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69. Shichiro Akiya*1 and Otomatsu Hoshino*2 : Studies on the Constitution of Muco-complex from *Micrococcus lysodeikticus*. II.[1] Physicochemical Properties of Muco-complex.

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In the previous paper[2] of this series, the authors reported on two fractions, named H and G, obtained from bacterial cells of *Micrococcus lysodeikticus* by acrinol (2-ethoxy-6,9-diaminoacridinium lactate) method, and the composition of these two fractions, H and G, was shown to be mainly composed of a polysaccharide containing D-mannose and muco-complex, respectively.

Recently, Gilby, Few, and McQuillen[3] reported the preparation of protoplasts of the same bacteria by lysozyme treatment according to Weibull's method[4] and they found that the separated protoplast membrane was a complex of lipid, protein, and carbohydrate. It is interesting that, similar to fraction H, the above carbohydrate was found to consist mainly of mannose.

On the other hand, there are several studies[5~7] on the isolation and properties of bacterial cell walls and, further, Meyer, *et al.*[8] reported a soluble mucopolysaccharide complex isolated from the bacterial cell wall of *M. lysodeikticus* without detailed informations on its physicochemical properties. This paper deals with several physicochemical properties of a soluble muco-complex (fraction G) obtained in the previous work.

Experimental

Electrodialysis of Fraction G—A solution of 1 g. of fraction G*3 in 50 cc. of water (pH 6.2) was electrodialysed against water, at 100 v, 50 mA, and after 10 hr. the current dropped to 5 mA. The main part of fraction G remained as a non-dialysable, high molecular, acid gel which was precipitated by successive addition of equivolumes of 0.4*N* acetate buffer and 3 volumes of EtOH. The precipitate was collected, washed with Me_2CO and Et_2O, and dried *in vacuo*. Yield, 0.9 g. (Fraction G').

Ethanol Fractionation of Fraction G'—To a solution of 1 g. of fraction G' in 50 cc. of 0.2*N* acetate buffer (pH 6.2), EtOH was added to bring the concentration to 50~60% and 60~70% of EtOH. Each of the precipitates (GI, GII) formed was centrifuged, washed with Me_2CO and Et_2O, and dried *in vacuo*. To the supernatant obtained after precipitation of GII, AcOK solution and EtOH were added to bring the respective concentration to 2% and 80%. The precipitate was collected, washed, and dried as above (GIII). The yield of each precipitate was 5~10% of GI, 70~80% of GII, and 10~15% of GIII.

Paper Electrophoresis of GI, GII, and GIII—The solutions applied : GI, GII, GIII (concn. 1%), CSA*4 (1%), HA*4 (0.2%). 5~10 μl of each solution was spotted on Toyo Roshi No. 51 (12×26 cm.)

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*3 Fraction G was prepared by the procedure described in Part I.[2]

*4 CSA and HA are abbreviations for chondroitinsulfuric acid and hyaluronic acid. CSA was isolated as K-salt from cartilage of *Prionace glauca* (a kind of shark) by the method of Einbinder and Shubert[9]; HA was isolated as K-salt by Suzuki's method[10] from human umbilical cord kindly supplied by the Mochida Pharmaceutical Company.

1) A part of this work was presented at the 2nd Kanto Local Meeting of the Pharmaceutical Society of Japan, October, 1958.
2) Part I. S. Akiya, O. Hoshino : Yakugaku Zasshi, **77**, 777(1957).
3) A. R. Gilby, A. V. Few, K. McQuillen : Biochim. et Biophys. Acta, **29**, 21(1958).
4) C. Weibull : J. Bacteriol., **66**, 688, 696(1953).
5) M. R. J. Salton : Biochim. et Biophys. Acta, **22**, 495(1956).
6) C. S. Cumins, H. Harris : J. Gen. Microbiol., **14**, 583(1956); **15**, ix(1956).
7) E. Work : Nature, **179**, 841(1957).
8) K. Meyer, E. Hahnel : J. Biol. Chem., **163**, 723(1946).
9) L. E. Einbinder, M. Schubert : *Ibid.*, **191**, 591(1951).
10) "Chemistry of Polysaccharides," 298(1955), Kyoritsu Shuppan Co., Tokyo.

and run with a mixed solvent of pyridine-AcOH-H_2O (10:2:488) (pH 5.8) for 2 hr. at 15 v/cm., and 0.5~1.0 mA/cm. The spots were detected by spraying Toluidine blue or bromophenol blue and the results are shown in Fig. 1.

