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Preparation of Yeast Mannan Derivatives by Stearoylation and Phosphorylation[1]

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The mannan from baker's yeast was modified by stearoylation and phosphorylation, and the water-soluble part of the resultant product, stearoyl mannan phosphate (SMP), was resolved by gel-filtration with Sephadex G-75 into two fractions, SMP-Fr. I and -Fr. II. The former fraction was found to contain three components, fatty acid, carbohydrate, and phosphate. The SMP-Fr. I was then fractionated by DEAE-Sephadex chromatography to give four subfractions, SMP-Fr. I -a, -b, -c, and -d. Each fraction except Fr. I-a was found to contain fatty acid, carbohydrate, and phosphate, and to be homogeneous in ultracentrifugal analyses.

It is well known that the lipopolysaccharides of gram-negative bacteria are capable of revealing a variety of biological activities including tumor-growth inhibitory activity.[3] With regard to the synthesis of polysaccharide derivatives possessing chemical structures related to those of the endotoxins, a few papers which described the preparation of fatty acid derivatives of simple polysaccharides have been published.[4] We have now attempted the preparation of various modified polysaccharides aiming at obtaining antitumor agents less toxic than bacterial lipopolysaccharides.

As the first step of this series, baker's yeast mannan was acylated with stearoyl chloride and subsequently phosphorylated with polyphosphoric acid in N,N-dimethylformamide (DMF) to give the corresponding modified product, stearoyl mannan phosphate (SMP). The product was isolated from the reaction mixture by the addition of a large amount of methanol, and was successively purified by dialyzing against distilled water, precipitating with methanol and by subsequent column chromatography using Sephadex G-75 and DEAE-Sephadex A-50.

Analytical data for the modified mannan synthesized in the present study are given in Table I. The crude stearoyl mannan phosphate (c-SMP) was found to contain three components, carbohydrate, phosphate, and fatty acid, 40.3, 10.5, and 9.5%, respectively. The c-SMP preparation was then extracted with water to separate water-soluble material. As will be seen in Fig. 1, the water-soluble fraction of c-SMP was resolved by Sephadex G-75 column chromatography into two subfractions, SMP-Fr. I and -Fr. II. Both subfractions were shown to contain carbohydrate and phosphate as common components, but the existence of fatty acid, 1.7%, was shown only in the former fraction.

The SMP-Fr. I was then fractionated over a column of DEAE-Sephadex A-50 by stepwise elution with water, 0.1, 0.5, and 1.0M sodium chloride solution to yield four subfractions designated as SMP-Fr. I-a, -b, -c, and -d as shown in Fig. 2. Subfraction I-a was composed only of

1) Main part of this study was presented at the 95th Annual meeting of Pharmaceutical Society of Japan, Nishi-no-miya, April 1975. This work was supported in part by a Grant in Aid from the Ministry of Health and Welfare of Japan.
2) Location: *Komatsushima 4-4-1, Sendai.*
3) *a*) M.J. Shear, *J. Natl. Cancer Inst.*, **4**, 461 (1944); *b*) S.R. Rosenthal, R.G. Crispen, M.G. Thorne, N. Raisys, and P.G. Retting, *J. Amer. Med. Assoc.*, **222**, 1534 (1972); *c*) F.C. Sparks, M.J. Silverstein, J.S. Junt, C.M. Haskell, Y.H. Pilch, and D.L. Morton, *New Engl. J. Med.*, **289**, 827 (1973); *d*) T. Tanaka, *GANN*, **65**, 145 (1974).
4) *a*) U. Hämmerling and O. Westphal, *European J. Biochem.*, **1**, 46 (1967); *b*) S. Hirano and Y. Ohe, *Carbohyd. Res.*, **41**, Cl (1975).

