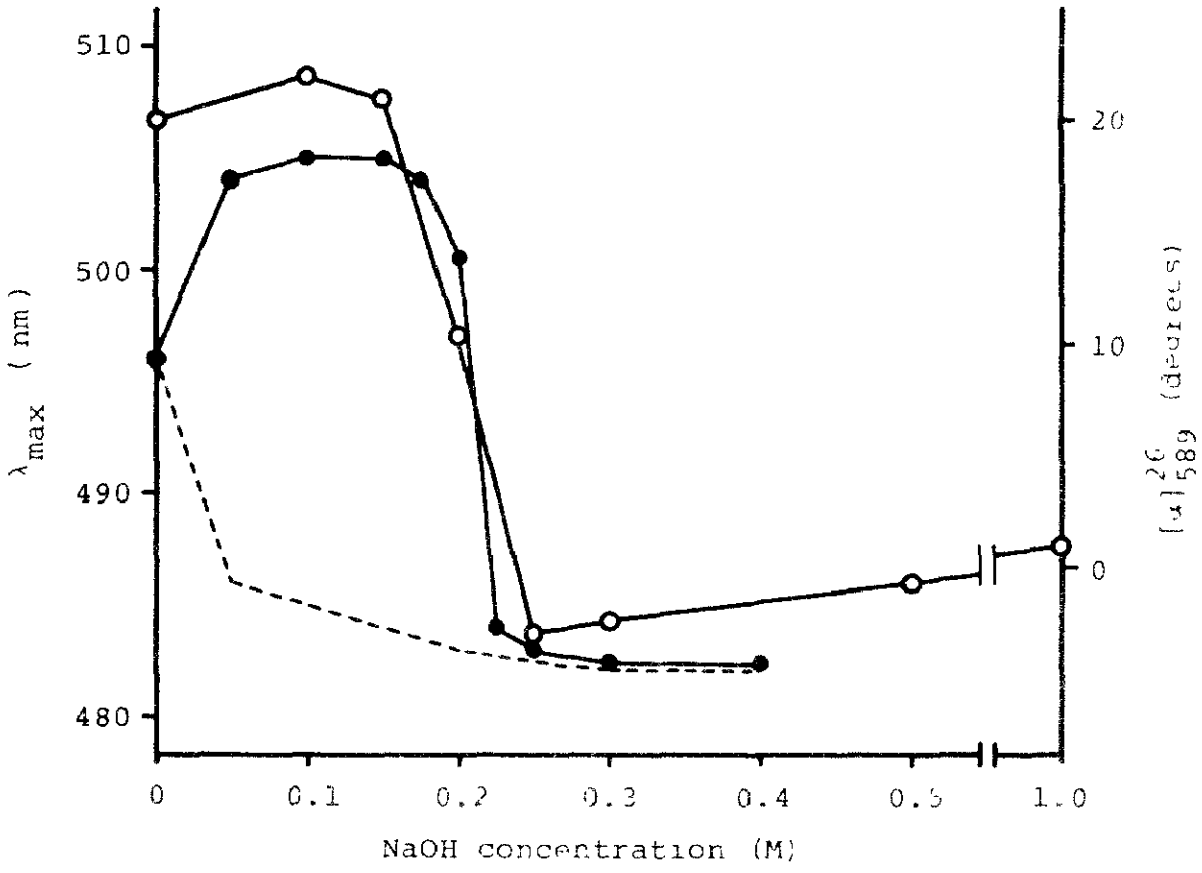

Fig. 6. Changes in the specific rotation of T-4-N at 589 nm, and in the absorption maximum (λ_{max}) of the Congo Red–polysaccharide complex, at various concentrations of sodium hydroxide [Key —○—, specific rotation; —●—, λ_{max} of the Congo Red–T-4-N complex ---, λ_{max} of Congo Red only]

absorption maximum (λ_{max}) of Congo Red were largely shifted to a longer wavelength (505 nm) by the presence of T-4-N at low concentrations of sodium hydroxide in the range of 0.05–0.20M, whereas it is not shifted at zero or at >0.25M concentrations of alkali (see Fig. 6). The shift in the value of λ_{max} of Congo Red was again observed, when, in a 0.4M sodium hydroxide solution of T-4-N, the concentration of alkali was lowered by addition of acid. These observations indicated that the reversible, conformational transition of T-4-N occurs at concentrations of sodium hydroxide in the range of 0.15–0.25M, and that T-4-N has an ordered, triple-helical structure in neutral or slightly alkaline solution (<0.15M NaOH), and has single chains in highly alkaline solution (>0.25M NaOH), as previously reported[5].

