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Studies on Basidiomycetes. I. Antitumor Polysaccharide from Bagasse Medium on which Mycelia of *Lentinus edodes* (BERK.) SING. had been grown

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From bagasse medium on which mycelia of *Lentinus edodes* (BERK.) SING. had been grown, an acidic polysaccharide (fraction C-1-2) was isolated by deproteinization, removal of nucleic acid, fractionation with barium hydroxide and DEAE-cellulose column chromatography. The purified polysaccharide consisted mainly of xylose, arabinose, mannose, galactose, glucose and uronic acid in a molar ratio of 1.4:0.7:2.0:0.9:1.0:1.5. The tumor inhibition ratio of the polysaccharide against solid sarcoma 180 was 87.9% in mice given doses of 100 mg/kg \times 10 d by intraperitoneal administration and the tumors in 5 of 10 mice completely regressed.

Keywords—antitumor effect; sarcoma 180; polysaccharide; bagasse; *Lentinus edodes*; isolation

Many papers have shown that polysaccharides isolated from various origins, such as Basidiomycetes,¹⁾ lichens²⁾ and higher plants³⁾ have a strong antitumor activity against transplanted solid tumors of mice. PS-K is a protein-bound polysaccharide extracted from *Coriolus versicolor* (L. ex Fr.) QUEL. and is used clinically in combination with other anticancer drugs.⁴⁾ It has been considered that the antitumor activity of the polysaccharides is not due to direct cytotoxicity on tumor cells but to host-mediated action.⁵⁾

In Japan, *Lentinus edodes* (BERK.) SING. (Shiitake in Japanese) is cultivated successfully on "hotagi".⁶⁾ Recently, owing to a shortage of "hotagi," attempts have been made to grow *L. edodes* on bagasse and sawdust.

This paper deals with the isolation of antitumor polysaccharide from bagasse medium on which mycelia of *L. edodes* had been grown.

The antitumor bioassay was carried out by observing the effect on the growth of subcutaneously implanted sarcoma 180 (solid form) for 5 weeks. Samples to be tested were administered by intraperitoneal injection once daily for 10 d, beginning 24 h after the implantation.

Chart 1 shows the fractionation of the aqueous extract of the autolyzed medium.

Fraction A was obtained by adding 5 volumes of ethanol to a solution of the hot water extract of the bagasse medium. Protein and nucleic acids were removed from fraction A by treatment with pronase E and cetyltrimethylammonium bromide (CTAB),⁷⁾ respectively, to obtain fraction C. Fraction C contained no nitrogen on elemental analysis (ash, 0.9%); thus, the presence of proteins, peptides and nucleic acids in fraction C was excluded. Saturated barium hydroxide solution was added to a solution of fraction C until precipitation was complete. The precipitated barium complex was decomposed with 2N acetic acid and barium ion was removed by treatment with CR-10 and Amberlite IR-120 resins to give fraction C-1. The supernatant (from fraction C-1) was dialyzed, passed through CR-10 and Amberlite IR-120 resins and precipitated by adding 5 volumes of ethanol to obtain fraction C-2. By this fractionation procedure, the antitumor-active fraction C-1 (16.7% from fraction C) was separated satisfactorily from non-active fraction C-2 (66.3% from fraction C). Table I shows the antitumor activities of the polysaccharide fractions against sarcoma 180.

