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Chemical Analysis of *Staphylococcus aureus*

II. Preparation and Chemical Analysis of the Antigenic Polysaccharides of *Staphylococcus aureus**

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Introduction

Since Jullianelle and Wieghard[1,2], numerous investigations on the chemical properties of specific polysaccharide of *Staphylococcus aureus* have been accumulating[3~9]. All of these results, however, seemed to be unsatisfactory with respect to the chemical analysis and evidence for homogeneity. Furthermore, there was no report on the comparative chemical analysis of somatic polysaccharides between both strains isolated from pathogenic and non-pathogenic origins.

The author extracted two polysaccharide preparations with hot formamide from *St. aureus* and analyzed their chemical compositions. In the present paper, the result of the comparative studies between the somatic polysaccharides of the strains isolated from pathogenic and non-pathogenic origins is described.

Materials and Methods

Bacterial Strains……Two strains of *Staphylococcus aureus*, No. 90 and No. 30, isolated in our laboratory were used. Strain No. 90 (P-strain) was isolated from a pathogenic origin (the head subcutaneous abscess of a patient) and No. 30 (N-strain) from a non-pathogenic origin (the forehead of a healthy person). Both strains are hemolytic, coagulase-positive, and show positive subcutaneous reaction in rabbits. Mannitol is fermented by P-strain, but not by N-strain. Although the biochemical properties of N-strain is closely similar to those of usual pathogenic strains, it is clearly distinguishable from P-strain in their serological properties by agglutination and absorption tests (Yamamoto, unpublished data).

Cultivation and Harvestion……The cultivation and harvestion were carried out as described in the previous report[10]. After several washings with ethyl ether-ethanol (3:1, v/v), dried white cell preparations of the both strains were obtained.

Extraction of Polysaccharide-I with Formamide……The cells were thoroughly ground in an agate mortar with repetitions of freeze-thawings until the complete disruption of the cell structure can be seen under the microscopy. To 15 g of the dried ground cells of P-strain, 150 mℓ. of formamide was added and the mixture was heated for 20 minutes at 150°C. After cooling, 400 mℓ. of acidified ethanol (to pH 4.5–5.0 by addition of HCl)

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was added and kept overnight in a refrigerator. The sediment was collected by centrifugation at 4,000 r.p.m. for 20 minutes after stirring for 1–2 hours and extracted twich with 350 mℓ. of 70% acidified ethanol (pH 4.5–5.0). The pooled extract which was very viscous and intensely red-brown colored was poured into 1,300 mℓ. of chilled acetone. Upon keeping the mixture in the cold room for 5 days, a white-gray muddy precipitate appeared, which was collected by centrifugation. After drying the precipitate *in vacuo*, a gray amorphous powder (F–4), polysaccharide-I (designated as Ps-I (P)), was obtained; yield being 1.65 g. While, Ps-I of N-strain (Ps-I (N)) was precipitated in a better yield by the addition of alkaline ethanol adjusted to pH 9.0–9.5 with NaOH rather than with the acidified one. Thus, 1.95 g of a dark-gray colored colored viscous Ps-I (N) was obtained from 15 g of dried ground N-strain cells.

Isolation of Polysaccharide-II……Clear yellow-orange supernatant (F–7) obtained after the removal of Ps-I (P) showed intense positive Molisch reaction and weakly positive biuret reaction. By the addition of 2.5 volumes of a mixture of acetone and ethanol (1:1, v/v) to F–7 which was concentrated until one sixth volume, a white cloudy sediment appeared. After keeping at least for a week in the cold room, precipitate was collected by centrifugation at 4,000 r.p.m. for 20 minutes, and washed with acetone, then twice with ethyl ether. Approximately 2.06 g of gray-white amorphous powder (F–5), polysaccharide-II preparation (designated as Ps-II (P)), was obtained. From a clear dark orange F–7 of N-strain, 2.11 g of Ps-II (N) was obtained by the addition of 3.5–5.0 volumes of the acetone-ethanol mixture.

