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Studies on the Anti-Tumor Activity of Polysaccharides. I. Isolation of Hemicelluloses from Yakushima-bamboo and Their Growth Inhibitory Activities against Sarcoma-180 Solid Tumor[1]

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In order to confirm the chemical entities of the anti-tumor substances in the extract of the leaves of a sort of bamboo, *Sasa senanensis* (Fr. et Sav.) Rehd, a series of fractionation was carried out on the macromolecular constituents of the extract. Two hemicelluloses were isolated as homogeneous states both in free boundary electrophoresis and ultracentrifugal analysis. One of them was a neutral polysaccharide which was precipitable with alkaline copper solution and consisted of L-arabinose, D-xylose and D-glucose, while the other contained D-galactose in addition to the three sugar components mentioned above and showed weak acidity due to a small amount of uronic acid. Both polysaccharides showed over 90% tumor-inhibitions when injected intraperitoneally into mice bearing Sarcoma-180 solid tumor.

It has been recognized that various kinds of extract from microorganisms[3a-h] or higher plants[4a-c] possess growth-inhibitory power against solid tumors. The chemical nature of these extracts was supposed to be polysaccharides. Recently Kuroki,[5] Sakai, *et al.*,[6] Nakahara, *et al.*[7] and Sugayama, *et al.*[8] reported that a polysaccharide fraction of the bamboo-extract showed a marked inhibitory effect against some kinds of transplantable tumor when the material was administered intraperitoneally into the host animal in either manner before or after the tumor implantation. The mode of action was different from cytotoxic anti-tumor agents and was nominated as "host-mediated action." Elucidation of the mechanism is of great interest as a novel type of biological activity of polysaccharide. However, no positive evidence was provided on the homogeneity of the polysaccharides which have been employed by the earlier workers as anti-tumor materials. Prior to the investigation of the

1) The second paper of the research series "Biological Activities of Polysaccharides." Part I: S. Suzuki, H. Sunayama and T. Saito, *Japan. J. Microbiol.*, **12**, 19, (1968). A part of this report was reported preliminary on *Ann. Rep. Tohoku Coll. Pharm.*, **14**, 13 (1967).

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3) *a*) H.B. Andervont, *Am. J. Cancer*, **27**, 77 (1936); *b*) L.C. Fogg, *Pub. Health Rep.*, **51**, 56 (1936); *c*) M.J. Shear, F.C. Turner, A. Perrault and T. Shovelton, *J. Nat. Cancer Inst.*, **4**, 81 (1943); *d*) I.C. Diller, *Cancer Res.*, **7**, 605 (1947); *e*) M.J. Shear and A. Perrault, *Cancer Res.*, **9**, 626 (1949); *f*) E.Y.L. Lasfargues, D.R.A. Wharton and J.C. Difine, *Cancer Res.*, **11**, 425 (1951); *g*) W.T. Bradner, D.A. Clarke and C.C. Stock, *Cancer Res.*, **18**, 347 (1965); *h*) H. Nakayoshi, *Nippon Saikingaku Zasshi*, **22** (11,12), 641 (1967).

4) *a*) M. Belkin, W.G. Hardy, A. Perrault and H. Sato, *Cancer Res.*, **19**, 1050 (1959); *b*) E.M.F. Roe, W. Galbraith and E. Mayhew, *Brit. J. Cancer*, **XVI**, 163 (1962); *c*) E.M.F. Roe, *Nature*, **184**, 1891 (1959).

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7) W. Nakahara, F. Fukuoka, Y. Maeda and K. Aoki, *Gann*, **55**, 283 (1964).

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reaction mechanism, it became important to isolate the polysaccharide fraction from bamboo in homogeneous state and to make a chemical estimation of the effective constituent.

The present paper describes the isolation of two himicelluloses from the crude extracts of the leaves of a bamboo, *Sasa senanensis* (Fr. et Sav.) Rehd, in homogeneous states both in free boundary electrophoresis and ultracentrifugal analysis by means of ion-exchange chromatography and zone elctrophoresis, and their anti-tumor activities against mouse Sarcoma-180 solid tumor.

Fractionation was carried out as follows: The water solution of the crude extract was dialyzed against water and the non-diffusables were applied on a column of diethylaminoethyl (DEAE)-cellulose (acetate type), and were separated into five fractions (Fig. 1).