We could not determine, in neutral solution, the molecular weight of T-4-N, owing to its high value and the high viscosity of the solution of the sample, although the molecular weight ($\sim 5.5 \times 10^6$) in 0.25M sodium hydroxide was estimated by gel filtration on Sepharose CL-2B, as already described.

The chemical and higher-order structure of the present β-D-glucan (T-4-N), isolated from a 2% sodium carbonate extract of *D. indusiata*, is essentially similar to that of β-D-glucan (T-5-N) obtained from a M sodium hydroxide extract of this fungus. However, some significant differences between T-4-N and T-5-N were observed in the chemical structure, that is, T-4-N has many more branches than T-5-N, and does 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 main chain, found[4] in

T-5-N. Furthermore, the molecular weight of T-4-N is much larger than that (3.3×10^5, in 0.25M NaOH[5]) of T-5-N. T-4-N differs from T-5-N in that the β-(1→6)-linked, D-glucosyl side-chains are mainly localized in the neighborhood of the non-reducing end of the β-(1→3)-linked main chain. In addition, it has been reported that such branched (1→3)-β-D-glucans as lentinan and schizophyllan have an anti-tumor effect[26]. T-4-N, as well as T-5-N, also exhibited antitumor activity against subcutaneously implanted Sarcoma 180 solid tumor in mice[6].

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 (dextran T-2,000, T-500, T-250, and T-150) were purchased from Pharmacia Fine Chemicals. Bio-Gel P-2 was purchased from Bio-Rad Laboratories. 4-Methylmorpholine *N*-oxide was purchased from ICN Pharmaceuticals, Inc. Partially *O*-methylated D-glucitol acetates, used as reference compounds for g.l.c. and g.l.c.–m.s., were prepared by the modified method of Haworth[27]. Laminarabiose was prepared by acetolysis of laminaran[16].

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 IRA-1 spectrometer. Ultracentrifugal analysis was conducted in 0.25M sodium hydroxide with a MOM 3170/b analytical ultracentrifuge at 60,000 r.p.m. at 20°. P.c. was performed by the ascending method, using Toyo No.51 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[31]. G.l.c. was performed in a JEOL JGC-1100 apparatus equipped with a flame-ionization detector. Glass columns (0.3 × 200 cm) were used, with nitrogen as the carrier gas at a flow rate of 43 mL/min. The columns used were (*1*) 3% of ECNSS-M on Gaschrom Q (100–120 mesh) at 171°, (*2*) 3% of Silicone OV-225 on Chromosorb W (80–100 mesh) at 187°, and (*3*) 2% of GE-XF 1105 on Chromosorb P (80–100 mesh) at 200°. G.l.c.–m.s. was conducted with a JEOL JMS-D 300 apparatus equipped with a glass column (0.2 × 100 cm) packed with 3% of ECNSS-M, at 186°, at a pressure of helium of 127.5 kPa (1.3 kg/cm^2). The mass spectra were recorded under the conditions previously reported[3].

Isolation of the polysaccharide. — After being extracted with hot 70% aqueous ethanol as already reported[2], the fruit bodies (100 g) were repeatedly washed with hot water. The residue was extracted 4 times with 2% sodium carbonate (1.5 L) overnight at room temperature, and the extracts were made neutral with M hydrochloric acid, and then dialyzed against distilled water for 5 days. The nondialyzable solution was concentrated to 1 L, the pH was adjusted to 7.8 with M sodium

hydroxide, and the mixture was treated with Pronase (42 mg) for 50 h at 40° (until there was no further drop in the pH). The mixture was further deproteinized by the Sevag procedure[7], and the aqueous phase was dialyzed against distilled water for 3 days. The nondialyzable solution was concentrated to 300 mL, and ethanol (1 vol.) was then added to the solution. The resulting precipitate was separated by centrifugation for 20 min at 3,000 r.p.m., dissolved in water, and the solution treated twice with ethanol as already described. A solution of the resulting precipitate in water was lyophilized, to afford the purified polysaccharide (T-4-N) as colorless flakes; yield 0.71 g.