Phenol-treatment of Polysaccharide-I……To the Ps-I solution in hot water (75°C), an equal volume of hot aqueous solution of 95% phenol (75°C) was added and extracted at 75°C for an hour with vigorous shaking[11]. The aqueous phase was dialyzed against distiled water for a week, poured into 3 volumes of acetone, and kept in the cold room for at least 5 days. Lyophilization of the aqueous solution of the sediments obtained by centrifugation gave a grayish white amorphous powder. The preparations extracted with aqueous phenol from Ps-I (P) and Ps-I (N) were designated as Ps-I (P)-PhOH and Ps-I (N)-PhOH, respectively, and their yields were only 0.51 and 0.56%.

Serological Properties and Antigenicity……Precipitation reactions were done between a series of ten-fold dilutions of saline solutions of Ps-I and Ps-II ranging from $1:10^3$ through $1:10^8$ and rabbit antiserum immunized with bacterial whole cells. Antigenicities of the Ps-I preparations were observed by their precipitin-forming abilities after intravenous injections into rabbit.

Chemical Properties……Molisch reaction was used for carbohydrates and biuret reaction for protein or peptide. Total N and P contents were estimated, respectively, by micro-Kjeldahl and Fiske-Subbarow methods. Carbohydrate content was measured by phenol-sulfuric acid method[12]. Dische's method[13] and orcinol test[14] were used for methylpentose and pentose contents, respectively. Protein content was measured by Lowry's procedure[15] using bovine serum albumin as standard. Paper electrophoresis was performed on Tōyō filter paper (4.5×20 cm.) in a M/100 Na-veronal/veronal buffer at pH 7.5, 8.2, and 8.8. After migration for 2 hours, the paper strips were stained with ninhydrin for amino acids or peptides, bromophenol blue for proteins, or Sudan black for

lipids. For detecting carbohydrates on the paper, alkaline potassium permanganate[16], β-naphthylamine[17], and fuchsin-periodate reagents[18] were used. The Ps-I and Ps-II preparations were dissolved in weak alkali, followed by neutralization, and their ultraviolet absorption spectra were measured. The ultraviolet absorption spectra of Ps-I-PhOH preparations were measured in their aqueous solutions. Sugar and amino acid compositions of the polysaccharide preparations were analyzed by paper chromatography. Twenty mg. of Ps-I was hydrolyzed with 2 mℓ. of *N* H_2SO_4 in a sealed tube at 100°C for 6 and 7 hours, whereas Ps-II for 3 and 7 hours. Hydrolysis of the Ps-I-PhOH preparation was carried out with *N* H_2SO_4 for 7 hours. Hydrolysis of Ps-I with *N* HCl for 8 hours was also performed. In order to know the amino acid compositions, Ps-II (P) and Ps-II (N) were hydrolyzed with *N* H_2SO_4 for 7 hours and with 6*N* HCl for 14 hours, then analyzed by two-dimensional paper chromatography in phenol and butanol solvent systems. Sugars were developed in phenol-water (4 : 1, v/v), *n*-butanol-acetic acid-water (9 : 1 : 7, v/v, upper phase)[19], or ethylacetate-acetic acid-water (3 : 1 : 3, v/v, upper phase). Two-dimensional developments were also performed[19]. Sugars on the paper were detected by spraying aniline phthalate. Ninhydrin and the Elson and Morgan reactions for amino sugars, naphthoresorcinol reaction for hexuronic acids, and diphenylamine reaction for ketoses[20] were also tested. For detecting amino acid spots on the paper, not only ninhydrin reaction, but also several specific reaction, such as isatin (proline and hydroxyproline), Sakaguchi's (arginine), sodium nitroprusside (arginine and lysine), periodate (hydroxy-amino acids), and hydrochloroplatinic acid reactions (S-containing amino acids), were applied[21].