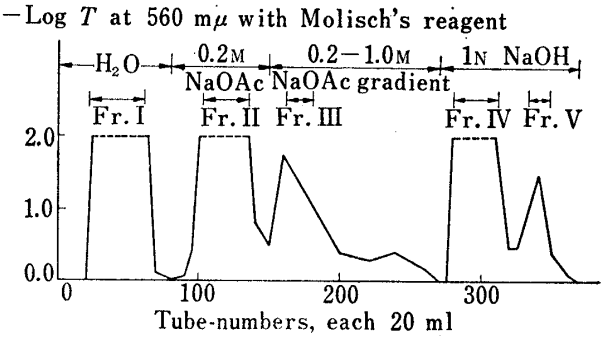

Fig. 1. Chromatographic Separation of Crude EtOH ppt. on DEAE-Cellulose Acetate Type Column

column size 5 × 30 cm, flow rate 60 ml/hr

Table I represents the analytical data on these fractions indicating that each fraction contained carbohydrate in high concentration.

Table I. Analyses of Each Fraction obtained by DEAE-Cellulose Column Chromatography

	Fr. I	Fr. II	Fr. III	Fr. IV	Fr. V
Total carbohydrate as xylose (%)	63.7	72.5	30.7	60.8	56.5
(as glucose)	(89.2)	(101.5)	(42.8)	(85.1)	(79.1)
Orcinol-$FeCl_3$ value as xylose (%)	40.4	39.8	21.2	22.7	52.3
Anthrone value as glucose (%)	38.1	27.6	15.5	19.3	22.8
N (%)	0.56	1.74	3.19	1.11	0.62
Yields from 10 g crude EtOH ppt. (g)	2.0	1.8	0.3	1.8	0.1
Appearance	pale yellow	brownish yellow	brown	brown	gray-white

The yields of Fr.I, Fr.II and Fr.IV were considerably higher than those of Fr.III and Fr.V. Since Fr. IV contained large amount of dark brownish pigmental impurities, further treatment of purification had not been carried out on this fraction.

Thus, two fractions, Fr.I and Fr.II, obtainable in high yields and contained relatively small amount of pigmental impurities, were subjected to further purifications. The results of the screening tests on Fr. I and Fr.II employing mice bearing Sarcoma-180 solid tumor indicated that these fractions possess marked anti-tumor activities (Table V). For the purpose of examination of homogeneities of Fr. I and Fr. II, free boundary electrophoreses were carried out in 0.05 M sodium tetraborate solution. As will be seen in Fig. 4-a and 4-b, both Fr. I and Fr.II were heterogeneous materials.

Since the results of qualitative analysis of the sugar components in both fractions by paper chromatography showed the presence of considerable amount of xylose, the copper-complex-precipitation method was applied for further purification of these fractions. Addition of Fehling's solution to the water-solution of Fr.I immediately caused a precipitation of copper-complex. The copper-complex was decomposed with hydrochloric a cid to recover the polysaccharide which was designated as Fr.I-(A). The supernatant solution was also treated to recover the non-precipitable polysaccharides, and was designated as Fr.I-(B). Thus, Fr.I was resolved into two fractions.

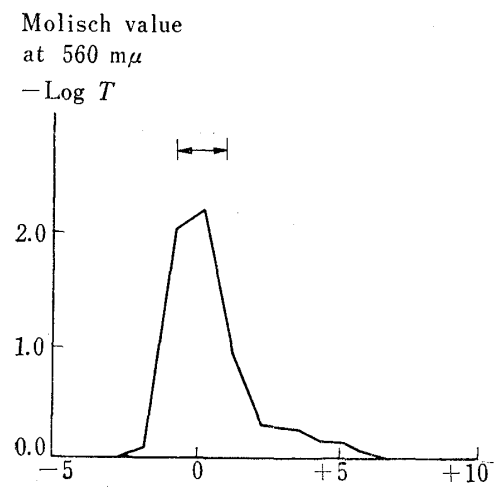

Fig. 2. Purification of Fr. I-(A) by Zone Electrophoresis

starch, 2×625cm, 0.05 M $Na_2B_4O_7$ buffer, 80 mA, 11 hr

Though Fr. 1-(B) was a heterogeneous material in electrophoresis (Fig. 4-d), it was employed for test of anti-tumor activity without further purification. No precipitation of copper complex occured by the addition of Fehling's solution to the water-solution of the other xylose-containing fractions including Fr. II.

