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Studies on Polysaccharides from *Serratia marcescens* I. Isolation and Characterization of Serratigen and Serratimannan

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A lipopolysaccharide, serratigen was isolated with weak acid extraction from the organisms of *Serratia marcescens*, and the purification and characterization were made. It had antigenic activity for rabbit antiserum and the total carbohydrate content was 75% and the molecular weight was 12600. A mannan was isolated from the same strain and had no antigenisity and the total carbohydrate content was 100% and the molecular was 10100.

Many studies have been made concerning polysaccharides and/or lipopolysaccharides from *Serratia marcescens*.[2] In the present paper we report the isolation and characterization of an antigenic lipopolysaccharide from *Serratia marcescens* red strain No. 51, which was newly isolated and characterized by M. Yamanaka, *et al.*[3]

Fractionation and purification were carried out following the antigenic activity for rabbit *anti-Serratia* serum by using the ring test and gel diffusion method.[4,5]

Organisms of *Serratia marcescens* were harvested after 24 hr cultivation on agar plates at 25°, and extracted with diluted acetic acid under refluxing at pH 3.5. The active component was precipitated with the addition of three volumes of ethanol after removing proteins by using the Sevag method. However, after inorganic salts were excluded with dialysis using cellophane tubes, the active component was no longer precipitated by ethanol addition. When a small amount of sodium chloride was added to the supernatant, it was precipitated and the white powder (SB) was obtained.

This white powder was dissolved in deionized water and the precipitates resulting from the addition of one volume ethanol were removed. Serratimannan was obtained by the addition of one more volume of ethanol and centrifugation. After removing the mannan the antigenic active component was precipitated by adding a small amount of sodium chloride solution to the supernatant. The salts were removed with dialysis against deionized water and after lyophilization the active component was subjected to an ion exchange column (Amberlite IRA 400, borate form), and was passed through with deionized water elution. The eluate was condensed to about one tenth volume under reduced pressure, dialysed and a purified active component was obtained by lyophilization of the non-dialysable solution, and yielded about 0.8% from the wet organism.

1) Location: *Tsukiji 5-chome, Chuo-ku, Tokyo.*

2) *a*) M.J. Shear and F.C. Turner, *J. Natl. Cancer Inst.*, **4**, 81 (1943); *b*) J.L. Hartwell, M.J. Shear and J.R. Adams, Jr., *ibid.*, **4**, 107 (1943); *c*) W.E. Q'malley, B. Achistein and M.J. Shear, *ibid.*, **29**, 1161 (1962); *d*) H.J. Creech, L.H. Koehler, H.F. Havas, R.M. Peck and J. Andre, *Cancer Res.*, **14**, 817 (1954); *e*) H.V. Srivastava and E. Breuninger, *J. Biochem. Physiol.*, **40**, 905 (1962); *f*) A. Nowothy, *Nature*, **210**, 278 (1966); *g*) G.A. Adams and R. Young, *Annals N.Y. Acad. Sci.*, **133**, 527 (1966); *h*) P. Alaupovic, A.C. Olson and J. Tsang, *Annals N.Y. Acad. Sci.*, **133**, 546 (1966); *i*) D. Tripodi and A. Nowothy, *Annals N.Y. Acad. Sci.*, **133**, 604 (1966); *j*) G.A. Adams and S.M. Martin, *Canad. J. Biochem.*, **45**, 477 (1967); *k*) T. Kayama, *Bull. Yamaguchi Med. School*, **8**, 161 (1961).

3) M. Yamanaka, Y. Okuda, H. Kuroda and J.K. Chang, *Jinsen-Igaku*, **12**, 1 (1970).

4) E.A. Kabat and M.M. Mayer, *Experimental Immunochemistry*, **1961**, 72.

5) O. Ouchterlony, *Acta Path. Microbiol. Scand.*, **32**, 231 (1953).