Results

Chemical Properties……Chemical properties of four kinds of polysaccharide (Ps-I (P), Ps-I (N), Ps-II (P), and Ps-II (N)) are summarized in Table 1. It shows that all of these preparations are polysaccharide-protein complex and contain nucleic acid. And the protein

Table 1 *Chemical Properties of Polysaccharide Preparations Derived from Staphylococcus aureus*

Properties	Polysaccharide preparation			
	Ps-I (P)	Ps-II (P)	Ps-I (N)	Ps-II (N)
Yield from dried ground cells (%)	11.0	13.7	13.0	14.1
Molisch reaction	##	#	##	#
Biuret r.	+	#	#	##
Ninhydrin r*.	+	#	#	##
Bromophenol blue staining*	+	#	+	#
Sudan black staining*	±	±	±	±
Hexosamine*#	##	#	#	#
β-Naphthylamine r*.	##	#	#	#
Alkaline $KMnO_4$ r*.	+	±	+	±
Fuchsin-periodate r*.	##	#	##	#
N (%)	8.81	11.66	7.37	11.04

* Indicates the tests which were done on filter paper. # was tested after hycrolysis with HCl.

Table 2 *Chemical Properties of Polysaccharide Preparations Obtained After Phenol Extraction*

Properties	Ps-I (P)-PhOH	Ps-I (N)-PhOH
Yield from Ps-I preparation	0.51%	0.56%
Total carbohydrate	3.7	4.4
Protein	0.13	0.08
Pentose*	1.9	2.3
Methylpentose*	2.2	1.6
Heptose*[13]	<0.5	<0.5

* Per cent / total carbohydrate.

and/or peptide moieties in Ps-II preparations were remarkably richer than in Ps-I preparations. Phenol treatment of Ps-I preparations eliminated most of their protein moiety, yielding only small quantities of Ps-I-PhOH (approximately 0.5% of Ps-I) (see Table 2). Furthermore, the results in Table 2 indicate that main portions of the preparations were chemically far different from the ordinary Ps-I preparations. These results showed that the phenol treatment of the Ps-I preparations failed to further purify the staphylococcal Ps-I preparations.

Serological Properties and Antigenicity……Ps-I (P) and Ps-I (N) showed positive precipitation reaction with respective homologous rabbit antiserum at high dilution of $1:3\times10^5$ and $1:10^5$, whereas both of Ps-II (P) and Ps-II (N) showed almost negative reaction. The intravenous injection of Ps-I (P) into rabbit evoked antibody formation. This antiserum agglutinated homologous bacterial cells, and precipitated Ps-I (P) at $1:3\times10^5$ dilution. However, neither Ps-I (N) nor phenol-treated preparations (both Ps-I (P)-PhOH and Ps-I (N)-PhOH) was antigenic.

Paper Electrophoretic Patterns……The antigenic polysaccharide preparations, Ps-I (P) and Ps-I (N), showed a paper electrophoretic pattern identical with each other at three pH's tested. As shown in Fig. 1, main component of Ps-I migrated toward cathodic end, which responded positively to all the reactions with bromophenol blue, ninhydrin, alkaline potassium permanganate, and fuchsin-periodate, and weakly to β-naphthylamine reaction. At the origin a band which was stained with bromophenol blue was observed and a few bands, reacting very faintly with ninhydrin, were also observed on anodic side. While, Ps-I (P)-PhOH and Ps-I (N)-PhOH showed only one band under the same condition. This component showed a migration rate slower than Ps-I preparations and was stained with the reagents for carbohydrate detection.

Ultraviolet Absorption Spectra……Fig. 2 shows ultraviolet absorption spectra of all polysaccharide preparations, of which concentrations were 0.1% in Ps-I (P), 0.03% in Ps-II (P), 0.05% in Ps-I (P)-PhOH, 0.05% in Ps-I (N), 0.025% in Ps-II (N), and 0.5% in Ps-I (N)-PhOH. Presences of protein and nucleic acid moieties were observed in both Ps-I and Ps-II, more remarkably in Ps-II than in Ps-I. However, it was found that the Ps-I preparations after phenol treatment were nearly free from nucleic acid and/or protein. Actually, the optical densities at 260 mμ of their 0.03% aqueous solutions were 0.815 in