The polysaccharide, Fr. I-(A), was again examined for the homogeneity by means of electrophoresis. It became obvious that Fr. I-(A) still contained small amount of the slower-migrating minor component (Fig. 4-c). Removal of this impurities in Fr. I-(A) was achieved by starch-block zone electrophoresis (see Fig. 2) and the material thus obtained, which gave single, symmetrical peak (Fig. 4-e) was designated as p-Fr. I-(A).

In the ultracentrifugal analysis, p-Fr.I-(A) gave a single peak having an approximate s value of 0.97 (Fig. 5-a).

Table II shows the analytical data of p-Fr.I-(A) and Fr.I-(B). They contained xylose, arabinose and glucose as the sugar components, and were completely free from nitrogen and

Table II. Analyses of p-Fr. I-(A), Fr. I-(B) and p-Fr. II

	p-Fr. I-(A)	Fr. I-(B)	p-Fr. II
Xyl (%)	63.4	29.1	34.5
Ara (%)	17.1	26.3	16.7
Glc (%)	19.5	12.2	21.8
Gala (%)	trace	32.4	26.7
Uronic acid (%)	non	—	1.2
$[\alpha]_D^{24}$ $C=1.0$, $l=1.0$, H_2O	−102.7°	—	−40.0°
N (%)	0.0	0.0	0.23
P (%)	0.0	0.0	0.0
D.P.	99	—	156
IO_4^- consumed after 192 hr mol/anhydro sugar residue	0.98	—	0.47
HCOOH formed after 192 hr mol/anhydro sugar residue	0.02	—	0.11

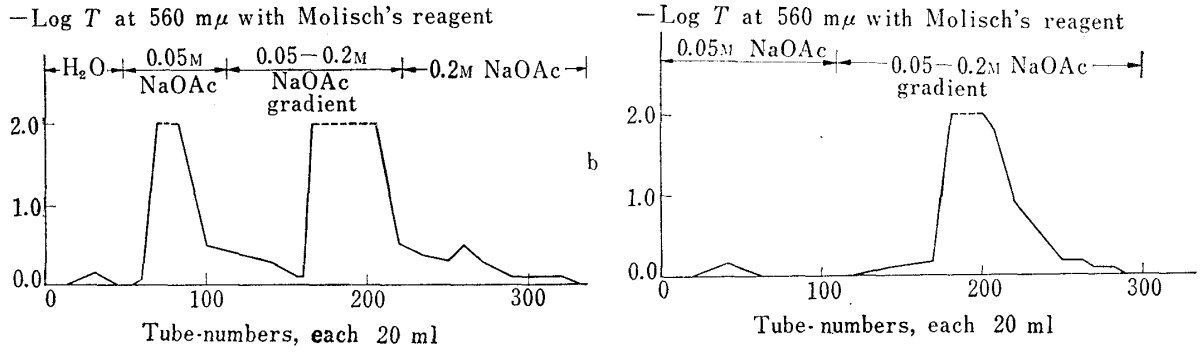

Fig. 3. Purification of Fr. II by Repetition of DEAE-Cellulose Column Chromatography

The left figure shows the elution pattern of the re-chromatography of Fr. II, and the right figure indicates the pattern of the twice-repetition of chromatography of Fr. II by the same procedure.

phosphorous. The facts that the values of periodate oxidation on p-Fr.I-(A) was lower than 1.0 M per anhydrosugar residues and that the formation of formic acid was negligible suggest that neither non-reducing terminals in pyranose form nor 1—6 linked hexopyranose residues present in the polysaccharide.

The other fraction, Fr.II, an heterogeneous acidic polysaccharide, was further purified by twice-repetition of column chromatography employing DEAE-cellulose acetate type. The elution patterns are represented in Fig. 3-a and 3-b.

The major component was isolated and designated as p-Fr.II. The electrophoretic and ultracentrifugal patterns of p-Fr.II are shown in Fig. 4-f and Fig. 5-b.

