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ANTI-INFLAMMATORY ACTIVITY AND CONFORMATIONAL BEHAVIOR OF A BRANCHED (1→3)-β-D-GLUCAN FROM AN ALKALINE EXTRACT OF *Dictyophora indusiata* FISCH.*

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ABSTRACT

A (1→6)-branched (1→3)-β-D-glucan (T-5-N), isolated from a M sodium hydroxide extract of the fruit bodies of *Dictyophora indusiata* Fisch., markedly exhibited anti-inflammatory effects on both carrageenan-induced edema and scalded edematous hyperalgesia in rat's hindpaws. The activities of T-5-N (25 mg/kg i.p. × 2) were more potent than those of phenylbutazone (25–50 mg/kg i.p. × 2). The conformational behavior of T-5-N was studied. Its molecular weight in neutral solution was about three times that in 0.25M sodium hydroxide. This finding, in addition to the results of optical rotatory measurement and complex-formation with Congo Red, indicated that T-5-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). The conformational transition occurs at concentrations of sodium hydroxide in the range of 0.15–0.25M.

INTRODUCTION

We have previously reported[2] structural investigations on the water-soluble, mucilaginous β-D-glucan (T-5-N) isolated from M sodium hydroxide extracts of the fruit bodies of *Dictyophora indusiata* Fisch. T-5-N is a highly branched, β-D-glucan that has a main chain composed of β-(1→3)-linked D-glucopyranosyl residues and has many side chains of two single β-(1→6)-linked D-glucopyranosyl units attached, on average, to every seventh sugar residue of the main chain.

In recent years, it has been reported that polysaccharide preparations produced by some bacteria[3–7] and some sulfated polysaccharides[8] possess anti-inflammatory activities. We have now found that the highly branched, (1→3)-β-D-glucan (T-5-N) shows significant, anti-inflammatory activity. On the other hand, much attention is being devoted to the conformational behavior of linear and branched (1→3)-β-D-

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glucans[9-15]. The present paper deals with the biological properties of, and conformational studies on, T-5-N.

RESULTS AND DISCUSSION

The main, structural features of the branched (1→3)-β-D-glucan (T-5-N) having side chains of single β-D-glucosyl groups at O-6 atoms seem to be essentially similar to those of other, branched (1→3)-β-D-glucans found in fruit bodies and in the culture broth of many fungi, such as *Lentinus edodes* (lentinan)[16], *Sclerotium glucanicum* (scleroglucan)[17], *Sclerotinia libertiana* (sclerotan)[18], and *Schizophyllum commune* (schizophyllan)[15,19]. However, some significant differences in the molecular weight and the fine chemical structure were observed between T-5-N and the aforementioned, branched (1→3)-β-D-glucans, as partly discussed previously[2].

In the course of investigations on the biological properties of polysaccharides obtained from the fruit bodies of several fungi, we found that T-5-N exhibits significant anti-inflammatory activities. As shown in Table I, T-5-N shows a marked, inhibitory effect on carrageenan-induced edema in rat's hindpaws at a dose of 12.5 or 25 mg/kg (i.p. × 2), and the effect is more potent than that of phenylbutazone

TABLE I

EFFECT OF CRUDE (T-5) AND PURIFIED (T-5-N) POLYSACCHARIDES ON THE RAT'S HINDPAW EDEMA INDUCED WITH CARRAGEENAN

Compounds	*Trial No.*	*Dose[a] (mg/kg × 2, i.p.)*	*Time (h) after the 2nd administration of sample* 0	1	2	*Integrated value of area[c] (0–2 h)*	*Change in area[c] under curve (%) (0–2 h)*
			Hindpaw thickness (in mm × 100)[b]				
Vehicle control	I	4 mL/kg	327 ±4	601 ±16	694 ±10	458 ±23	—
	II	5 mL/kg	336 ±4	684 ±9	701 ±19	531 ±20	—
	III	5 mL/kg	343 ±7	708 ±34	767 ±32	577 ±59	—
	IV	5 mL/kg	320 ±6	529 ±15	540 ±10	320 ±20	—
T-5	I	50	315 ±6	525 ±25[c]	546 ±25[d]	325 ±35[d]	−29[c]
T-5-N	III	6.25	338 ±4	670 ±23	683 ±16	505 ±25	−12.5
	II	12.5	333 ±6	586 ±25[d]	590 ±19[d]	381 ±25[d]	−28.2[d]
	III	25	340 ±3	552 ±17[d]	601 ±32[d]	349 ±38[d]	−39.5[d]
Phenyl-butazone	IV	12.5	315 ±3	496 ±11	508 ±10	278 ±17	−13
	IV	25	311 ±2	477 ±11[c]	488 ±12[c]	255 ±16[c]	−20[c]
	IV	50	318 ±4	462 ±11[d]	464 ±9[d]	216 ±10[d]	−32[d]