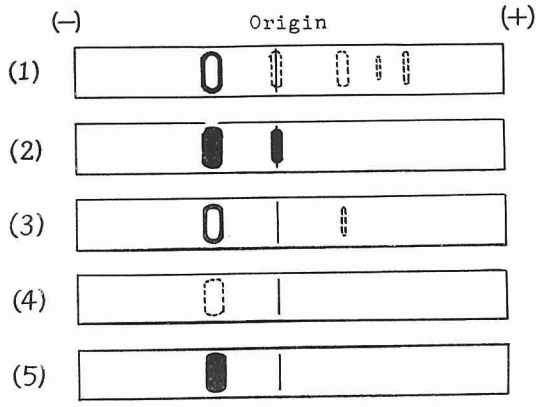

Fig. 1 Paper electrophoretic patterns of polysaccharide-I preparations derived from pathogenic and non-pathogenic strains of *Staphylococcus aureus*. On the condition for migration, see text. Staining: (1) ninhydrin; (2) bromophenol blue; (3) β-naphthylamine; (4) alkaline potassium permanganate; and (5) fuchsin-periodate.

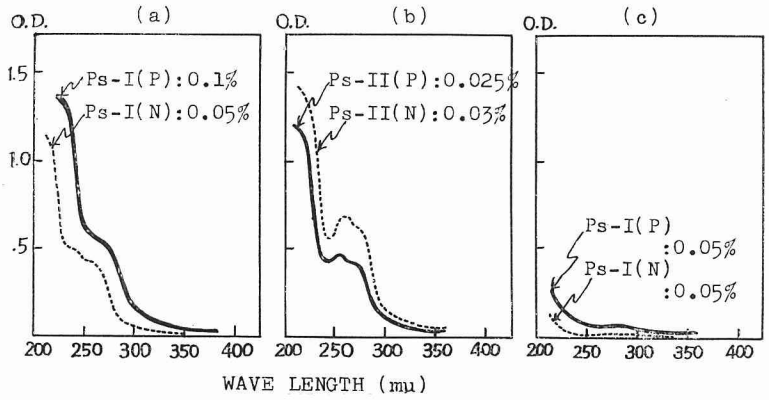

Fig. 2 Ultraviolet absorption spectra of the polysaccharide preparations derived from the pathogenic and non-pathogenic strains of *Staphylococcus aureus*. (C): Ps-I (P): Ps-I (P)-PhOH; Ps-I (N): Ps-I (N)-PhOH

Ps-I (P), 0.230 in Ps-I (N), 0.315 in Ps-II (P), 0.806 in Ps-II (N), 0.048 in Ps-I (P)-PhOH and 0.012 in Ps-I (N)-PhOH.

Monosaccharide Compositions of the Polysaccharides……Paper chromatograms of the acid hydrolysate of each polysaccharide preparation are shown in Fig. 3 and their constituent sugars were summarized in Table 3. The paper chromatograms showed that Ps-I (P) was composed of glucosamine, glucose, galactose, and xylose, while Ps-I (N) was lacking xylose out of these four. When incompletely hydrolyzed, a spot (spot A) with a similar R_f value to mannose was also found (Ps-I (6P) and Ps-I (6N) in Fig. 3). Spot A developed a bleaching white spot with aniline phthalate and a blue purple spot with ninhydrin, but did not show positive Elson and Morgan reaction. However, on the paper chromatograms of Ps-I (7P) and Ps-I (7N) (hydrolysates of Ps-I (P) and Ps-I (N) with *N*