Table II shows the analytical data on p-Fr.II. The presence of small amount of uronic acid residues, assumed to be the cause of the acidity of Fr.II, were detected by carbazole-sulphuric acid method. It was assumed that the lower values of the specific rotation and the

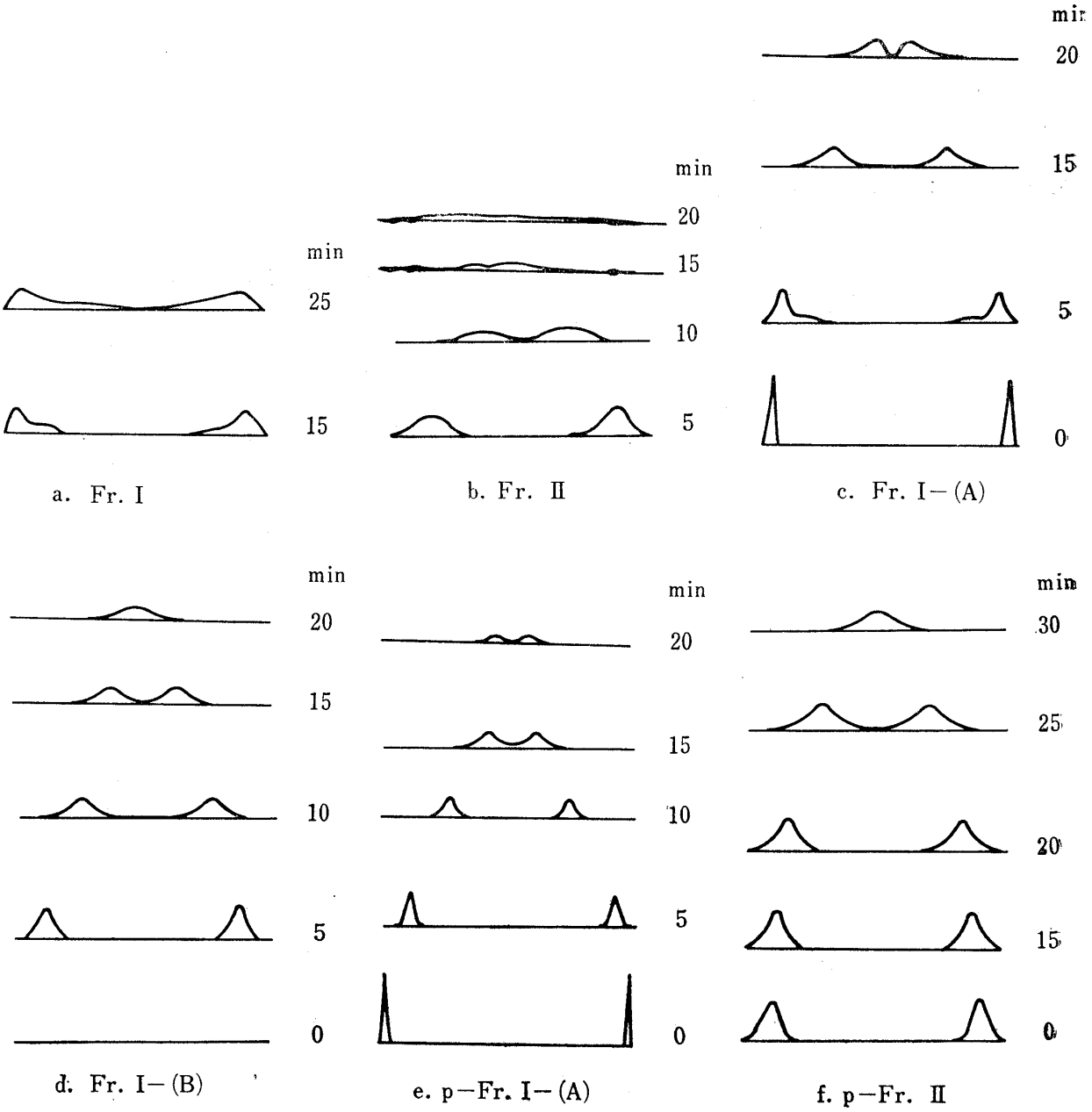

Fig. 4. Electrophoretic Patterns of Fr. I, Fr. II, Fr. I-(A), Fr. I-(B), p-Fr. I-(A) and p-Fr. II

