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Studies on Polysaccharides from *Serratia marcescens*. II. On the Structures of Serratigen and Serratimannan

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An antigenic lipopolysaccharide, serratigen isolated from *Serratia marcescens* was mainly made up of galactose and contained 18.4% of galacturonic acid. It had α-(1→3) galactan moiety in the molecule.

The chemical structure of serratimannan isolated from the same strain was shown to have α-(1→2) and α-(1→3) linkages in mannan.

In our previous paper isolation and characterization of serratigen and serratimannan from *Serratia marcescens* were reported.[2] Serratigen had antigenic activity for rabbit antiserum against *S. marcescens*, but serratimannan was not antigenic. From the immunological studies it might be suggested serratigen to be a purified component of the antitussive agent (SMC) for coughing caused by *Dilofilaria immitis* as previously reported.[2]

We report in this paper on the structures of serratigen and serratimannan. Sugar components of each polysaccharide were determined by liquid chromatography, gas chromatography and thin-layer chromatography. Liquid chromatography was applied for quantitative analyses for sugar components.[2-4] Total carbohydrate content of serratigen was 75% by phenol sulphuric acid method.[5]

It was demonstrated by liquid chromatographic analyses of the complete acid hydrolyzates that serratigen contained 94.9% of galactose and 4.2% of mannose when assumed total neutral carbohydrate content as 100%.

Table I. Neutral Sugar Components[a]

	Serratigen (%)	Serratimannan (%)
Galactose	94.9	1.3
Mannose	4.2	94.3
Glucose	—	3.2

a) The numerical values showed the calculated values from square intensity in liquid chromatograms on assumption of the total neutral carbohydrate content as 100.

Serratigen had a carbonyl band in infrared (IR) spectra at 1708 cm^{-1} but it disappeared by alkaline treatment and appeared by acid treatment again. Further fully methylated serratigen had an ester band at 1740 cm^{-1}, and therefore, it was suggested to have carboxylic acid groups in the molecule. Acidic sugars of the hydrolyzate of serratigen were analysed by liquid chromatography using strong basic resin.[3,4] Retention time of the hydrolyzate of serratigen was coincident with that of galacturonic acid in the liquid chromato-

1) Location: *5-chome, Tsukiji, Chuo-ku, Tokyo.*
2) *a*) T. Ikekawa and Y. Ikeda, *Chem. Pharm. Bull.* (Tokyo), **22**, 78 (1974); *b*) T. Ikekawa and Y. Ikeda, *Jap. J. Vet. Sci.*, **35**, 269 (1973).
3) Y. Yoshioka, T. Ikekawa, M. Noda and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **20**, 1175 (1972).
4) Y. Yoshioka, T. Sano and T. Ikekawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 1772 (1973).
5) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

graphy as shown in Fig. 1. Uronic acid content of serratigen estimated by carbazole reaction[6] was 18.4%.

Now we determined binding position of galactose in serratigen by fully methylation, following methanolysis and gas chromatographic analysis. Methylation of serratigen was repeated with Hakomori's method[7] until no hydroxide absorption was observed in IR spectra. Fully methylated serratigen thus obtained was hydrolyzed with 5% methanolic hydrochloride solution, and the hydrolyzate was analysed by GC and GC-Mass spectrometry using neopentylglycol succinate column. In gas chromatography relative retention times of the hydrolyzates were identical with those of the authentic sample of methyl 2,4,6-tri-O-methyl galactoside. Moreover, fragmentation of those peaks were identical with those of the authentic sample in GC-Mass analysis. As reported in our previous paper[2] serratigen was illustrated by IR spectra and optical rotatory dispersion (ORD) curve to have α-linkage. Thus it was demonstrated that serratigen had α-(1→3) galactan moiety in the molecule.

Total carbohydrate content of serratimannan which was isolated from the same strain was 100% by phenol sulphuric acid method.[54,] The sugar component of serratimannan was 94.3% of mannose and 3.2% of glucose by liquid chromatographic analysis. Furthermore, no carbonyl band was detected in IR spectral analysis and it gave negative carbazole reaction, and in liquid chromatographic analysis for acidic sugars[3,4] it contained no acidic sugars. It was fully methylated by repeating Hakomori's method,[7] and fully methylated serratimannan was hydrolyzed by the same procedure as described in serratigen. The hydrolyzate was analysed by GC and GC-Mass spectrometry. Fragmentation of the hydrolyzate in Mass