Electrophoresis of GII and GIII—Each of 1% solution of 40 mg. of GII and GIII in veronal (pH 8.60), phosphate (pH 6.25), acetate (pH 5.50), or glycine-HCl (pH 4.20) buffer ($\mu=0.1$) was submitted to Tiselius electrophoresis (4°, 95~110 v, 10 mA). The patterns observed at intervals of 15 min. are shown in Fig. 2a and b. In another run, a 1% solution of 40 mg. of GIII dissolved in acetate buffer was digested with 1 mg. of lysozyme*5 at 37° for 15 hr., and the digested GIII was submitted to electrophoresis, the pattern of which is given in Fig. 2c.

Zone Electrophoresis of GII—A 4% aqueous solution of 100 mg. of GII was applied to zone electrophoresis on cellulose powder (Toyo Roshi, 100~200 mesh) (6×27×1 cm.) in a mixed solvent of pyridine-AcOH-H_2O (pH 5.8), at 300 v, 30~50 mA (Fig. 3). After migration of GII for 6 hr. the patterns were printed on paper and the band of muco-complex was detected with bromophenol blue reagent. The band of cellulose powder containing muco-complex was eluted with water. After concentration of the eluate, EtOH was added to separate the precipitate which was washed with Me_2CO and Et_2O, and dried; yield, 85 mg. (fraction GIIs).

Electrophoresis of GIIs—A 1% solution of 30 mg. of fraction GIIs in phosphate buffer (pH 5.9, $\mu=0.1$) was submitted to electrophoresis. Another similar solution of GIIs was digested with lysozyme (0.5 mg.) and the digest obtained was also submitted to electrophoresis as above (Fig. 4). Mobility of muco-complex : -5.6×10^{-5} $cm^2/v^{-1}/sec^{-1}$.

Ultracentrifugal Analysis of GIIs—Fraction GIIs was dissolved in a mixture of $M/15$ phosphate buffer and $0.2M$ NaCl (pH 7.8) in concentration of 0.2, 0.4, 1.0%, and the solutions were examined by Spinco (analytical) ultracentrifuge (Model E). The rotor speed in all experiments was 59,780 r.p.m.

Viscosity of GIIs—GIIs was dissolved in a mixture of $0.1N$ acetate buffer (pH 6.2) and $0.1M$ NaCl to make concentrations of 0.16, 0.22, 0.32% and the viscosity of each solution was measured by the Ostwald viscometer (vol. 1.5~2.0 cc.) at 25°.

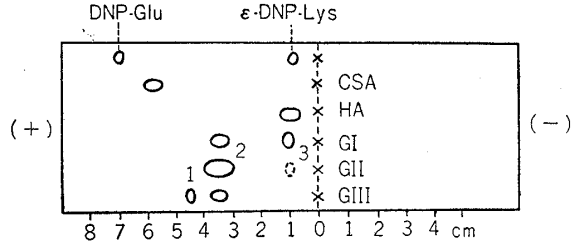
Electrometric Titration and Optical Rotation of GIIs—GIIs (9.40 mg.) was dissolved in 2 cc. of water and the solution was titrated electrometrically. $[\alpha]_D^{15}$ +31.2°(c=2.0, H_2O).

Osmotic Pressure of GIIs—GIIs (104 mg.) was dissolved in 10.4 cc. of water and osmotic pressure of the solution was measured by Bull-Curie apparatus at 27°; h=2.50±0.1 cm.

Results and Discussion

The fraction G, obtained in the previous work and mainly consisting of muco-complex, was found to contain a small amount of two contaminants which moved on electrophoresis a little faster (MuA) and slower (MuN) towards the anode than the main muco-complex. For the purpose of further purification, fraction G was electrodialysed to obtain fraction G′ which was further fractionated by ethanol precipitation to give three fractions, GI, GII, and GIII, precipitated at the respective ethanol concentrations of 50~60%, 60~70%, and 70~80%. Of the three fractions, the main one, fraction GII, was obtained in a yield of ca. 70~80%.