buffer: 0.05 M sodium tetraborate pH 9.6

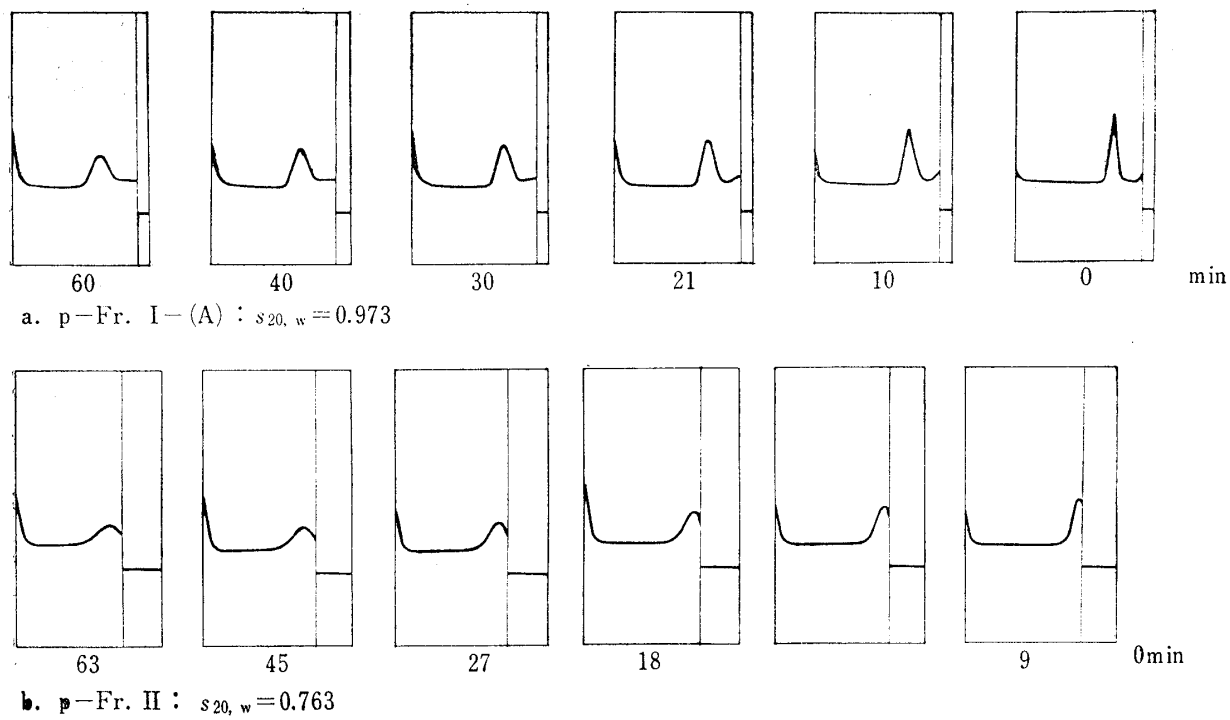

Fig. 5. Ultracentrifugal Patterns of the Purified Polysaccharides

periodate oxidation of p-Fr.II than those of p-Fr.I-(A) were due to the presence of considerable amount of periodate-resistant D-galactose residues.

Since both polysaccharides contained xylose as the major sugar-component and showed negative specific rotations, these were assumed to be hemicelluloses. In order to ascertain the above assumption the content of arabinose released by partial acid-hydrolysis and the specific rotation of the complete acid-hydrolyzates were determined. On partial acid hydrolysis, liberation of arabinose was significantly faster than xylose in both polysaccharides (Table III).

Table III. Quantitative Paper Chromatography of Acid-Labile Arabinose in the Purified Polysaccharides

Ara/Xyl	p-Fr. I-(A)	p-Fr. II
Partial hydrolysis	1/0.39	1/1.31
Complete hydrolysis	1/3.68	1/2.18

Therefore, it is quite reasonable that the arabinose residues existed as furanose form in both polysaccharides. The specific rotation of p-Fr.I-(A) and p-Fr.II after complete acid-hydrolysis were +32.9° and +41.3° (Table IV), and the theoretical values correspond to the ratio of the sugar components were +40.0° and +47.3°, respectively.

Table IV. Specific Rotations of Complete Hydolyzates of the Purified Polysaccharides

	p-Fr. I-(A)	p-Fr. II
Calcd.	+40.0°	+47.3°
Found	+32.9°	+41.3°

$[\alpha]_D^{22}$, $c=1.0$, $l=1.0$, 1 N H_2SO_4

These results indicate that former polysaccharides were consisted of D–xylose, L–arabinose and D–glucose, and the latter D–xylose, L–arabinose, D–glucose and D–galactose. Therefore, predominant linkages in their polysaccharides are the β–types of D–series–sugar. The above results gave the evidences that the polysaccharides possess essentially similar structures with the hemicelluloses, which have been isolated from various higher plants.