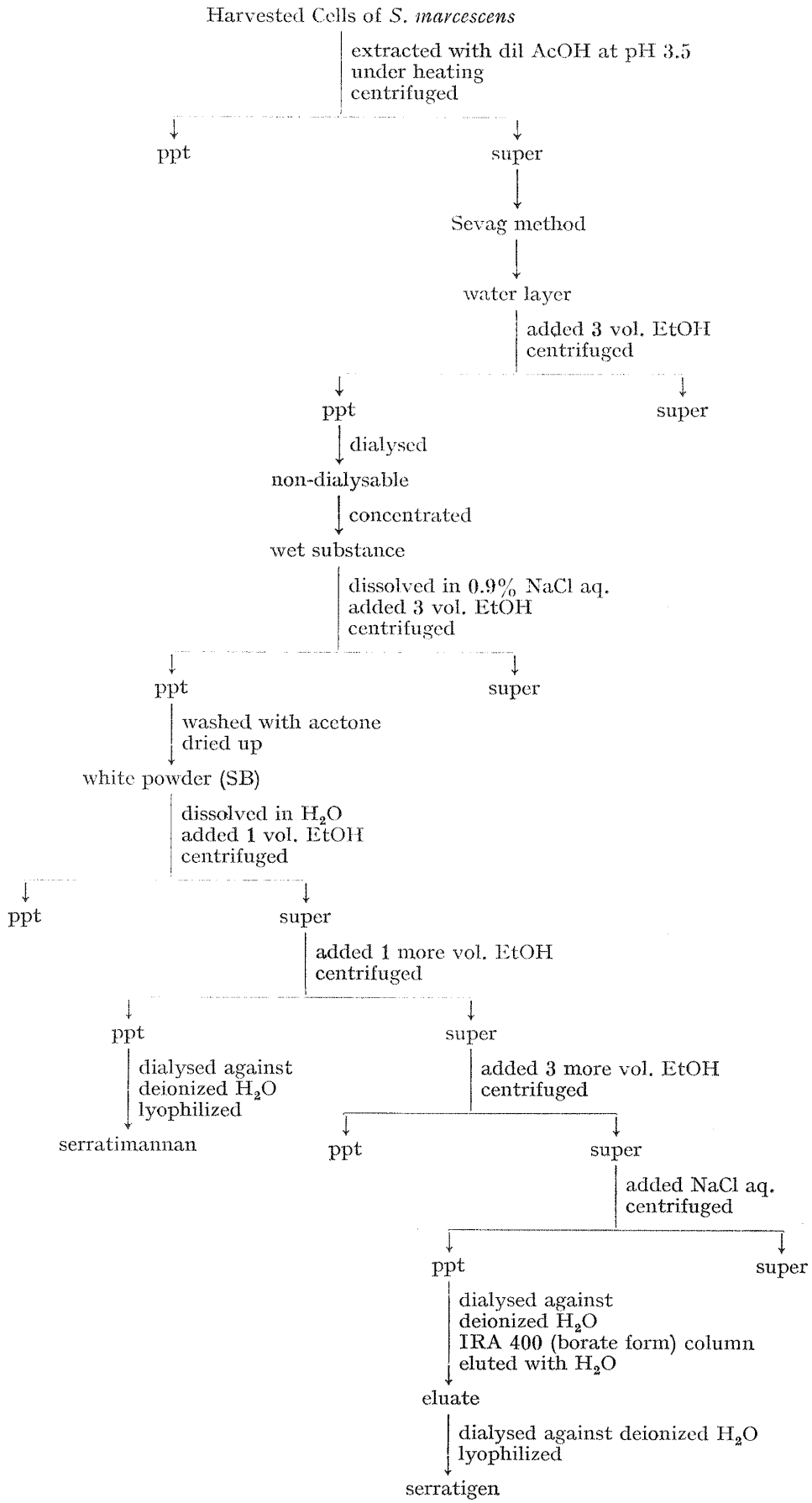

Chart 1. Isolation of Serratigen and Serratimannan

The antigen thus obtained (serratigen) gave a single spot by high voltage electrophoresis using glass fiber paper in 0.1M borate buffer. After development of the paper electrophoresis of SB and serratigen, the papers were placed in a 30 cm×10 cm plastic dish and a dissolved agar solution was poured into the dish so that a trench paralleled for the paper could be made and the antiserum was filled. In this experiment only one immunoprecipitin band was given corresponding with the spot of serratigen on the paper as shown in Fig. 1. For immunoelectrophoresis using the agar gel diffusion method, the white powder (SB) gave one precipitin band for rabbit antiserum of *S. marcescens*. An Ouchterlony gel diffusion test showed a completely fusing reaction between a precipitin line of the SB and that of serratigen.

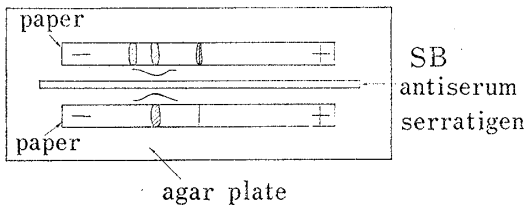

Fig. 1. Immunoelectrophoresis

high voltage electrophoresis
glass fiber paper (Whatman GF 83)
60 volt/cm, 20 min
0.1M borate buffer (pH 9.4)
rabbit antiserum

On the other hand the *anti*-tussive agent (SMC)[6] which is used against dogs coughing caused by *Dilofilaria immitis* infections was isolated from *S. marcescens* by M. Yamanaka, *et al.*[3,7] The mode of action of the *anti*-tussive agent might be due to an *anti*-allergic cross rinking reaction or desensitizing action against *D. immitis* antigen. Serratigen showed slightly positive Schwarzmann reaction on rabbit skins though SMC was positive in the reaction, and gave a completely fused precipitin reaction with SMC by the Ouchterlony gel diffusion method.[8] This seems to suggest it may be a principle of the *anti*-tussive agent.