[a]The polysaccharides dissolved in water were given i.p. twice immediately, and 1 h after injection of 0.1 mL of 5% carrageenan suspension into the right hindpaw. [b]The data shown indicate the means ±s.e.m. of paw thickness (mm × 100) obtained from 5 rats per group. [c]A significant difference from the corresponding vehicle controls, $p < 0.05$. [d]$p < 0.01$.

TABLE II

EFFECT OF CRUDE (T-5) AND PURIFIED (T-5-N) POLYSACCHARIDES ON HYPERALGESIA[a] OF THE RAT SCALDED HINDPAW

Compounds	*Trial No.*	*Dose[b] (mg/kg × 2, i.p.)*	*Time (h) after the 2nd administration of sample* 0	0.5	1	2	*Change (at peak effective time) (%)*	*Integrated value of area (0–2 h)*	*Change in area under curve (%) (0–2 h)*
			Pain threshold (in g of pressure on the inflamed paw)[c]						
Vehicle control	I	4 mL/kg	339 ±7	243 ±12	227 ±7	216 ±8	—	179 ±19	—
	II	5 mL/kg	365 ±10	259 ±11	240 ±17	213 ±17	—	223 ±17	—
	III	5 mL/kg	362 ±7	235 ±2	224 ±5	209 ±11	—	243 ±11	—
	IV	5 mL/kg	338 ±10	222 ±6	222 ±9	222 ±4	—	203 ±22	—
T-5	I	50	329 ±5	280 ±3[d]	272 ±6[e]	223 ±19	−49[e] (1 h)	120 ±22	−33
T-5-N	III	6.25	345 ±8	236 ±12	201 ±12	227 ±15	−22.4 (2 h)	221 ±19	−9.1
	II	12.5	361 ±5	260 ±9	264 ±13	243 ±17	−22.9 (1 h)	182 ±15	−18.4
	III	25	358 ±9	322 ±10[e]	317 ±12[f]	277 ±11[e]	−71.5[e] (0.5 h)	100 ±13[f]	−58.8[f]
Phenylbutazone	IV	12.5	324 ±9	329 ±7	321 ±7	208 ±8	−27 (0.5 h)	172 ±21	−15
	IV	25	353 ±8	289 ±8[e]	267 ±8[e]	236 ±6	−45[f] (0.5 h)	151 ±15	−26
	IV	50	352 ±12	308 ±10[e]	283 ±14[e]	241 ±5[d]	−62[f] (0.5 h)	129 ±18[d]	−36[d]

[a]Scalded edema. [b]The polysaccharides dissolved in water were given i.p. twice, 1.5 and 2.5 h after immersion of the left hindpaw for 12 s in water at 54°. [c]The data shown indicate the means ±s.e.m. of pain threshold (g/pin) obtained from 5 rats per group. [d]A significant difference from the corresponding vehicle controls, $p < 0.05$. [e]$p < 0.01$. [f]$p < 0.001$.

TABLE III

ANTI-INFLAMMATORY ACTIVITIES[a] OF THE POLYSACCHARIDE (T-5-N) IN BOTH INFLAMMATORY MODELS

Compound	*Dose (mg/kg i.p. × 2, at 1-h interval)*	*Scalded hyperalgesia ED50 (in mg/kg, i.p. × 2; 95% confidence limits) Time (h) after the 2nd sample administration* 0.5	1	2	*Area* (0–2 h)	*Carrageenan edema ED30 (in mg/kg, i.p. × 2; 95% confidence limits) Area* (0–2 h)
T-5-N	6.25–25	19.9 (13.4–70.6)	18.1 (15.1–23.2)	28.8 (17.3–58.2)	22.8 (16.8–43.7)	14.6 (10.6–25.5)
Phenylbutazone	12.5–50	29.8 (18.6–73.9)	>50	>50	>50	45 (30–206)

[a]Anti-inflammatory ED50 (scalded hyperalgesia) and ED30 (carrageenan edema) values indicate the doses required to increase the pain threshold by 50%, or to decrease the thickness of the hindpaw by 30%, respectively. These values were determined by 3 doses (5 animals per group) and by the dose–response curve.