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Table I. Chemical Analyses of Modified Mannans

Sample	Sugar (%)	Phosphate (%)	Fatty acid (%)	S_{20}	$[\alpha]_D^{20}$
Yeast mannan	98.5	0.0	0.0	3.2	+76.0°
Stearoyl mannan phosphate (*c*-SMP)	40.3	10.5	9.5		
SMP-Fr. I	58.0	4.4	1.7		
SMP-Fr. II	88.6	3.9	0.0		
SMP-Fr. I-a	92.0	0.0	0.0		+74.0°
SMP-Fr. I-b	81.0	2.4	trace	4.3	+66.0°
SMP-Fr. I-c	72.0	6.0	0.2	4.6	+61.0°
SMP-Fr. I-d	69.0	7.6	1.9	4.8	+53.0°

Total carbohydrate and total phosphate were determined by Anthrone method,[5] and Allen-Nakamura method,[6] respectively.
Fatty acid content was determined by GLC.

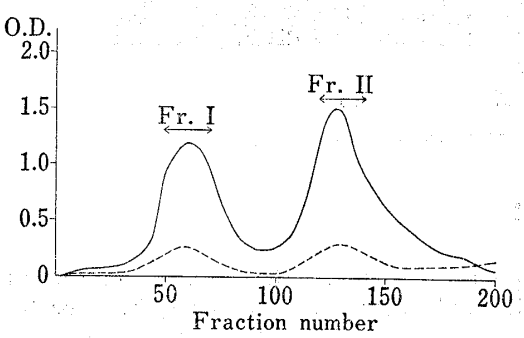

Fig. 1. Gel-filtration Pattern of Water-soluble Part of SMP on Sephadex G-75 (6×100 cm)

5 ml/tube, 5 ml/min, sample 1.0 g
——: sugar (O.D. at 550 nm)
------: phosphorus (O.D. at 750 nm)

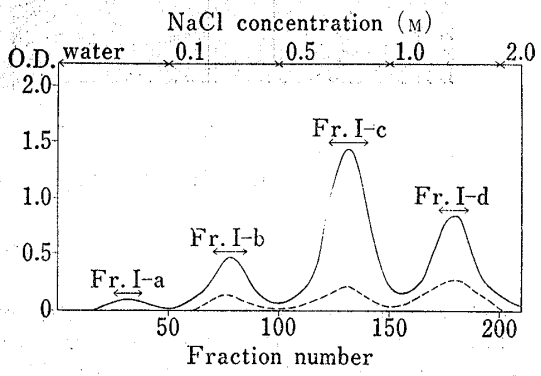

Fig. 2. Elution Pattern of SMP-Fr. I in Chromatography on DEAE-Sephadex A-50 Acetate Type (6×100 cm; 10 ml/tube)

stepwise elution with 0.1, 0.5, 1.0, and 2.0M NaCl solution, sample 1.0 g
——: sugar (O.D. at 550 nm)
------: phosphorus (O.D. at 750 nm)

carbohydrate, 92.0%, and therefore regared to be the unreacted yeast mannan. The SMP-Fr. I-b was composed of carbohydrate, phosphate, and trace amounts of fatty acid. A small amount of fatty acid was detected in both SMP-Fr. I-c and -Fr. I-d, 0.2 and 1.9%, respectively. As shown in Fig. 3, SMP-Fr. I-b, -c, and -d were found to be homogeneous in ultracentrifugal analyses. The S_{20} values of SMP-Fr. I-b, -c, and -d were 4.3, 4.6, and 4.8, respectively. The specific rotations of these fractions were +66.0°, +61.0°, and +53.0°, respectively.

In order to assess the structural feature, the purified SMP-Fr. I-d was treated with 0.01N hydrochloric acid solution at 100° for 1 hr. Neither release of fatty acid nor inorganic phosphoric acid was observed from SMP-Fr. I-d under the above condition. Thus, the existence of any acid-labile linkages such types as acylphosphoryl and pyrophosphoryl was entirely excluded.

The yields and chemical compositions of SMP-Fr. I-d and other modified polysaccharide fractions were all highly reproducible.