It has been generally accepted for the chemical structure of hemicellulose that the skeletons were composed by 1→4 linked D–xylose residues to which the other components, L–arabinose and the other hexoses or uronic acids, were linked.

In the assay of anti–tumor activity of three polysaccharides, p–Fr.I– (A), an arabinoxyloglucan, showed the highest potency, as will be seen in Table V.

Table V. Effect of Bamboo Polysaccharide Fractions on Subcutaneoulsy Implanted Sarcoma–180.

Expt. No.	Group	No. of mice	Average tumour weight (g)	Regression	Tumour growth inhibition (%)
1	control	10	6.68	—	—
2	Fr. I	10	1.30	6/10	80.6
3	control	10	9.61	—	—
4	Fr. II	10	1.52	5/10	84.9
5	control	9	11.40	—	—
6	p–Fr. I–(A)	9	0.04	7/9	99.6
7	Fr. I–(B)	9	0.79	3/9	93.2
8	p–Fr. II	9	0.78	5/9	93.2

The other two polysaccharide fractions, Fr.I–(B) and p–Fr.II, also gave almost identical tumor–inhibitory activities with that of p–Fr.I–(A). Thus, the chemical entities of the effective substances of the extract of the bamboo leaves are clarified as hemicelluloses, although the difference of the compositions of sugar components was observed among these materials. So it would be possible to assume that arabinoxylan, the common moiety throughout these polysaccharides, may play an important role in the tumor–inhibiting mechanism such as enhancement of production of serum γ–globulin in the host–animal which was described by Tanaka[9a,b] or other possible mechanisms discussed by Belkin, *et al.*[4a]

Experimental

Materials——The crude, saturated–lime–water extract of the leaves of bamboo was kindly provided from the Japan Contra–Cancer Council, Chihayacho, Toshimaku, Tokyo.

General Procedures——Paper chromatography was done on Toyo Roshi No. 51–A filter paper in descending methods, at room temperature for 48 hr. Solvents employed were as follows; (a) butanol, ethanol, water 4:1:1, (b) butanol, pyridine, water 5:3:1, (c) phenol, water 5:1. Aniline hydrogen phthalate was used as the spray reagent.

For the quantitative determination of carbohydrates, following colorimetric methods were employed. (a) total carbohydrate—α–naphthol–sulfuric acid,[10] (b) pentose—orcinol–hydrochloric acid,[11] (c) hexose—anthrone–sulfuric acid,[12] (d) uronic acid—carbazole–sulfuric acid.[13] Nitrogen contents were analyzed by Dumas' method.

Total phosphorous was determined by Allen–Nakamura's modified method.[14] Periodate oxidation was carried out essentially similar method described by Akiya and Okui[15] as follows. Each 20 mg of polysaccharide was dissolved into 15 ml of 3% sodium chloride solution separatedly and 0.25 M sodium meta-

9) *a*) T. Tanaka, F. Fukuoka and W. Nakahara, *Gann*, **56**, 529 (1965); *b*) T. Tanaka, *Gann*, **58**, 1 (1967).
10) A.W. Devor, *Anal. Chem.*, **24**, 1626 (1948).
11) W.J. Mejbaum, *Z. Physiol. Chem.*, **258**, 117 (1939).
12) T.A. Scott and E.H. Melvin, *Anal. Chem.*, **25**, 1656 (1953).
13) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).

periodate (1 ml) was added to each, than the total volumes were made to 25 ml. Reagent blank was also prepared and oxidations were carried out at room temperature in the absence of light. At the intervals of 24 hr, samples (each 1 ml) were withdrawn and were titrated with N/100 arsenite solution after addition of 10 ml of borate buffer and 0.2 g of potassium iodide. For the estimation of formic acid, each 10 ml aliqout of the solution was withdrawn after completion of the oxidation, and the excess periodate was destroyed by the addition of ethyleneglycol (1 ml) and titrated with N/50 NaOH. The results were shown in Table II. Free boundaty electrophoreses were carried out using a Hitachi HTD-I apparatus. Prior to the examination, all the samples were dialyzed against 0.05M $Na_2B_4O_7$ for overnight. Ultracentrifugal analyses were done in 1% water solution with a Hitachi UCA-I ultracentrifuge with a standard cell at 60000 rpm.