(25–50 mg/kg, i.p. × 2). T-5-N was also found to inhibit scalded edematous hyperalgesia in rat's paws at a dose of 25 mg/kg (i.p. × 2), and the effect was stronger than that of phenylbutazone at a dose of 25 or 50 mg/kg (i.p. × 2) (see Table II). Furthermore, study of the results in Table III shows that the anti-inflammatory potency of T-5-N (given i.p.) on carrageenan edema (ED30) and scalded edematous hyperalgesia (ED50) is stronger than that of phenylbutazone. These effects were confirmed for the crude polysaccharide fraction (T-5) containing a small proportion of acidic polysaccharide but no nitrogenous substance. The activity of the purified polysaccharide (T-5-N) in both of the inflammatory models was more potent than that of its crude polysaccharide (T-5). In oral administration (200 mg/kg × 1), however, these polysaccharides inhibited neither carrageenan edema nor scalded edematous hyperalgesia in rat's hindpaws.

Recently, it has been reported that some preparations of polysaccharide produced by *Aerobacter cloacae*[3], *Sabal serrulate*[4], *Serratia piscatorum*[5,6], and *Streptomyces fradiae*[7], and some sulfated polysaccharides[8], exhibit anti-inflammatory activities. These anti-inflammatory-active polysaccharide preparations contain proteins, or lipids, or both, as minor components[3–7], or have sulfate groups in the molecule[8], and so it is feasible that these minor components, or particular sulfate groups, may participate in the anti-inflammatory activities. T-5-N, however, is very pure as a polysaccharide, as previously reported[2], and it is noteworthy that the simple, branched (1→3)-β-D-glucan (T-5-N) exhibits marked anti-inflammatory activity. There are reports of attempts to clarify the mechanism of the anti-inflammatory action of polysaccharide preparations[5,8], but the mechanism is not yet clear.

The conformational behavior of linear, and branched, (1→3)-β-D-glucans 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[9,12,15]. As may be seen in Fig. 1, T-5-N shows positive specific rotations ($[\alpha]_D^{25}$ +25.1 to +28.7°) at concentrations of sodium hydroxide lying between 0 and 0.15M, whereas T-5-NL ($\overline{d.p.n}$ 15), prepared by partially hydrolyzing T-5-N with acid, has negative specific rotations ($[\alpha]_D^{25}$ −14.4 to −6.5°). Thus, at alkaline concentrations lower than 0.15M, both revealed a large difference in their specific rotations. The values of specific rotations of T-5-N were abruptly decreased at alkaline concentrations in the range of 0.15–0.25M, but T-5-NL did not show such a change. At concentrations of alkali higher than 0.3M, the values were almost equal to each other.