Electrophoresis. — Tiselius-type electrophoresis of T-4-N was performed with a Hitachi HID-1 boundary-electrophoresis apparatus in 0.05M sodium tetraborate buffer (pH 9.3) for 50 min at 43 V; electrophoretic mobility (u) = 0.54 × 10^{-4} $cm^2/V.s$.

Gel filtration. — A solution of the sample (1.2 mg) in 0.25M sodium hydroxide (0.5 mL) was applied to a column (1.5 × 98 cm) of Sepharose CL-2B which was then eluted with 0.25M sodium hydroxide at a flow rate of 5 mL/h. Fractions (3.9 mL each) were collected, and an aliquot of each fraction was analyzed by the phenol–sulfuric acid method[10].

Estimation of molecular weight. — Gel filtration on a column of Sepharose CL-2B was conducted with 0.25M sodium hydroxide as already described. A calibration curve, constructed by use of dextran T-2,000 (mol. wt., 2,000,000), T-500 (495,000), T-250 (253,000), and T-150 (154,000) is shown in Fig. 3, and therefrom the molecular weight was estimated.

Analysis of component sugars. — T-4-N (3 mg) was heated with 90% formic acid (2 mL) in a sealed tube for 6 h at 100°. After removal of the formic acid by evaporation, the residue was hydrolyzed with 0.25M sulfuric acid (2 mL) for 15 h at 100°. The hydrolyzate was made neutral with barium carbonate, the suspension filtered, the filtrate passed through a column of Amberlite CG-120 (H^+) ion-exchange resin, and the eluate evaporated. By p.c. (solvents *A* and *B*), the syrupy residue was found to be glucose. An aqueous solution of the hydrolyzate was reduced with sodium borohydride, the alditol acetylated[8], and the resulting alditol acetate determined as glucitol by g.l.c. (column *1*).

The specific rotation of the hydrolyzate of T-4-N, obtained by a proceduresimilar to that already described, was $[\alpha]_D^{17}$ +50.0° (*c* 0.056, 0.06M sulfuric acid). Authentic D-glucose showed $[\alpha]_D^{17}$ +52.8° (*c* 0.2, 0.06M sulfuric acid).

Methylation analysis. — T-4-N (4 mg) was methylated twice by Hakomori's method[11], as previously described[28]. The final, methylation product showed no hydroxyl absorption-band in the i.r. spectrum. The fully methylated glucan was successively heated in a sealed tube with 90% formic acid (3 mL) for 4 h at 100°, and 0.25M sulfuric acid (4 mL) for 20 h at 100°. After the acid had been neutralized with Amberlite CG-400 (carbonate) ion-exchange resin, the hydrolyzate was converted into the alditol acetates[8]. The resulting, partially methylated alditol acetates were analyzed by g.l.c. (columns *1* and *2*) and g.l.c.–m.s.; the results are shown in Table I

Periodate oxidation and Smith degradation. — The polysaccharide (28 mg) was subjected to periodate oxidation with 2.5mM sodium metaperiodate (240 mL), with stirring, at 4–7° in the dark. After various times, the periodate consumption was measured by the spectrophotometric method (290 nm) used by Ikenaka[29], and the formic acid produced was determined by titration with standard, 5mM sodium hydroxide[30]. The oxidation was complete after 28 days. The oxidation mixture was treated with ethylene glycol, dialyzed, and the contents reduced with sodium borohydride (60 mg) for 48 h at 5°. The mixture was treated with acetic acid, dialyzed, and then lyophilized, to afford the polyalcohol (yield 25 mg); a sample (2 mg) was treated with M sulfuric acid (2 mL) in a sealed tube for 33 h at 100°. The hydrolyzate was converted into the corresponding alditol acetates as already described, and the product was analyzed by g.l.c., using dual columns of 3% of ECNSS-M (column *1*), the column temperature being increased by 6°/min from 60 to 185°. The retention times of the acetates of glycerol and glucitol were 16.1 and 50.8 min.

Controlled, Smith degradation and methylation analysis. — The polyalcohol (23 mg) was hydrolyzed with 50mM sulfuric acid (20 mL), with stirring, for 26 h at 25°, the mixture was centrifuged for 30 min at 8,000 r.p.m., and the precipitate washed with water, and dried, to give the controlled, Smith-degradation product (T-4-NOI) (17 mg).