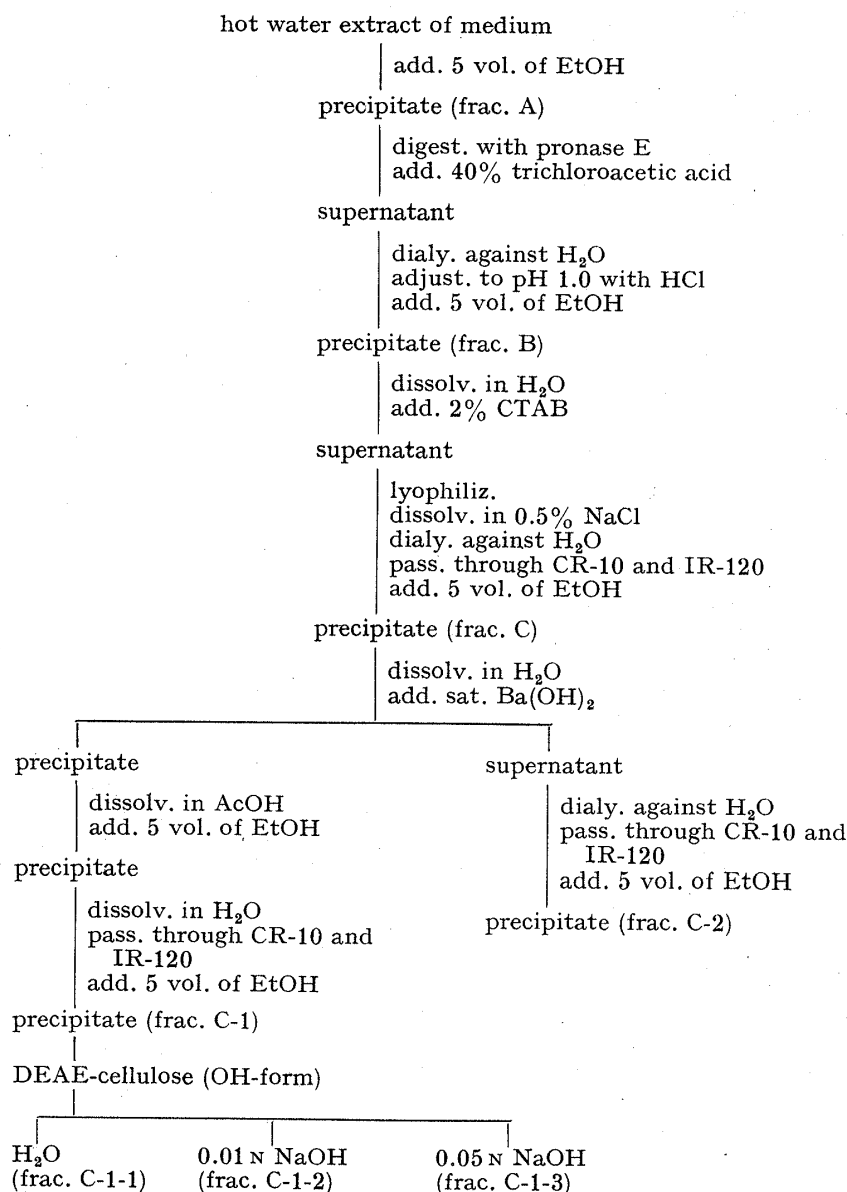


Chart 1. Fractionation of Aqueous Extract of Bagasse Medium

Fraction C-1 showed antitumor activity giving a 76.2% inhibition ratio at 100 mg/kg \times 10 doses, but fraction C-2 gave a ratio of -11.1% at the same dose. Fraction C-1 ($[\alpha]_D^{25} -7^\circ$) was readily soluble in water to give a solution of pH 3.0-4.0, and its acid hydrolysate gave a distinct carbazole-sulfuric acid reaction. The infrared spectrum (IR) of fraction C-1 showed a characteristic absorption at 1710 cm^{-1} which seemed to be due to the carbonyl group of uronic acid residues. Fraction C-1 was hydrolyzed completely with 1 N sulfuric acid to analyze the sugar components. In paper chromatography (PPC), six neutral sugars, rhamnose, arabinose, xylose, mannose, galactose and glucose were detected and their contents were determined by gas liquid chromatography (GLC) as the alditol acetates (Table II). The presence of galacturonic and glucuronic acid residues in fraction C-1 was confirmed by GLC as the trimethylsilylated aldonolactones.⁸⁾ These analytical results indicate that fraction C-1 was an acidic polysaccharide containing uronic acid residues.

Further, fraction C-1 was applied to a column of DEAE-cellulose (OH-form) and eluted stepwisely with water, 0.01 N and 0.05 N sodium hydroxide. It was found that fraction C-1 could be mainly separated into two fractions, C-1-2 ($[\alpha]_D^{25} +32^\circ$) and C-1-3 ($[\alpha]_D^{25} -29^\circ$), in a

TABLE I. Antitumor Activities of the Polysaccharide Fractions against Sarcoma 180