Furthermore, the complex-formation of T-5-N with Congo Red was evaluated

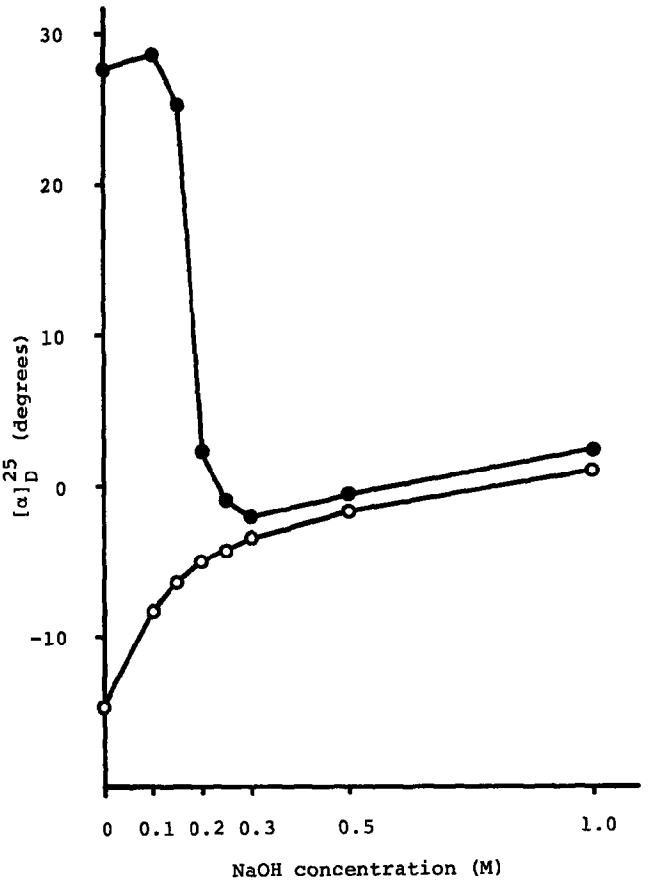

Fig. 1. Dependence of specific rotation of T-5-N, and T-5-NL, at 589 nm on the concentration of sodium hydroxide: ●—●, T-5-N; ○—○, T-5-NL.

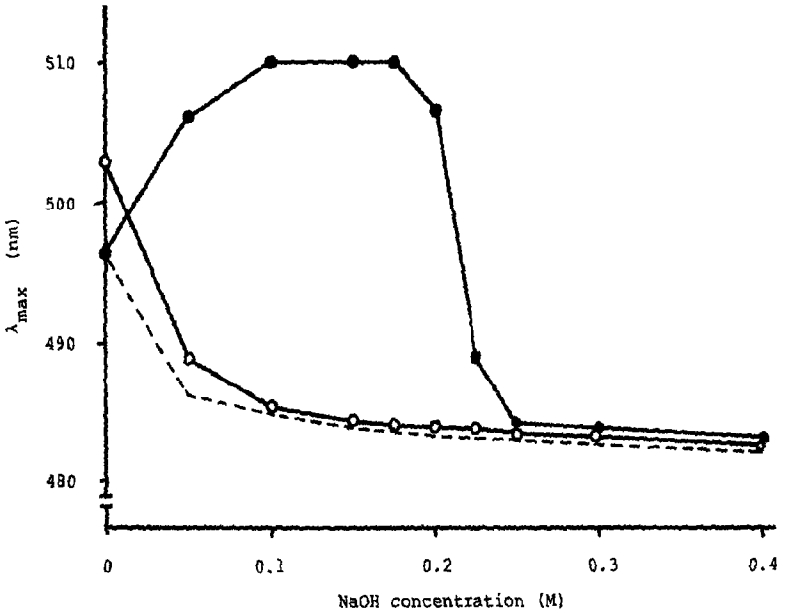

Fig. 2. Change in the absorption maximum of the Congo Red–polysaccharide complex at various concentrations of sodium hydroxide: ●—●, T-5-N; ○—○, T-5-NL; – – – –, Congo Red only.

TABLE IV

PHYSICAL PROPERTIES OF T-5-N

Sedimentation coefficient $(s_0)^a$	7.35 S
Partial specific volume $(\bar{\nu})^a$	0.635 (mL/g)
Intrinsic viscosity $[\eta]^a$	7.55 (dL/g)
Molecular weight $(\bar{M}_w)$	
from the equation[b] used by Eyring and Yang[20] and Pancake and Karnovsky[21]	1.0×10^6
from gel chromatography[c]	3.3×10^5

[a]These measurements were conducted at 20° with 0.1M NaCl–0.33mM phosphate buffer (pH 7.0). [b]See text, and refs. 15 and 16. [c]Sepharose CL-2B, with 0.25M NaOH as the eluant.

from the shift in the visible absorption maximum (λ_{max}) of Congo Red at various concentrations of sodium hydroxide, according to the method of Ogawa *et al.*[9]. As shown in Fig. 2, the values of λ_{max} of Congo Red (dotted line) are largely shifted by the presence of T-5-N to a longer wavelength (510 nm) at low concentrations of alkali (in the range of 0.05–0.20M), whereas it is not shifted at zero or at >0.25M concentrations of alkali. As the shift in the λ_{max} of Congo Red was again observed when, in a 0.4M sodium hydroxide solution of T-5-N, the concentration of alkali was lowered by addition of acid, it was suggested that such shift was reversible. On the