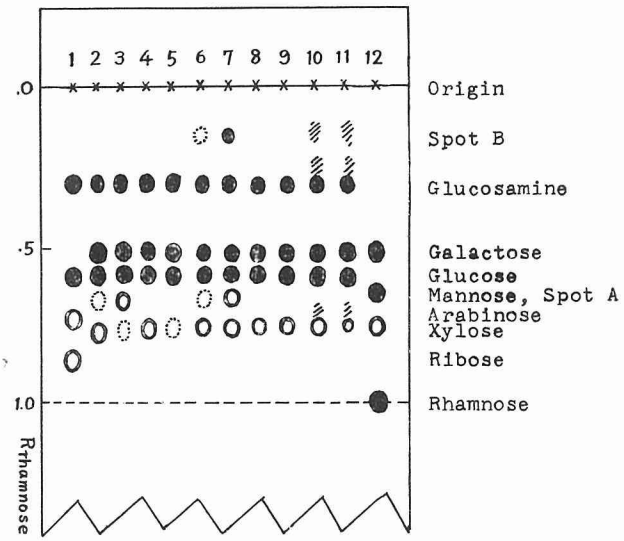

Fig. 3 Paper chromatograms of the acid hydrolysates of Ps-I (P), Ps-I (N), Ps-I (P)-PhOH and Ps-I (N)-PhOH preparations of *Staphylococcus aureus*. Ethylacetate-acetic acid-water (3:1:3, v/v, upper phase) was used as a solvent by the folded filter paper method for descending development[19]. 1: glucosamine+glucose+arabinose+ribose; 2: Ps-I (6P); 3: Ps-I (6N); 4: Ps-I (7P); 5: Ps-I (7N); 6: Ps-II (3P); 7: Ps-II (3N); 8: Ps-II (7P); 9: Ps-II (7N); 10: Ps-I (P)-PhOH; 11: Ps-I (N)-PhOH hydrolysates; and 12: galactose+mannose+xylose+rhamnose. Ps-I (6P) indicates a hydrolysate of Ps-I (P) for 6 hours, and others are same explanations.

Table 3 *Paper Chromatographic Patterns on the Sugar Components of Ps-I (P), Ps-I (N), Ps-II (P), Ps-II (N), Ps-I (P)-PhOH, and Ps-I (N)-PhOH Preparations Derived from Staphylococcus aureus*

Preparations	Hydrolyzed With	For	Sugar Components Detected* (NA)GlcN	Glc	Gal	Xyl	Rib	Spot A	Spot B
Ps-I (P)	*N* H_2S_4O	6 Hrs.	+	++	++	++	−	−	−
	"	7	++	++	++	++	−	−	−
	N HCl	8	++	++	++	++	−	−	−
Ps-I (N)	*N* H_2SO_4	6 Hrs.	+	++	++	±	−	−	−
	"	7	+	++	++	±	−	−	−
	N HCl	8	+	++	++	±	−	−	−
Ps-II (P)	*N* H_2SO_4	3 Hrs.	+	±	++	++	+	+	+
	"	7	+	+	+++	+++	±	−	−
Ps-II (N)	*N* H_2SO_4	3 Hrs.	+	+	++	++	±	++	++
	"	7	+	+	+++	+++	±	−	−
Ps-I (P)-PhOH	*N* H_2SO_4	7 Hrs.	+++	+++	+++	+++	±	−	−
Ps-I (N)-PhOH	*N* H_2SO_4	7 Hrs.	+++	+++	+++	+	±	−	−

* (NA)GlcN, Glc, Gal, Xyl, and Rib indicate (N-acetyl-) glucosamine, glucose, galactose, xylose, and ribose, respectively.

H_2SO_4 for 7 hours or with N HCl for 8 hours) no spot like A was found and instead a few ninhydrin-reactive spots appeared intensively. It is, therefore, suggested that spot A is a peptide. Two-dimensional paper chromatography revealed the same results on the sugar compositions.

On the other hand, no qualitative difference in the sugar components was fonud between Ps-II (P) and Ps-II (N), both of which contained glucosamine, glucose, galactose, and xylose. When Ps-II (N) was hydrolyzed with N H_2SO_4 for 3 hours (Ps-II (3N)), two peptide spots, A and B, were found in addition to these sugar spots. After prolonged hydrolysis, both spots disappeared (Ps-II (7P) and Ps-II (7N)). Although a faint spot of ribose was observed in Ps-II (3N), neither hexuronic acid nor ketose was detected by specific color reactions in any preparation. By the Elson and Morgan reaction on the paper, not only glucosamine but also trace of N-acetylglucosamine was detected. Two-dimensional paper chromatograms of Ps-II (7P) and Ps-II (7N) showed an identical sugar composition to Ps-I (7P). The phenol-treated Ps-I preparations contained (N-acetyl-) glucosamine, galactose, glucose, and xylose, however, the xylose content in Ps-I (N)-PhOH was strikingly lower than in Ps-I (P)-PhOH. These results were summarized in Table 3.