These three fractions were submitted to paper electrophoresis with chondroitinsulfuric acid and hyaluronic acid as control. The results obtained are given in Fig. 1. Thus it was found that muco-complex was mainly contained in GII and travelled to the distance



Solvent : pyridine-acetic acid buffer (pH 5.8); 300 v, 5~10 mA, 2 hr.
Migration distance from origin (mm.) : CSA 58. HA 8.5, MuA (spot 1) 45, MuC (spot 2) 36, MuN (spot 3) 9.5
DNP-Glu : N-(2,4-dinitrophenyl)glutamic acid
ε-DNP-Lys : ε-N-(2,4-dinitrophenyl)lysine

Fig. 1. Diagram of Paper Electrophoresis of CSA (Chondroitin sulfate), HA (Hyaluronate), and Fractions containing Muco-complex (GI, II, III)

*5 Lysozyme was prepared from egg-white by the bentonite adsorption method.

between chondroitinsulfuric acid and hyaluronic acid. GI and GIII were established to contain respective contaminant of MuN and MuA besides muco-complex.

GII and GIII were checked by electrophoresis and shown in Fig. 2a and b. Parallel to the result of paper electrophoresis, muco-complex moved faster toward the anode when pH of the buffer used was 6.25 than at pH 5.50. A preparation of fraction GIII digested with lysozyme revealed an electrophoretic pattern shown in Fig. 2c. In this case the peak corresponding to muco-complex in Fig. 2b diminished in height and thus, the component corresponding to that peak was the true substrate for the enzyme.

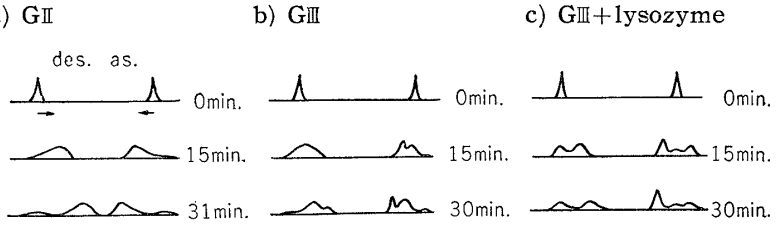


Fig. 2. Electrophoretic Patterns of GII and GIII
Buffer : a) phosphate, pH 6.25 (μ=0.1)
b), c) acetate, pH 5.50 (μ=0.1)

From the above results, it is known that fraction GII contains muco-complex as its main component but it is still contaminated with a trace of MuN which is hardly removed by ethanol fractionation. Further trials of several methods, i.e. electrodialysis, adsorption with Hyflo Supercel, and Celite for removal of MuN, were unsuccessful because of high viscosity of the muco-complex. The removal was achieved by using zone electrophoresis of the crude muco-complex on cellulose block. The part of cellulose powder containing muco-complex (see Fig. 3) was eluted to obtain GIIs consisting only of muco-complex.

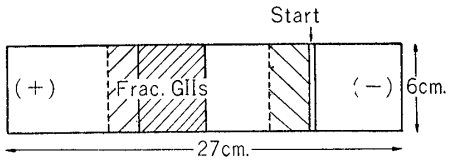


Fig. 3.
Zone Electrophoresis of GII
Solvent : Pyridine-AcOH buffer (pH 5.8). Run on cellulose powder, 300 v, 30~50 mA, 6 hr.

The homogeneity of fraction GIIs was revealed in electrophoretic pattern (Fig. 4a) and a similar pattern on GIIs after digestion with lysozyme showed that one simple peak (in Fig. 4a) separated into more than two peaks with smaller heights (Fig. 4b).