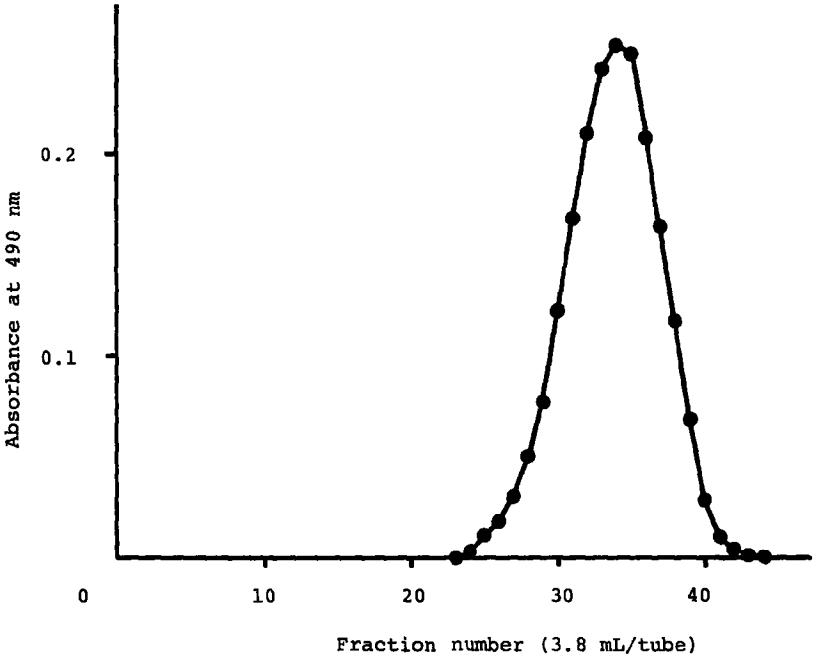

Fig. 3. Chromatogram of T-5-N on Sepharose CL-2B. [The column (1.5 × 98 cm) was eluted with 0.25M sodium hydroxide.]

other hand, in the presence of T-5-NL, a smaller shift was observed, and only in the range of 0 to 0.05M sodium hydroxide. These observations suggest that a conformational transition of T-5-N might occur at concentrations of sodium hydroxide in the range of 0.15 to 0.25M, as stated by Ogawa *et al.*[9], and that it might be reversible.

Such physical properties of T-5-N as the sedimentation coefficient (s_0), the partial specific volume ($\bar{v}$), and the intrinsic viscosity [η], were determined with 0.1M sodium chloride–0.33mM phosphate buffer (pH 7.0) at 20°, as summarized in Table IV. On the basis of these physical data, the molecular weight was calculated by means of the equation used by Eyring[20] and Pancake[21] and their co-workers, which more precisely reflects the molecular weight of a nonelectrolytic, linear substance of high molecular weight. The molecular weight of T-5-N in the buffer was thus estimated to be $\sim 1.0 \times 10^6$, which indicates the weight-average molecular weight ($\overline{M}_w$). Furthermore, the molecular weight of T-5-N was determined by gel chromatography on Sepharose CL-2B by using 0.25M sodium hydroxide as the eluant. T-5-N gave a single peak (see Fig. 3), and its molecular weight ($\overline{M}_w$) was estimated to be $\sim 3.3 \times 10^5$, by means of the calibration curve for standard dextrans (see Fig. 4).

In these two experiments, an appreciable difference in the molecular weight of T-5-N was observed, and its molecular weight in neutral solvent (0.1M NaCl–0.33mM phosphate buffer, pH 7.0) was about three times that in 0.25M sodium hydroxide. This finding firmly indicated occurrence of a conformational transition of T-5-N.