Assay of Anti-tumor Activity——Mice of the Swiss albino strain, initially weighing about 20 g, were used. Sacroma-180 ascites cells was implanted in dose of 0.1 ml (*ca.* 5×10^6 cells) subcutaneously into the right groin. After 1 day of tumor implantation, each polysaccharide, in a solution of distilled water, was injected intraperitoneally in dose of 200 mg/kg/day for ten days.

The tumor were weighed on the 35th days after implantation. The growth-inhibiting ratio was calculated by the following formula: Inhibition $\% = (A-B)/A \times 100$ where A is the average tumor weight of control group, and B is the average tumor weight of the administered group.

Fractionation of the Crude Extract——Ten percent water solution of the crude extract was dialyzed in a cellophane bag against tap water for 48 hr. The non-diffusables were concentrated *in vacuo* to 1/20 of the original volume. Four volumes of EtOH were added with stirring and the resulted precipitates were collected by centrifugation. The solid mass was washed several times with EtOH, then dried *in vacuo* over P_2O_5. The yield in this stage was about 10% in weight of the crude extract. Ten g of the ethanol-precipitate was dissolved into water at 10% concentration and applied on a top of a column of DEAE-cellulose (acetate type: 5×30 cm), then eluted by following sequence; (1) 1600 ml of water, (2) 1400 ml of 0.2M NaOAc, (3) 2200 ml of 0.2—1.0M NaOAc linear gradient elution, (4) 2000 ml of N NaOH. Each fractions were examined on carbohydrate contents with Molisch's reagent and the peaks (Fig. 1) were collected seperately, then concentrated *in vacuo* and dialyzed against tap water. To each non-diffusables, 4 volume of abs. EtOH was added. The precipitates were collected and dried *in vacuo* after washing with abs. EtOH.

Separation of Fr. I-(A) and Fr. I-(B) from Fr.I——To 20 ml of 10% aqueous solution of Fr.I (2.0 g) was added 50 ml of Fehling's solution. Blue copper-complex precipitated immediately. The precipitate was collected by centrifugation and washed twice with water. To the insoluble residue, 6 N HCl was added dropwise to just dissolve the complex. Several volumes of abs. EtOH was added to the solution and the resultant white precipitate was collected by centrifugation, then dried *in vacuo* after washing with abs. EtOH. In order to remove trace amount of copper contamination, the dried material was dissolved into water at 5% concentration, then passed through a small column (2×15 cm) of CM-Sephadex C-50 NH_4^+ type. The eluate was dialyzed against water, then precipitated and dried in similar manner described above. Yield of Fr.I-(A) was 650 mg or 32.5% of Fr. I.

The supernatant solution of the copper complex was neutralized by the addition of conc. HCl, then dialyzed against tap water for 72 hr and passed through a column of CM-Sephadex C-50 NH_4^+ type. The eluate, completely freed from copper, was concentrated *in vacuo* and the polysaccharide precipitated by the addition of 4 volumes of abs. EtOH. The precipitate was thoroughly washed with abs. EtOH, then dried *in vacuo* over P_2O_5. Yield of this fraction was 1.1 g.

Purification of Fr.I-(A) to p-Fr.I-(A) by Starch-Block Zone Electrophoresis——Starch block was prepared in the size of $2 \times 6 \times 25$ cm with potate starch equilibrated with 0.05 M $Na_2B_4O_7$ for 48 hr. One hundred mg of Fr.I-(A) was dissolved into 2 ml of 0.05 M $Na_2B_4O_7$, then the solution was applied on starch block. After electrophoresing at 80 mA for 11 hr, the starch block was cutted off into 1 cm length. The pieces were eluted with water to make each volume to 50 ml, which were examined on carbohydrate contents. The eluate from -1 to $+1$ were combined, passed through a small column of Amberlite IR-120 H^+ type, then the eluate was dialyzed against in water. The non-diffusable was concentrated *in vacuo* to about 2 ml, precipitated by the addition of 4 volumes of abs. EtOH, collected by centrifugation, washed with abs. EtOH and dried *in vacuo*. In order to removal of remaining borate, dried material was suspended in 5 ml of 1%-methanolic hydrogen chloride and evaporated in diminished pressure at room temperature. To the residue, was added methanol and evaporated repeatedly. Finally, insoluble material was collected by centrifugation and dried *in vacuo*. Yield of p-Fr.I-(A) was about 60 mg. $[\alpha]_D^{24}$: $-102.7°$ ($c=1.0$, $l=1.0$, H_2O).