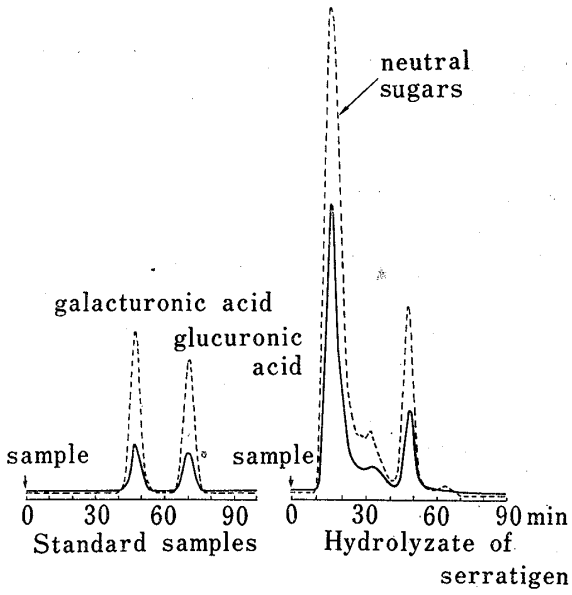

Fig. 1. Liquid Chromatogram of Acidic Sugar in Hydrolyzate of Serratigen

stationary phase: JEOL resin LC-R-3 (Cl^-)
mobile phase: 0.005M HCl
coloration: orcinol-sulphuric acid and measured the optical density at 423 mμ
——: X 1, ——: X 3

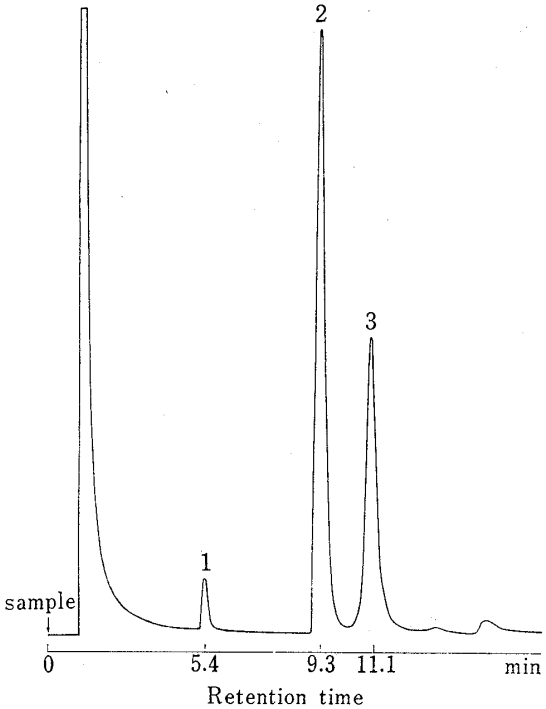

Fig. 2. Gas Chromatogram in Methylation Analysis of Serratimannan

1: methyl 2,3,4,6-tetra-O-methyl-D-mannoside
2: methyl 3,4,6-tri-O-methyl-D-mannoside
3: methyl 2,4,6-tri-O-methyl-D-mannoside
stationary phase: 1% neopentylglycol succinate
temp. of column: 130°
carrier gas: N_2 gas, He gas in GC-Mass spectrometry

6) T. Bitter and H.M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
7) S. Hakomori, *J. Biol. Chem.*, **55**, 205 (1964).

spectrometry showed *m/e* 205, 191, 187, 161 and so on, and this result illustrated that the compound was methylated at 6-position of mannose and one of 2, 3 or 4-position in mannose was not methylated.[8]

After fully methylated serratimannan was hydrolyzated by methanolysis, the methanolyzate and trimethylsilyl (TMS) derivatives of the methanolyzate were analysed by gas chromatography. It was shown that the relative retention times of the peaks were identical with those of the methanolyzate and TMS derivatives of fully methylated mannan isolated from *Saccharomyces cerevisiae*,[9] and the latter gave patterns identical with those of the methanolyzate of permethylated mannotetraose prepared from mannan of *S. cerevisiae*, which was reported by Lee, *et al.*[10] Therefore, serratimannan was shown to have (1→2) and (1→3) linkages in the molecule.

In our previous paper[2] serratimannan was suggested by IR spectra and ORD curve to have α-linkage, and it was supported by enzymatic hydrolysis of serratimannan with α-mannosidase. On the other hand, serratimannan consumed 0.6M of periodate per one anhydro sugar unit. Ratio of square intensity of peak 2 and 3 in Fig. 2 was approximately 3: 2. From these results it might be deduced that serratimannan was composed of α-(1→2) and α-(1→3) linkages in a ratio of 3: 2.