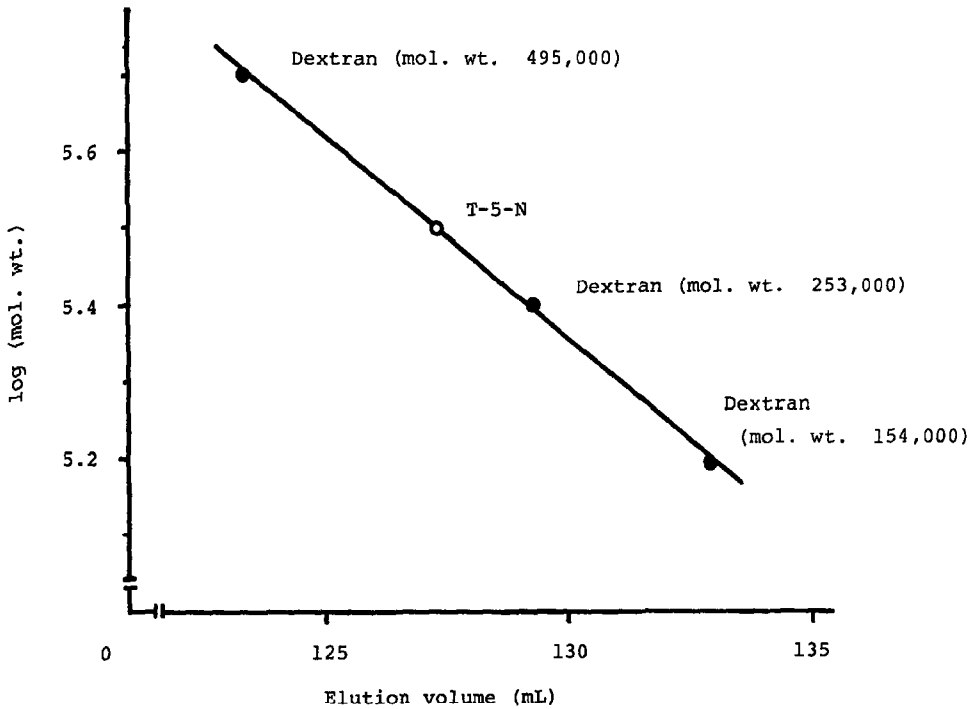

Fig. 4. Determination of molecular weight of T-5-N by gel chromatography on Sepharose CL-2B. [The elution volume is plotted against the logarithm of the molecular weight of dextrans T-500, T-250, and T-150.]

A similar, significant difference in the molecular weight of a branched (1→3)-β-D-glucan (schizophyllan) in water and in dimethyl sulfoxide was reported by Norisue *et al.*[14]. Thus, from the present study, it was concluded that T-5-N has an ordered, triple-helical structure in neutral or weakly alkaline solution (<0.15M NaOH), and has single chains (random coils) in highly alkaline solutions (>0.25M NaOH). Such triple-strand helices are probably formed by hydrogen bonds between β-D-(1→3)-linked D-glucosyl main-chains[10,11,14]. On the other hand, it is probable that T-5-NL ($\bar{d}.\bar{p}.n$ 15) does not take such an ordered structure, because it shows no changes in specific rotation, and in absorption maximum with Congo Red, at various concentrations of alkali.

Some linear and branched (1→3)-β-D-glucans, *e.g.*, curdlan (isolated from *Alcaligenes faecalis*[22]) and schizophyllan, have been reported to have triple-helical structures from the results of X-ray diffraction analysis, and viscosity and other studies[10,11,13,14]. We have demonstrated that T-5-N also has a similar, triple-helical structure, and have found that such a branched (1→3)-β-D-glucan (T-5-N) possesses significant anti-inflammatory activities on both carrageenan edema and scalded edematous hyperalgesia in rat's hindpaws. Such (1→6)-branched (1→3)-β-D-glucans as lentinan and schizophyllan had been found to exhibit prominent antitumor activity against Sarcoma 180 solid tumor in mice[12,23].

EXPERIMENTAL

Materials. — The purified, branched (1→3)-β-D-glucan (T-5-N) and its crude preparation (T-5) used in this study were prepared as reported previously[2]. T-5-NL (low-molecular-weight T-5-N) was prepared as follows. T-5-N (100 mg) was partially hydrolyzed with 50% sulfuric acid (21 mL) for 25 h at 3–5°; the acid was neutralized with barium carbonate, the suspension filtered, the filtrate treated with Amberlite CG-120 (H^+) ion-exchange resin, and the solution submitted to ultrafiltration using a UH-1 ultrafilter (Toyo Roshi Co., Ltd.) to remove mono- and oligo-saccharides. The fraction left on the filter was lyophilized, to afford low-molecular-weight T-5-N (T-5-NL) in 68% yield. The molecular weight of T-5-NL was determined by gel chromatography on a column (1.5 × 98 cm) of Sephadex G-50 with the use of standard dextrans supplied by Meito Sangyo Co., Ltd., and was estimated to be ~2500 ($\overline{d.p.n}$ 15).