The purified SMP-Fr. I-d was found to be effective against mouse bearing sarcoma 180 and Ehrlich ascites carcinoma *in vivo*, and more effective by a combination treatment with Mitomycin C which produced no acute toxic symptoms. Detailed descriptions on this aspect are now in preparation.

5) M. Schramm and A. Loyter, *Methods Enzymol.*, **8**, 533 (1966).
6) M. Nakamura, *Nippon Nogei Kagaku Kaishi*, **24**, 1 (1950).

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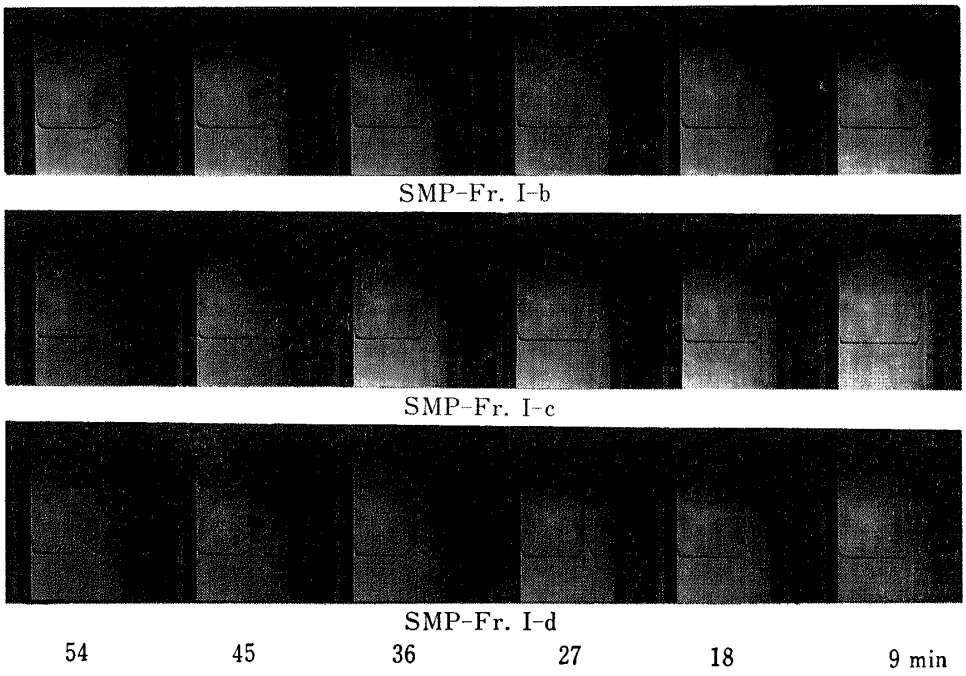

Fig. 3. Ultracentrifugal Patterns of SMP-Fr. I-b, -c, and -d

Experimental

General Procedures——Total carbohydrate, total phosphorus, and total nitrogen were determined by the modified Anthrone method,[5] Allen-Nakamura method,[6] and microelementary analysis, respectively. Fatty acid content was determined by gas-liquid chromatography (GLC): Sample (10 mg) was heated with 5 ml of 2N hydrogen chloride at 100° for 2 hr. At this time oleic acid (1.0 mg) was added in the solution as the internal standerd. After being cooled, the mixture was extracted with ethyl ether (50 ml). The solvent layer was dehydrated with anhydrous sodium sulfate, concentrated *in vacuo* to a small volume, and then added an excess amount of diazomethane in ether. After being kept at room temperature for 1 hr, the solution was evaporated and the residue was dissolved in hexane (0.5 ml). A Shimadzu GC-4BMPFFP apparatus equipped with a hydrogen flame ionization detector and a glass column (0.3×200 cm) packed with 15% diethylene glycol succinate on 60—80 mesh Celite 545 was used for analysis of fatty acid methyl ester. Ultracentrifugal analyses were done using a Hitachi UCA-6A ultracentrifuge at 60000 rpm. Optical rotation was measured using an applied electric polarimater; $c=1.0$, $l=1.0$, water.