Table I. Physico-chemical Properties

		Serratigen	Serratimannan
Elemental analysis	C	40.48 %	38.18 %
	H	6.64	6.92
	N	1.97	—
$[\alpha]_D^{22}$ (c=1.0, in H_2O)		+216°	+43°
Molecular weight[a]		12600	10100
Total carbohydrate content[b]		75.0 %	100.0 %

a) by osmotic pressure method
b) by phenol sulpuric acid method

Sedimentation patterns of serratigen in ultracentrifugal analysis showed one peak, and molecular weight was determined by the osmotic pressure method to be 12600. It gave $[\alpha]_D^{22}$ +216° (c=1.0 in H_2O) and had no ultraviolet (UV) absorption except end absorption. Optical rotatory dispersion (ORD) curve (positive plane curve) and infrared (IR) spectra suggested α-linkage. The nitrogen content by elemental analysis was 1.97% and the total carbohydrate content was determined by the phenol sulphuric acid method[9] to be 75.0%.

6) SMC is an antigenic polysaccharide preparation for veterinary use and now commercially available (Kanebo-Nakataki Pharm. Co., Ltd., Tokyo).
7) *a*) M. Yamanaka, Y. Okuda, H. Kushida and J.K. Chang, *J. Vet. Med.*, **478**, 971, **483**, 1270 (1968); *b*) H. Kimata and K. Sato, *ibid.*, **490**, 259 (1969); *c*) T. Iwahashi, S. Ishimura, S. Kuwahara, N. Sato, M. Yamamoto, N. Yamamoto, H. Michitsugi and Y. Okuda, *ibid.*, **498**, 749 (1969); *d*) M. Nonogaki, *ibid.*, **498**, 751 (1969); *e*) Y. Okuda, *ibid.*, **516**, 369 (1970), **527**, 969 (1970); *f*) T. Okuyama, *ibid.*, **522**, 707 (1970).
8) T. Ikekawa and Y. Ikeda, *Jap. J. vet. Sci.*, **35**, 269 (1973).
9) *a*) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956); *b*) *Idem*, *Nature*, **168**, 167 (1951).

The compound was shown to have lipid moiety in the molecule by nuclear magnetic resonance (NMR) measurement. The physical and chemical analysis demonstrated that it was different from any other polysaccharides of *Serratia marcescens*.

Serratimannan gave a single spot by high voltage electrophoresis and one peak in sedimentation patterns of ultracentrifugal analysis. Total carbohydrate content by the phenol sulphuric acid method[9] was 100%. Molecular weight was 10100 by the osmotic pressure method and $[\alpha]_D^{22}$ +43° (c= 1.0 in H_2O). ORD curve and IR spectra suggested α-linkage. In color reactions serratigen and serratimannan gave positive anthrone sulphuric acid and Molisch reactions, but they were negative in Ninhydrin, Biuret and iodine reactions. In Fehling and aniline-phthalate reactions serratigen was negative, but serratimannan was positive. It was different from any other polysaccharides of *S. marcescens*. Furthermore no precipitin band was observed at 200 mcg/ml of serratimannan using the ring test, but at the level of 1 mcg/ml of serratigen the precipitin band was obtained.

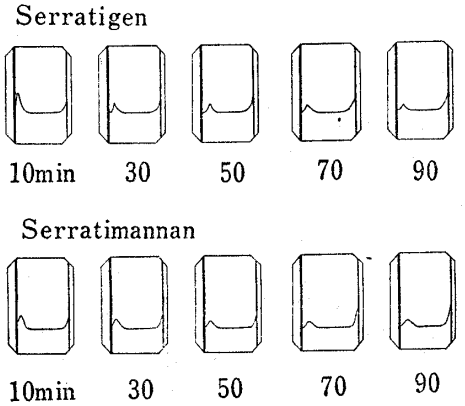

Fig. 2. Sedimentation Patterns in H_2O, at 30°, 44770 rpm

Experimental

The NMR spectra were obtained with a Japan Electron Optics Lab. JNM-3H-60, IR spectra with a Japan Spectroscopic Co. Model DS 402G Spectrophotometer and the UV spectra with a Cary Spectrophotometer Model 14. The specific rotations were measured with a Japan Spectroscopic Co. Model DIPS Polarimeter and the ORD curves with a Japan Spectroscopic Co. Model ORD/UV Spectrometer using H_2O as a solvent. Measurement of sedimentation patterns were made using a Spinco Model E analytical ultracentrifuge with a Schlieren optical system and H_2O as a solvent.