Sample	Dose (mg/kg)	Route	Average Tumor wt. (g)	Inhibition ratio (%)	Complete regression
Exp-1					
A	100 × 10	<i>i.p.</i>	6.7	27.2	0/10
Control	Saline		9.2	—	0/9
Exp-2					
C-1	100 × 10	<i>i.p.</i>	1.5	76.2	0/8
C-2	100 × 10		7.0	-11.1	0/10
Control	Saline		6.3	—	0/10
Exp-3					
C-1	100 × 10	<i>i.p.</i>	3.6	53.2	0/9
C-1-2	100 × 10		2.0	74.0	3/9
C-1-3	100 × 10		4.3	44.2	2/10
PS-K	100 × 10		0.8	89.6	1/10
Control	Saline		7.7	—	0/10
Exp-4					
C-1-2	20 × 10	<i>i.p.</i>	4.7	48.4	0/10
	50 × 10		1.5	83.5	2/9
	100 × 10		1.1	87.9	5/10
Control	Saline		9.1	—	0/10
Exp-5					
C-1-2	100 × 15	<i>p.o.</i>	6.8	23.6	0/10
	500 × 15		7.8	12.4	0/10
PS-K	100 × 15		8.9	0	0/10
	500 × 15		6.8	23.6	0/10
Control	Saline		8.9	—	0/11

Exp-1, Exp-2 and Exp-3; Samples were administered 24 h after implantation, once daily for 10 d.

Exp-4; Samples were administered 7 d after implantation, every other day for 10 d.

Exp-5; Samples were administered 24 h after implantation, once daily for 7 d and then every other d for 8 d.

TABLE II. Sugar Compositions of Fractions C-1, C-1-2, C-1-3 and BA-1

Sugar	Composition (wt %)			
	C-1	C-1-2	C-1-3	BA-1
Rha	1.5	1.8	2.1	2.5
Ara	12.8	10.1	20.2	9.1
Xyl	25.9	20.1	34.4	15.2
Man	28.5	35.3	18.2	20.2
Gal	13.0	15.2	11.0	19.7
Glc	18.2	17.3	16.0	33.3
Uronic acid ^{a)}	17.7	11.7	20.5	19.2
GlcUA/GalUA ^{b)}	1.29	1.37	0.78	1.78

a) Estimated in terms of glucuronic acid.

b) GlcUA: glucuronic acid.
GalUA: galacturonic acid.

ratio of 1.2:1 by DEAE-cellulose chromatography as shown in Fig. 1. On high voltage electrophoresis using glass fiber paper and phosphate buffer (pH 6.5), fraction C-1-2 and C-1-3 each gave a single spot, as shown in Fig. 2. As shown in Table II, the major carbohydrate constituents of fraction C-1-2 are mannose, xylose, galactose and glucose in a molar ratio of 2.0:1.4:0.9:1.0, while fraction C-1-3 consists mainly of xylose, arabinose, mannose and glucose in a molar ratio of 2.6:1.5:1.1:1.0. Rhamnose is a minor component in these fractions. Fraction C-1-2 differs from fraction C-1-3 in uronic acid content and the ratio of glucuronic acid to galacturonic acid.

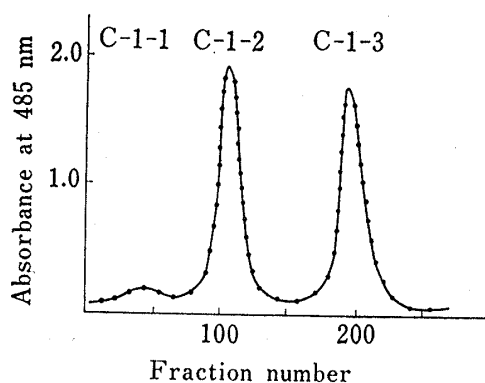


Fig. 1. Chromatogram of Fraction C-1 on DEAE-cellulose

Sugar (—●—) was detected by the phenol- H_2SO_4 method.

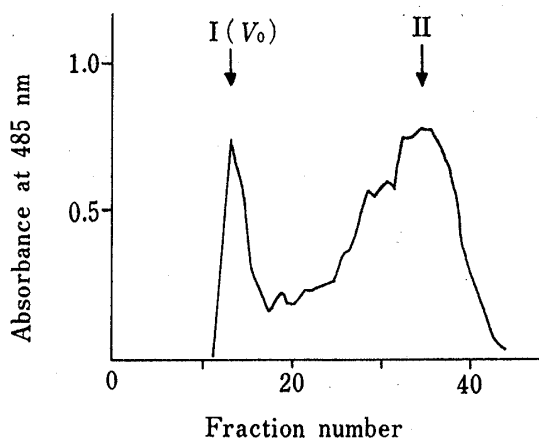


Fig. 3. Chromatogram of Fraction C-1-2 on Sephadex G-200

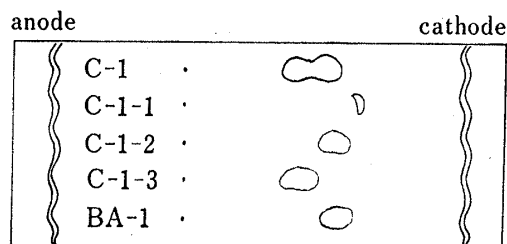


Fig. 2. Paper Electrophoresis of Fractions prepared from Fraction C-1 and Bagasse

Paper: Whatman glass fiber GF/C.
Solvent: 0.1 M phosphate buffer, pH 6.5.
Field strength: 1000 V, for 15 min.
Reagent: anisidine- H_2SO_4 .