Assay of anti-inflammatory activity. — Scalded hyperalgesic and carrageenan edemas were induced in hindpaws of the same Sprague Dawley rats weighing 190–200 g (5 rats per group), as described previously[24-26]. The rat's left hindpaw was immersed for 12 s in water at 54°, and, 1.5 h later, the animals received a subcutaneous injection of 5% carrageenan suspension (0.1 mL) into the right hindpaw. The polysaccharides, dissolved in water (6.25–50 mg/kg), were given intraperitoneally (i.p.) twice immediately, and 1 h after the injection of carrageenan. The inhibitory effects on scalded edematous hyperalgesia and carreegeenan-induced edema of each hindpaw were determined by using a Randall–Selitto instrument at 0.5, 1, and 2 h, and at 1 and 2 h after administration of the second sample, respectively. For oral administration, 200 mg of sample per kg was given once. Phenylbutazone (12.5–50 mg/kg) was used as the reference drug.

Specific rotations in aqueous sodium hydroxide. — T-5-N (15.4 mg) and T-5-NL (12.5 mg) were each dissolved in water (10 mL), and the concentration of alkali was increased from 0 to M by stepwise addition of 4M sodium hydroxide. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter at 25° at each concentration of alkali.

Complex-formation with Congo Red in aqueous sodium hydroxide. — The complex-formation with Congo Red was evaluated from the shift in the visible absorption maximum of Congo Red that was induced by the presence of polysaccharide at various concentrations of alkali, according to the method of Ogawa *et al.*[9]. T-5-N (2.8 mg) and T-5-NL (2.5 mg) were each dissolved in water (2.5 mL) containing Congo Red (91 μM). The concentration of alkali was increased from 0 to 0.4M by stepwise addition of 4M sodium hydroxide to the sample solutions. Visible absorption spectra were recorded with a Hitachi 323 recording spectrophotometer at each concentration of alkali.

Physical analyses. — The physical measurements described were conducted with 0.1M sodium chloride–0.33mM phosphate buffer (pH 7.0) at 20°. Relative viscosities [η_{rel}] were measured by using an Ubbelohde type of viscometer at four concentra-

tions of sample (25, 48, 72, and 96 mg/100 mL). The intrinsic viscosity $[\eta]$ was estimated by extrapolating the reduced viscosities $[\eta_{sp}/c]$ and $\ln[\eta_{rel}/c]$ to infinite dilution of the solution of the sample. The partial specific volume ($\bar{v}$) was determined with an Anton Paar DMA-45 density meter at a concentration of the sample of 0.608 g/100 mL. The sedimentation velocities were determined, at 50,700 r.p.m., at four concentrations of the sample (0.098, 0.146, 0.198, and 0.246%), with a Hitachi UCA-1 analytical ultracentrifuge equipped with a Schlieren optical system. The sedimentation coefficient (s_0) was calculated by extrapolating the reciprocals of the sedimentation coefficients obtained to infinite dilution of the solution of the sample. On the basis of these physical data, the molecular weight (M) was calculated by means of the equation used by Eyring[20] and Pancake and co-workers[21]: $M^{2/3} = N[\eta]^{1/3}\eta_0 S/2.5 \times 10^6 (1 - \bar{v}\rho)$, where N is Avogadro's number; $[\eta]$, the intrinsic viscosity; η_0, the solvent viscosity; S, the sedimentation coefficient: $\bar{v}$, the partial specific volume; and ρ, the solvent density, respectively.

Gel chromatography on Sepharose CL-2B with aqueous sodium hydroxide. — Gel chromatography on Sepharose CL-2B (Pharmacia Fine Chemicals) was conducted with 0.25M sodium hydroxide as the eluant. T-5-N and standard dextrans (Pharmacia Fine Chemicals) (1.0–1.2 mg) were each dissolved in 0.25M sodium hydroxide (0.5 mL), and applied to a column (1.5 × 98 cm) of Sepharose CL-2B. The column was eluted with 0.25M sodium hydroxide at a flow rate of 5 mL/h. Fractions (3.8 mL each) were collected, and an aliquot of each fraction was analyzed for carbohydrate by the phenol–sulfuric acid method[27]. For estimation of the molecular weight ($\bar{M}_w$), a calibration curve was constructed by use of Dextran T-500 (mol. wt., 495,000), Dextran T-250 (253,000), and Dextran T-150 (154,000). The results are shown in Figs. 3 and 4.

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