Purification of Fr.II——Ten percent solution of Fr.II was applied onto a column of DEAE-cellulose (acetate type: 5×30 cm). The column was succesively eluted with following eluants; 1000 ml of water, 1200 ml of 0.05 M NaOAc, 2200 ml of 0.05—0.2 M NaOAc in linear gradient and 2250 ml of 0.2 M NaOAc. The eluates, obtained by the gradient elution, were combined and concentrated under diminished pressure to 1/20 of the orginal volume, then dialyzed against tap water for 24 hr. Four volumes of abs. EtOH were added to the solution of non-diffuzables and the precipitates occured collected by centrifugation and dehyd-

14) M. Nakamura, *Nippon Nogeikagaku Kaishi*, **24**, 1 (1950).
15) S. Akiya and S. Okui, *Yakugaku Zasshi*, **71**, 865 (1951).

rated by washing with abs. EtOH. After drying over P_2O_5, the material was again subjected to chromatography by the same procedure described above. Yield of p–Fr.II was 27% calculated from the weight base of Fr. II.

Detection of the Sugar Components and Their Relative Molar Ratio in p-Fr.I-(A), Fr.I(B) and p-Fr.II—Each polysaccharide (3 mg) was hydrolyzed by heating with 1N H_2SO_4 (1 ml) at 98° for 4 hr. The solutions were neutralized with finely powdered $BaCO_3$ and centrifuged. The supernatants were passed through a small column of IR–120 H^+ type. The eluates obtained were chromatographed with solvent (a). By spraying AHP reagent, xylose, arabinose and glucose were detected on the chromatograms orginated from p–Fr.I–(A) and Fr.I–(B). In addition to these three sugar components, p–Fr.II gave the spot identical with galactose. For determination of relative molar ratio of sugar components, corresponding areas of each sugar components of the other paper strips were cutted off seperately and eluted with water. Then the eluates correspond to xylose and arabinose were subjected to orcinol–$FeCl_3$ method, and glucose contents were determined by anthrone–H_2SO_4 method. The ratios, thus obtained, were showed in Table II. The presence of 1.2% of uronic acid (as glucuronolactone) in p–Fr.II was detected by carbazole–H_2SO_4 method.

Partial Hydrolysis of p-Fr.I-(A) and p-Fr.II——Both polysaccharides (each 1 mg) were hydrolyzed with 1 N H_2SO_4 at 98° for 35 min. After neutralization, the hydrolyzates were subjected to paper chromatography. Xylose, arabinose and several oligosaccharides were detected on both chromatograms. Relative ratios (Table III) of arabinose to xylose were determined by the same method described in the case of the detection of molar ratio of the sugar–components.

Specific Rotations of Complete Acid–Hydrolyzate of p-Fr.I-(A) and p-Fr.II——Each polysaccharide (20 mg) were hydrolyzed with 1 N H_2SO_4 at 98° for 4 hr. After centrifugation the supernatant were made up to 2 ml with water. Measurements of specific rotations using a Rex electrophotopolarimeter gave following values; p–Fr.I–(A) +32.9°, p–Fr.II +41.3°. The theoretical values of mixtures of D–xylose, L–arbinose and D–glucose in the ratios of the sugar components of each polysaccharide were +40.0° and +47.3° respectively (Table IV).

Determination of the Polymerization Degree of p-Fr.I-(A) and p-Fr.II——Each 50 mg of polysaccharides in 5 ml water were heated with 5 ml of Somogyi's reagent in boiling water bath for 30 min. At the same time, 0.5 mg of D–xylose in 5 ml of water solution was run in the same manner. 0.01 N $Na_2S_2O_3$ consumed were as follows; p–Fr.I–(A) 3.81, p–Fr.II 2.54, D–xylose 3.33 ml respectively. Thus, D.P. of each polysaccharides were as follows; p–Fr.I–(A) 99, p–Fr. II 156.

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