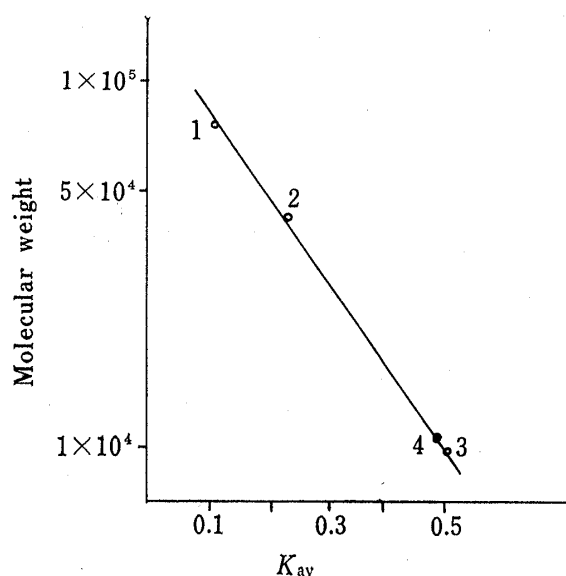


Fig. 4. Estimation of the Molecular Weight of Fraction C-1-2 (II) by Gel Filtration on Sephadex G-200

1: Dextran T-70.
2: Dextran T-40.
3: Dextran T-10.
4: C-1-2 (II).

Gel filtration using a column of Sephadex G-200 revealed that fraction C-1-2 consisted of two components, I and II, as shown in Fig. 3. Since I was eluted at the void volume (V_0) and II had a partition coefficient (K_{av}) of 0.47, the molecular weight of I was greater than 200000, and that of II corresponded to *ca.* 10000 as shown in Fig. 4.

The antitumor activities of the polysaccharide fractions are summarized in Table I. Although the inhibition ratio of fraction C-1-2 was 74.0% at 100 mg/kg \times 10 doses, that of fraction C-1-3 was 44.2% in the same dose. This difference in antitumor activity of these two fractions may depend on the difference in sugar composition, including uronic acids. PS-K, a positive control, showed a strong antitumor activity with complete regression of 1/10 at 100 mg/kg \times 10 doses. In exp-3, fraction C-1-2 inhibited the tumor growth until the 3rd week after transplantation but had no effect after that. As shown in Exp-4, fraction C-1-2 gave inhibition ratios of 83.5% and 87.9% at 50 mg/kg and 100 mg/kg daily doses,

respectively, when treatments were begun 7 d after implantation. However, as shown in Exp-5, when fraction C-1-2 was administered orally to mice 7 d after implantation for 15 d, the fraction was hardly effective at 100 mg/kg and 500 mg/kg doses. Tsukagoshi *et al.* reported that PS-K showed inhibition ratios of 57.1% and 75.9% at 500 mg/kg and 1000 mg/kg doses, respectively, when PS-K was given orally to mice 7 d after inoculation of sarcoma 180, once daily for 20 d.⁹⁾ Under our experimental conditions, PS-K was hardly effective at 100 mg/kg and 500 mg/kg \times 15 doses; the lack of effect was probably due to the low doses of PS-K.

Sakai *et al.* reported that polysaccharide fraction B2 prepared from bagasse showed remarkable antitumor activity against sarcoma 180.^{3b)} Oka *et al.* reported that fraction B2 was composed mainly of galactose, arabinose, xylose and mannose and a smaller amount of glucose, although they did not describe the uronic acid content, if any.¹⁰⁾ Glucose (17.3%) in fraction C-1-2 was apparently detected on PPC and GLC, while glucose in fraction B2 was detected with difficulty on PPC according to Oka and co-workers. Fujii *et al.* isolated an antitumor and antiviral peptide mannan, KS-2, from culture mycelia of *Lentinus edodes* KSLE007.¹¹⁾ The fraction C-1-2 differs from KS-2 and fraction B2 in the content of glucose. In order to clarify the difference between fraction C-1-2 and bagasse polysaccharide, bagasse which had no mycelia of *L. edodes* was extracted. Fraction BA-1 corresponding to fraction C-1 was prepared in 0.02% yield from bagasse. Though fraction BA-1 was identical with fraction C-1-2 on electrophoresis, the sugar compositions were different, especially in the contents of mannose, glucose and uronic acid. This result suggested that bagasse polysaccharide, BA-1, might be modified by *L. edodes* to form fraction C-1-2. It is reported that many Basidiomycetes can digest wood components directly to satisfy their carbohydrate requirement.¹²⁾ Hashimoto *et al.* reported that an enzyme in *Agaricus bisporus* hydrolyzes hemicellulose of rice straw to xylooligosaccharides.¹²⁾ Further chemical and biological studies are required to elucidate the nature of the modification produced by *L. edodes* to form fraction C-1-2.