When Ps-II preparations were hydrolyzed with N H_2SO_4 for 7 hours, paper chromatographic separations of ninhydrin-reactive substances were complicated owing to their incomplete hydrolysis. Whereas, seventeen amino acids could be identified after the hydrolysis with 6N HCl for 14 hours (see Fig. 4). The applications of several kinds of specific color reactions on the paper confirmed the presences of proline, arginine, lysine, serine, threonine, and cystine. Furthermore, three unidentified spots with lower R_f values, one of which showed similar R_f value to α, ϵ-diaminopimelic acid, were also found. The other two unidentified spots were observed in a case of Ps-II (N), but not in Ps-II (P).

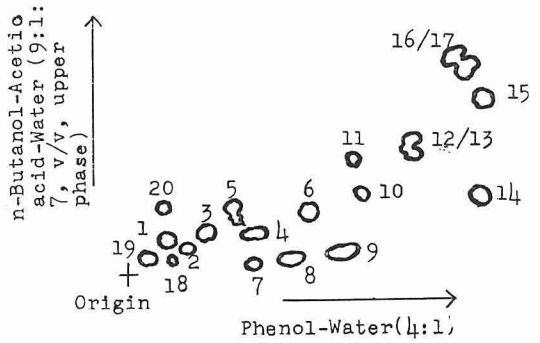

Fig. 4 Amino acid composition of the Ps-II preparation derived from pathogenic and non-pathogenic strains of *Staphylococcus aureus*. 1: Aspartic acid; 2: cystine; 3: glutamic acid; 4: serine; 5: glycine; 6: threonine; 7: lysine; 8: histidine; 9: arginine; 10: alanine; 11: tyrosine; 12/13: valine/methionine; 14: proline; 15: phenylalanine; 16/17: leucine/isoleucine; 18; 19; and 20: unidentified spots.

Discussions

Due to the chemical and serological complexities of the antigens of *St. aureus*, satisfactory results could not yet been obtained by any other authors on sugar compositions

of the antigenic polysaccharide[9,22].

Jullianelle and Wieghard[1,2] demonstrated the presences of carbohydrate A and B in *St. aureus* and *St. albus*, one of which is clearly different from the other in respect of the optical rotation and sugars liberated on hydrolysis.

A carbohydrate, similar to carbohydrate A, was extracted from *St. aureus* and purified by Verwey[23]. This substance which was group specific contained no pentoses. Inoue[24] had isolated a similar substance which liberated 28.5% reducing substance after hydrolysis and contained pentoses, but not glucosamine.

In 1943, Kawakami[4] identified acetylglucosamine, galactose, sulfate and phosphate (1:3:0.25:0.25 molar ratio) as the constituents of a somatic polysaccharide of a pathogenic *St. aureus*.

Fellowes and Routh[5] and recently Oeding[7,8] recommended a usage of hot formamide as the most satisfactory method for extraction of the specific polysaccharide. The preparation obtained from a pathogenic strain by Fellowes and Routh, which contained 6.5–8.0% N and was antigenic, yielded 20% reducing sugars (pentoses and glucosamine) after hydrolysis.

Using hot formamide I have has extracted a specific antigenic polysaccharide (Ps-I) from the both P- and N-strains of *St. aureus*. Ps-I (P) was precipitated from the formamide extract with acidified ethanol, whereas Ps-I (N) with alkaline ethanol. Similary, additions of 2.5 and 3.5–5.0 volumes of acetone-ethanol (1:1) were enough to precipitate the serologically inert polysaccharides, Ps-II (P) and Ps-II (N), respectively.

With regard to the qualitative reactions and ultraviolet absorption spectra, Ps-I and Ps-II of P-strain seemed to contain less amount of protein or peptides moieties than those of N-strain. But, N contents of the formers are nearly equal to the latters and there is no remarkable difference in their hexosamine contents. Therefore, further investigations should be carried out for characterization of the N-containing materials in Ps-II.

All of these polysaccharide preparations migrated toward cathodic direction and detected as a polysaccharide-protein complex on the paper electropherograms.