T-4-NOI (5 mg) in 4-methylmorpholine *N*-oxide (500 mg) was heated, with stirring, for 2 h at 120° in a tightly stoppered tube under nitrogen[14]. After complete solubilization of the sample, Me_2SO (5 mL) was added, the mixture was cooled to room temperature, and the polysaccharide was methylated twice by the Hakomori method[11]. The fully methylated product was hydrolyzed, the sugars were reduced with sodium borohydride, the alditols acetylated, and the acetates analyzed by g.l.c. (columns *1* and *2*) as already described.

Partial acetolysis. — T-4-N (20 mg) was suspended in a mixture of acetic anhydride (5 mL), acetic acid (3 mL), and sulfuric acid (0.5 mL), and the suspension was allowed to stand, with occasional shaking, for 3 days at room temperature. The reaction mixture was poured into ice–water (50 mL), made neutral with sodium hydrogencarbonate, and then extracted with chloroform. The acetates were deacetylated in methanol (10 mL) containing 0.05M sodium methoxide as previously reported[4], and the products analyzed by p.c. (solvent *C*). The disaccharide fraction isolated from the paper chromatograms was treated with sodium borohydride, followed by (trifluoroacetyl)ation[18] with 1:1 trifluoroacetic anhydride–*N*,*N*-dimethylformamide for 5 min at room temperature. The resulting trifluoroacetate of the disaccharide-alditol was analyzed by g.l.c. (column *3*). The retention time of the derivative of the sample and of that of laminarabiose was 25.1 min.

Enzymic hydrolysis. — (*1*) T-4-N (5 mg) was treated with exo-(1→3)-β-D-glucanase (Lysing Enzymes; 2.5 mg) in 17mM McIlvaine buffer, pH 4.9 (20 mL) for 45 h at 38°. Then, the mixture was heated for 15 min at 100°, cooled, dialyzed against distilled water (1.5 L), and the outer solution concentrated. After treat-

ment with Amberlite CG-4B (OH^-) and Amberlite CG-120 (H^+) resins (to remove the buffer), the products were analyzed by p.c. (solvent C). Two spots, corresponding to glucose and gentiobiose (R_{Glc} 0.74), were detected. The molar ratio of the sugars isolated from the paper chromatograms was determined by the phenol–sulfuric acid method[10]. The gentiobiose was further identified by g.l.c. (column *3*) as the trifluoroacetate of the disaccharide-alditol, as already described, by comparing the retention time (26.7 min) with that of an authentic sample.

T-4-NOI (3 mg) was digested with the enzyme (1.5 mg) in the same way, and the product in the dialyzable fraction was found by p.c. to be glucose only.

(*2*) T-4-N (7.2 mg) was treated with the enzyme (3.6 mg) by a procedure similar to that just described. An aliquot (3–5 mL) of the reaction mixture was taken at different periods (5, 10, 22, 33, and 46 h), and each was heated for 15 min at 100°. Then ethanol (4 vol.) was added to the solution, and the supernatant liquor obtained by centrifugation was evaporated to dryness, the residue dissolved in water (0.3 mL), and the solution applied to a column (1.5 × 98 cm) of Bio-Gel P-2. The column was eluted with water, and fractions (1.5 mL each) were collected, and analyzed by the phenol–sulfuric acid method[10]. On the other hand, the material (T-4-R22) (1 mg containing the denatured enzyme), that had been obtained as a precipitate by centrifugation from the ethanolic reaction-mixture at 22 h, was again digested with the enzyme (0.2 mg) for 42 h at 38°, and the product was analyzed by gel filtration on Bio-Gel P-2 as already described. T-5-N (7.1 mg) was digested with the enzyme (3.5 mg) in the same way. The molar ratio (Gen/Glc) of the products was calculated from the peak-areas.

Specific rotations in aqueous sodium hydroxide. — Specific rotations of T-4-N (1.3 mg/mL) were measured at 26° at various concentrations of sodium hydroxide in the range of 0–1.0M, as previously reported[5].

Interaction with Congo Red in aqueous sodium hydroxide. — The complex-formation of T-4-N (1 mg/mL) with 38μM Congo Red was evaluated from the shift in the visible absorption maximum of Congo Red induced by the presence of T-4-N at various concentrations of sodium hydroxide in the range of 0–0.4M, as previously reported[5].

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