Experimental

The IR spectra were measured with a JASCO (Japan Spectroscopic Co.) IRA-1 spectrophotometer, and the specific rotations with a JASCO DIP-4 automatic polarimeter. Paper chromatography was carried out on Toyo Roshi No. 51A (2 \times 40 cm) paper by the ascending method with the following solvent; *n*-BuOH-pyridine-H₂O (6:4:3) for 15 h. Sugars were detected by spraying anisidine reagent and heating at 100°. High voltage paper electrophoreses were performed on Whatman glass fiber GF/C paper at 1000 V, using 0.1 M phosphate buffer of pH 6.5. The spots were detected by spraying anisidine-H₂SO₄ reagent and heating at 100°. GLC analyses were carried out with a Hitachi 063 gas chromatograph with a hydrogen flame ionization detector.

Materials—The bagasse medium on which mycelia of *L. edodes* were grown was prepared according to the method of Iizuka and Fumoto.¹³⁾ The medium used was composed of bagasse (90%) and rice bran (10%). Mycelia of *L. edodes* were inoculated into the medium and cultivated at 20–22°C for 60 d. After autolysis, the bagasse medium was extracted with hot water and the extract was lyophilized. The lyophilized extract was a gift of Japan Create Co., Ltd., Tokyo.

Assay Method for Antitumor Effect—Seven-day-old sarcoma 180 ascites, 0.1 ml (1×10^6 cells), were transplanted subcutaneously into right groins of ICR mice (male), weighing 25 ± 3 g. Test samples, dissolved in 0.85% sodium chloride solution, were injected intraperitoneally or given orally at 20–500 mg/kg \times 10 or 15 doses, beginning 24 h or 7 d after the implantation, daily or every other day. After observation of the tumor growth for 5 weeks, the tumor weights of treated mice were compared with those of untreated mice. The inhibition ratios were calculated by means of the following formula: Inhibition ratio (%) = $(C - T)/C \times 100$; where *C* is the average tumor weight of the control group, and *T* is that of the treated group. Complete regression of the tumors was also recorded. The results are shown in Table I.

Sugar Analysis—Uronic acid content was determined by the carbazole-sulfuric acid method.¹⁴⁾ Complete acid hydrolysis was carried out by heating a sample with 1 N H₂SO₄ for 8 h at 100°C. The reaction mixture was neutralized with Ba(OH)₂ and centrifuged. The supernatant was treated with Amberlite IR-120 (H-form) and applied to a column (1 \times 10 cm) of Duolite A-4 (OH-form) to adsorb uronic acids. Neutral sugars were eluted from the column with distilled water, and then uronic acids were eluted with 10% HCO₂H. Neutral sugars were converted into the corresponding alditol acetates and analyzed by GLC

on a column of ECNSS-M.¹⁵⁾ Uronic acids were converted to their salts with BaCO₃. The uronic acid salts were reduced by aqueous sodium borohydride to the corresponding aldonic acids which were subsequently converted to the 1,4-lactones by treatment with conc. HCl. The aldono-1,4-lactones thus obtained were trimethylsilylated and analyzed by GLC on a column of SE-52. Retention times: GlcUA: 35.2 min, GalUA: 28.4 min. GlcUA/GalUA was calculated from the peak areas.