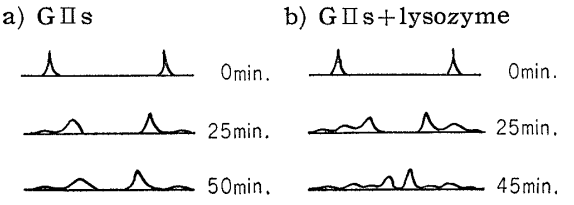


Fig. 4.
Electrophoretic Patterns of GIIs
Solvent : Phosphate buffer, pH 5,90 (μ=0.1)

Thus, GIIs is electrophoretically homogeneous and a substrate for the enzyme. Several physicochemical properties of GII were investigated by ultracentrifugal analysis and this gave a sedimentation pattern shown in Fig. 5. It shows a sharper and slower moving peak for GIIs than crystalline ribonuclease measured in the same concentration. These properties of GIIs in ultracentrifugation should be caused by the larger viscosity of muco-complex than that of RNase. The observed $S_{20,w}$ for the GIIs was 3.5 S (Fig. 6).

The viscosity of GIIs measured was $[\eta]$=3.06 (c=g./100 cc.). The neutralization equivalence of GIIs was 626 and its isoelectric point calculated from mobility in electrophoresis with glycine-hydrochloric acid or veronal buffer was close to 3.0 (Fig. 7).

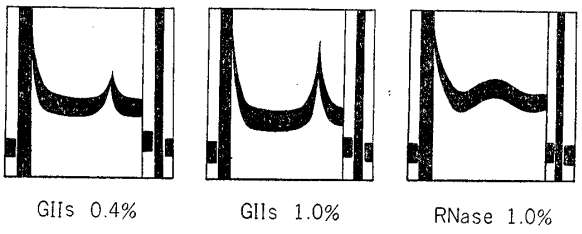


Fig. 5.
Sedimentation Diagrams
Phosphate buffer+0.2*M* NaCl, pH 7.8, 128 min. Spinco Model E ultracentrifuge (59,780 r.p.m.). Sedimentation occurs from right to left.

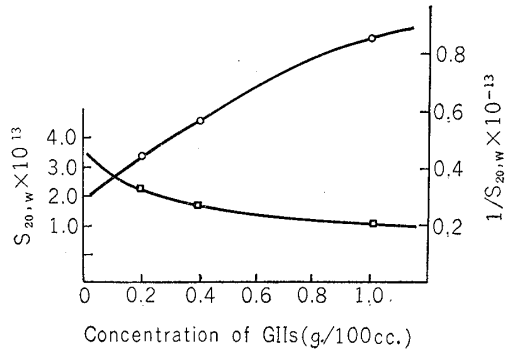
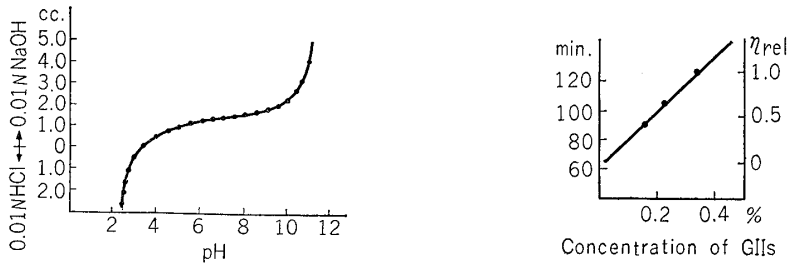


Fig. 6.
Sedimentation Constant
$S_{20,w=3.5}$ S(v=0.750)



a) Titration curve (GIIs, 9.40 mg.) b) Viscosity

Fig. 7.

The molecular weight of GIIs obtained from osmotic pressure method was 101,700, but that obtained from both its sedimentation constant and intrinsic viscosity was 94,400.

From these results the muco-complex obtained this time from *Micrococcus lysodeikticus* is a substrate for lysozyme and it is a kind of mucopolysaccharide with molecular weight about 100,000.

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Summary

The fraction G previously isolated from *Micrococcus lysodeikticus* mainly consists of a muco-complex. This fraction was further purified by both electrodialysis and zone electrophoresis to separate a small amount of impurities and to obtain a single muco-complex, GIIs. The latter gave a single spot which migrated towards the anode in paper electrophoresis.

The homogeneity of GIIs was further investigated by Tiselius electrophoresis and ultracentrifugation. Several measurements of physicochemical properties of GIIs, viscosity, molecular weight, osmotic pressure, and electrometric titration, were carried out. From these results, the muco-complex, GIIs, was established to be a mucopolysaccharide-like substance having a molecular weight of nearly 100,000.

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