The protein moieties in Ps-II (P) and Ps-II (N) liberated the same amino acids after acid hydrolysis……these are 17 usual amino acids and a ninhydrin-reactive substance which has a R_f value similar to α, ϵ-diaminopimelic acid. In a case of Ps-II (N), but not in Ps-II (P), two additional spots were also detected. The amino acid composition is quite identical with that of the whole cell protein of the both strains[25].

In order to obtain protein-free lipopolysaccharide, the author treated the Ps-I with hot phenol[11]. However, only a few per cent was recovered after the phenol extraction and these were composed mainly of other than carbohydrate or protein. Therefore, the phenol extraction did not result in good recovery of the protein-free lipopolysaccharide at least from the Ps-I preparation.

In 1954, Oeding[7] isolated two carbohydrates by alcohol fractionation of the formamide extract, which did not contain free protein but still some N and indicated that the both carbohydrates contain pentoses. The author extracted an serologically inert polysaccharide (Ps-II) which are polysaccharide-protein complex in addition to the specific antigenic polysaccharide (Ps-I) from the both strains. It has been demonstrated by the

author also in cases of *salmonellae* (Sasaki, unpublished) and *Pasteurella pseudotuberculosis*[26] that a serologically inert polysaccharide could be isolated from the supernatant after recovery of an antigenic polysaccharide.

The specific polysaccharide of P-strain contained glucosamine, galactose, glucose, and xylose, whereas that of N-strain was lacking in xylose out of them. Also Yamamoto in our laboratory get same result in a preliminary experiment (unpublished data). In addition to glucosamine, a trace of acetylglucosamine was also detected simultaneously. As pointed out already by Kawakami[4], all or most of glucosamine might be in an acetylated form in the native somatic polysaccharides. The inert polysaccharides of the both strains showed identically above four sugars as their components.

The presences of pentoses in the specific polysaccharide preparation extracted from *St. aureus* were demonstrated by Inoue[24], Fellowes and Routh[5], or Oeding[7], while Verwey[23] and Kawakami[4] did not find out pentose in their preparations. The author indicated that a specific antigenic polysaccharide of P-strain contains pentose but not in that of N-strain, and identified it as xylose. Although it is not certain whether this definite difference between P- and N-strains can be generalized to the other cases of *St. aureus* or not, a presence of pentose (xylose) in the specific polysaccharide moiety of the former may be significant.

Davies (personal communication) have pointed out that xylose occurs in the antigenic polysaccharide preparations of young cultures when it is absent from older ones, and it was suggested that the presence of xylose might be susceptible to youth and/or vitality of the bacteria. With regard to these points, the fact that P-strain, vitality of which would reflect its serious biochemical activities for the host, contains xylose in its specific antigenic polysaccharide, whereas N-strain is lacking xylose in it would be very interesting and suggestive to consider the pathogenicity of *St. aureus*[27,28].

Summary

1. From two strains of *Staphylococcus aureus* which were isolated from pathogenic and non-pathogenic origins, two kinds of somatic polysaccharides were prepared by extraction with hot formamide, followed by alcohol fractionation. One was a specific antigenic polysaccharide and the other serologically inert.

2. By chemical analysis, paper electrophoresis and ultraviolet absorption spectroscopy it was indicated that these polysaccharide preparations are polysaccharide-protein complex. The serologically inert polysaccharide preparations were abundant in protein moiety.

3. The antigenic polysaccharide of the strain isolated from pathogenic origin was composed of (N-acetyl-) glucosamine, galactose, glucose, and xylose, whereas that from non-pathogenic origin lacked in xylose out of above four sugars. The inert polysaccharides of the both strains contained all of there four sugars.

4. Seventeen kinds of usual amino acids were identified in the protein moieties of the inert polysaccharide preparations from the both strains. This amino acid composition was precisely agree to that of whole cell proteins of these strains.

5. On the significations of that xylose is present in the antigenic polysaccharide preparation isolated from the strain of pathogenic origin, it was discussed making some references to the pathogenicity of *Staphylococcus aureus*.

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