Experimental

General Method——IR spectra were made by a Japan Spectroscopic Co., Model DS 402G spectrophotometer, gas-liquid chromatography was performed with a Shimadzu Model GC 4A gas chromatograph attached a hydrogen flame detector, and liquid chromatographic analysis was carried out with a Japan Electron Optics Lab., liquid chromatograph Model JLC-3BC.

Complete Acid Hydrolysis——The sample (15 mg) was dissolved in 15 ml of 5% H_2SO_4 aq. and heated in a sealed tube for 5 hr. After complete acid hydrolysis it was neutralized by $BaCO_3$ solution. $BaSO_4$ was removed with filtration and the hydrolyzate was obtained by drying up.

Methylation by Hakomori's Method[7]——One hundred mg of the sample was dissolved in 20 ml of dimethylsulfoxide under vigorous stirring. A mixture of NaH (200 mg) and dimethylsulfoxide (5 ml) was refluxed at 65—70° for 45 min, and the solution was added to the sample solution and the mixture was stirred for 6 hr at room temperature. Methyl iodide (1.2 g) was added carefully and stirred at room temperature for 16 hr. Fully methylated serratigen gave $[\alpha]_D^{22}+173°$ ($c=0.25$, $CHCl_3$), and fully methylated serratimannan gave $[\alpha]_D^{22}+44°$ ($c=0.25$, $CHCl_3$).

Methanolysis of Fully Methylated Polysaccharides——Fully methylated serratigen and serratimannan was hydrolyzed with 5% methanolic hydrochloric acid solution in a sealed tube at 100° for 4 hr. The methanolyzate was analysed with gas chromatography using 5% or 1% neopentylglycol succinate column. The peaks on the gas chromatograms were subjected to GC-Mass analysis with GC-Mass spectroscope, Shimadzu-LKB 9000.

Methanolysis of Fully Methylated Mannan of *S. cerevisiae*——Fully methylated mannan isolated from *Saccharomyces cerevisiae*, which was kindly supplied by Dr. S. Suzuki, was carried out methanolysis by the conditions identical to fully methylated serratimannan as described above. The methanolyzate was analysed with gas chromatography comparing with the methanolyzate of fully methylated serratimannan. The methanolyzate of fully methylated serratimannan (*ca.* 1 mg) and that of fully methylated mannan of *S. cerevisiae* (*ca.* 1 mg) were dissolved in 0.1 ml of dried pyridine, respectively. Hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.1 ml) were added to the sample solution and heated at 60° for 15 min.[11] TMS derivatives thus obtained were chromatographed to 1% neopentylglycol succinate or 0.75% OV-17 column of gas chromatography.

Acidic Sugar Analysis——It was performed with liquid chromatography. Stationary phase was strong basic ion exchange resin, JEOL resin LC-R-3 (Chlorate form), and mobile phase, 0.005M HCl, and colored with orcinol-H_2SO_4 reagent and heating.[3,4] Quantitative analysis of acidic sugars was made with carbazole method,[6] and serratigen had 18.4% of acidic sugar content, using galacturonic acid as standard.

8) *a*) N.K. Kochetkov, N.S. Wulfson, O.S. Chizhov and B.M. Zolotarev, *Tetrahedron*, **19**, 2209 (1963); *b*) K. Heyns, K.R. Sperling and H.F. Grutzmacher, *Carbohydrate Res.*, **9**, 79 (1969).
9) H. Sunayama and S. Suzuki, *Jap. J. Microbiol.*, **14**, 197 (1970).
10) Y. Lee and C.E. Ballou, *Biochemistry*, **4**, 257 (1965).
11) C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

Enzymatic Hydrolysis of Serratimannan——To 4.5 ml of 0.05M citrate buffer (pH 6.0) of serratimannan (5 mg), the citrate buffer solution of α-mannosidase,[12] which was kindly supplied by Dr. H. Adachi, and NaCl (5.9 mg) were added, and the mixture was incubated at 25°. Samples were taken out at intervals for 48 hr to determine mannose content by Somogyi-Nelson method[13] using mannose as standard. At 3 hr 70% of serratimannan and more than 80% within 24 hr was hydrolyzed by the enzyme.

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12) T. Muramatsu, *Arch. Biochem. Biophys.*, **115**, 427 (1966).
13) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).