Chemicals——The mannan from baker's yeast was prepared by the method described previously.[7] Neither nitrogen nor phosphorus was detected in this specimen. Stearoyl chloride of practical grade was purchased from Nakarai Chemicals Co., Osaka, Japan. It consisted of *ca.* 7: 3 ratio mixture of stearoyl and palmitoyl chloride as examined by GLC after conversion of it to the corresponding methyl ester. Unless otherwise stated, other chemicals used were the highest grade reagents commercially available.

Preparation of Stearoyl Mannan Phosphate——Yeast mannan (1.0 g), dried over P_2O_5, was dissolved into a 10: 3 mixture of DMF (100 ml) and tri-*n*-butylamine (30 ml) by stirring at room temperature. To the solution was added stearoyl chloride (5 ml) under vigorous stirring, then the stirring was continued for 2 hr at room temperature. Polyphosphoric acid (5 g) was added subsequently to the above solution, then the mixture was gently heated at 40° for 30 min. This mixture was diluted with methanol (500 ml), then saturated aqueous solution of sodium acetate was added dropwise into the methanol solution until no more precipitation occurred. After being collected by centrifugation and washed 5 times with methanol, the precipitate was suspended in distilled water (200 ml) and adjusted to pH 9.0 with 1N sodium-hydroxide. The suspension was dialyzed against distilled water for 48 hr. The inner fluid was concentrated *in vacuo* to 50 ml, and after centrifugation, the supernatant was poured into 4 volumes of methanol. Precipitate formed was collected by centrifugation, washed 5 times with methanol, and dried over anhydrous calcium chloride (c-SMP). One gram of c-SMP was homogenized with distilled water (100 ml), then the resultant suspension was filtrated on Toyo Roshi No. 2 filter paper by suction and the filtrate was concentrated *in vacuo* to 20 ml. After removal of small amounts of precipitate by centrifugation, the supernatant was applied over a column (6×100 cm) of Sephadex G-75 (medium) (Fig. 1). Elution was carried out with water and each eluate (5 ml/tube) was

7) *a*) S. Peat and W.J. Whelan, *J. Chem. Soc.*, 3862 (1958); *b*) S. Suzuki, H. Hatsukaiwa, H. Sunayama, M. Uchiyama, F. Fukuoka, M. Nakanishi, and S. Akiya, *GANN*, **60**, 65 (1969).

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assayed for the contents of carbohydrate and phosphate. Two fractions, the first and the second peaks, were pooled and designated as SMP-Fr.I and -Fr. II, respectively. The fractions were concentrated under diminished pressure to a small volume, and then poured into methanol. The resultant precipitate was dried over anhydrous calcium chloride. The yields of SMP-Fr. I and -Fr. II were 0.35 and 0.49 g, respectively. One gram of the former was dissolved in distilled water (10 ml) and was applied on a column (6 × 100 cm) of DEAE-Sephadex A-50 (acetate type). A stepwise elution was carried out using each 500 ml of water, 0.1, 0.5, and 1.0M sodium chloride (Fig. 2). The eluates containing carbohydrate were pooled, and dialyzed against distilled water for 48 hr. The polysaccharide derivative was then recovered by the method-described above. These eluates were designated as SMP-Fr. I-a, -b, -c, and -d, and yields of these fractions were 0.01, 0.15, 0.43, and 0.25 g, respectively.

Treatment of SMP-Fr. I-d with 0.01N Hydrochloric Acid——SMP-Fr. I-d (100 mg) was dissolved in 0.01N hydrochloric acid (10 ml), and the clear-solution was heated on a boiling water bath for 1 hr. After being cooled, a part of the solution (2 ml) was withdrawn and assayed for the content of inorganic phosphate. Only a negligible amount of phosphorus was detected in this solution. Remaing acid solution was extracted with hexane (5 ml × 2), and the extract was assayed for fatty acid content. No peak of fatty acid methyl ester other than the internal standard, methyl oleate, was detected by GLC.

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