Isolation of Serratigen and Serratimannan——*Serratia marcescens* red strain No. 51 isolated by M. Yamanaka, *et al.*[3] was cultivated on agar plates containing 1.0% peptone, 1.0% meat extracts, 1.0% glycerine and 0.3% NaCl at 25° for 24 hr. The organisms were harvested and extracted with diluted acetic acid solution at pH 3.5 under refluxing. After removing the cells by centrifugation the extract was neutralized and evaporated *in vacuum*, and the proteins were removed with the Sevag method using $CHCl_3$. To the water layer three volumes of EtOH were added and the precipitate was collected with centrifuging and dialysed against tap water using cellophane tubes. The non-dialysable part was active in the immunological ring test and Ouchlerlony gel diffusion methods. After dialysis a few ml of NaCl aq. and three volumes of EtOH were added to the non-dialysable solution, and then the precipitate was isolated by centrifugation. Thus the crude active white powder (SB) was obtained (Yield was *ca.* 1.8% from the wet organisms).

SB (4 g) was dissolved in 300 ml of H_2O and one volume of EtOH was added to the solution and centrifuged at 10000 rpm for 10 min to remove a small amount of precipitate. To the supernatant one more volume of EtOH was added, and the precipitate was isolated by centrifuging. It was dialysed against deionized water and lyophilized, and serratimannan (457 mg) was obtained.

After removing inorganic salts antigenic active substances were no more precipitated, but when a few ml of NaCl solution was added to the supernatant solution, serratigen was precipitated and centrifuged at 10000 rpm for 10 min and dialysed against deionized water for 3 days. Then it was passed through a column of Amberlite IRA 400 (borate form, 5 cm×60 cm). The eluate was condensed and dialysed against tap water and deionized water for 3 days, respectively. Serratigen (1.596 g) was obtained by lyophilization of the non-dialysable solution.

High Voltage Paper Electrophoresis——High voltage paper electrophoresis was carried out using glass fiber paper (Whatman GF 83) in 0.1 M borate buffer (pH 9.45). It was developed at 60 volt/cm for 20 min and colored by spraying ammonium vanadate–H_2SO_4 reagent and then heated. *Rg* value of serratigen was 2.4 and that of serratimannan was 3.2, when development was done using glucose as a standard.

Immunoelectrophoresis——a) After development of high voltage electrophoresis using glass fiber paper (Whatman GF 83) in 0.1 M borate buffer (pH 9.4) at 60 volt/cm for 20 min, the paper was dried up and placed on the agar plate in a 30 cm×10 cm plastic dish. Beforehand a trench was made in the center of the dish and the dried paper was placed parallel to the trench, which was filled with the rabbit *anti-Serratia*

serum as shown in Fig. 2. Then it was left to stand for a few days, and the precipitin band could be observed if immunologically active.

b) The sample was dissolved in a Veronal buffer (pH 8.60) and dropped in spots on the agar plate. It was developed in the buffer at 100 volts for 2 hr, and the antiserum was poured into the trench and left to stand for a few days. In SB and serratigen only one precipitin arc was observed, respectively.

Immunoprecipitin Reaction——a) The ring test was applied to quantitative analysis for precipitin reaction. The minimum concentration value that would give a visible precipitin ring was determined with a rabbit antisera against *S. marcescens* as follows: Dilution of the sample (serratigen, serratimannan, SB *etc.*) was doubled in successive tubes and each sample was gently added to the tube containing 0.1 ml of the antisera along the wall, and left to stand for over 5 hr at room temp. The serological precipitin ring between the sample solution layer and the antisera layer could be found if it would have antigenic activity against the antisera. Serratigen showed a positive precipitin ring in less than 1 mcg/ml by this test, but serratimannan was negative in more than 200 mcg/ml.

b) Ouchterlony gel diffusion method was applied to the qualitative precipitin test. *Anti-Serratia* serum was poured into the central well and antigens (ex. SMC, serratigen, serratimannan) in the surrounding wells of the agar plate and left to stand at room temp. and diffused through the agar plate and encountered each other. Then white lines were observed at the zone of the optimal concentration between the antibody and the antigen. The active precipitin lines were observed in serratigen, SB and SMC. Further, when SMC and serratigen were used as antigens the precipitin bands made complete fusion, and this finding indicated the serological homology of these two polysaccharides.

Molecular Weight Determination——The osmotic pressure method was applied to determine the molecular weight. Water was used as a solvent. Serratigen was 10100 and serratimannan was 12600.

Total Carbohydrate Content[9]——The sample was dissolved in 2 ml of deionized water containing 0.05 ml of 80% phenol solution, and immediately 5 ml of conc. H_2SO_4 was added. After standing for 30 min, the optical density was measured at 490 mμ.

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