Preparation of Fractions A, B and C—The hot water extract of the bagasse medium (400 g) was dissolved in 2 l of water and five volumes of EtOH was added to the solution. The precipitate was collected by centrifugation and washed with EtOH, and fraction A (76 g) was obtained by drying it. Fraction A (76 g) was dissolved in 2.7 l of water and digested with pronase E (Kaken Kagaku, 380 mg) overnight at 37°C after the solution had been adjusted to pH 7.8 with 1 N NaOH. The reaction mixture was adjusted to pH 7.8 and pronase E (190 mg) was again added to the reaction mixture. Afterwards, the reaction mixture was boiled for 5 min and 930 ml of 40% trichloroacetic acid was added. The whole was allowed to stand overnight in a refrigerator (about 4°C). The supernatant obtained by centrifugation was dialyzed against water for two days. Non-dialyzable liquor was concentrated under reduced pressure, adjusted to pH 1.0 with 6 N HCl and precipitated by the addition of five volumes of EtOH. Fraction B (12.4 g) was obtained as a brownish powder, which was dissolved in 500 ml of water; 110 ml of 2% CTAB was then added to the solution with stirring. The precipitate was removed by centrifugation and the supernatant was lyophilized. The residue was dissolved in 500 ml of 0.5% NaCl and the solution was dialyzed against water for two days. The non-dialyzable liquor was passed through columns of CR-10 (500 ml) and Amberlite IR-120 (500 ml) resins. The effluent was concentrated and five volumes of EtOH was added to the concentrate to give fraction C (6.5 g). Elemental analysis: C, 41.9%, H, 6.1%, N, nil, ash, 0.9%.

Fractionation of Fraction C with Ba(OH)₂—Fraction C (2.58 g) was dissolved in 130 ml of water and 80 ml of sat. Ba(OH)₂ solution was added to the solution with stirring. The mixture was kept for 1 h at room temperature. The precipitate was collected by centrifugation, washed with dilute Ba(OH)₂ solution and dissolved in 20 ml of 2 N AcOH. The solution was poured into five volumes of EtOH. The precipitate obtained by centrifugation was dissolved in 50 ml of water and the solution was passed through columns of CR-10 (250 ml) and Amberlite IR-120 (250 ml) resins. The effluent was concentrated and five volumes of EtOH was added to the concentrate to obtain fraction C-1 (0.43 g) as a pale brownish powder. $[\alpha]_D^{25} - 7^\circ$ ($c=0.74$, H₂O). The residual solution, which did not give any precipitate with sat. Ba(OH)₂ solution, was dialyzed against water and treated with CR-10 (500 ml) and Amberlite IR-120 (500 ml) resins. Fraction C-2 (1.71 g) was obtained after being precipitated with five volumes of EtOH.

Preparation of Fraction BA-1 from Bagasse—Bagasse (2.8 kg) was extracted three times with 13.3 l of distilled water for 5 h at 55°C. The extract was concentrated and five volumes of EtOH was added to the concentrate to give fraction BA (11.63 g) as a brownish powder. After being treated with pronase E and CTAB, fraction BA was fractionated with Ba(OH)₂ to obtain fraction BA-1 (0.44 g, corresponding to fraction C-1 from bagasse medium). $[\alpha]_D^{25} + 33^\circ$ ($c=0.97$, H₂O). Electrophoresis and sugar composition: see Fig. 2 and Table II, respectively.

DEAE-Cellulose Column Chromatography of Fraction C-1—Fraction C-1 (3.08 g) was dissolved in 100 ml of water and applied to a column (3.5 × 30 cm) of DEAE-cellulose (DE-52, OH-form) which was eluted stepwisely with 700 ml of water, 1080 ml of 0.01 N NaOH and 450 ml of 0.05 N NaOH. The positive fraction as detected by the phenol-sulfuric acid method¹⁶⁾ was collected and dialyzed against water for two days. The non-dialyzable liquor was treated with CR-10 (500 ml) and Amberlite IR-120 (500 ml) resins, concentrated and lyophilized. Fraction C-1-1 was obtained in trace amount, fraction C-1-2 (1.24 g) was obtained as a somewhat cream-colored powder and fraction C-1-3 (1.04 g) was obtained as a brownish powder. C-1-2: $[\alpha]_D^{25} + 32^\circ$ ($c=0.72$, H₂O). C-1-3: $[\alpha]_D^{25} - 29^\circ$ ($c=0.75$, H₂O).

Gel Filtration¹⁷⁾ of Fraction C-1-2—Fraction C-1-2 (15 mg) dissolved in 2 ml of 0.1 M ammonium formate was applied to a column (1.8 × 81 cm) of Sephadex G-200 and eluted with 0.1 M ammonium formate. As shown in Fig. 3, fraction C-1-2 was separated into two fractions whose molecular weights were estimated to be greater than 200000 (I) and ca. 10000 (II) by gel filtration on a Sephadex G-200 column using standard dextrans of known molecular weight.

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