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Occurrence of 2-Amino-2-deoxy-hexuronic Acids as Constituents of *Vibrio parahaemolyticus* K15 Antigen

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1. Acidic capsular polysaccharide of *Vibrio parahaemolyticus* A55 (05:K15) was esterified with ethylene oxide and then reduced with sodium borohydride. Hydrolysis of the reduced material gave two hexosamines identical with mannosamine (2-amino-2-deoxy-mannose) and gulosamine (2-amino-2-deoxy-gulose) on paper and ion-exchange chromatograms. This fact suggested that the K15 antigen contained mannosaminuronic acid (2-amino-2-deoxy-mannuronic acid) and gulosaminuronic acid (2-amino-2-deoxy-guluronic acid).

2. Direct acid hydrolysis of the antigen gave two 2-amino-2-deoxy-hexuronic acids. These two were identified as mannosaminuronic and gulosaminouronic acids by paper chromatography and paper electrophoresis, being compared with synthesized standard samples.

3. Titration of acid form of the polysaccharide indicated that about 91.5% of the polysaccharide was composed of the *N*-acetyl-hexosaminuronic acids.

Capsular K-antigen of *Vibrio parahaemolyticus* A55 (05:K15) was isolated by Omori *et al.* [1] and extensive studies on its purification and properties were also carried out by the same authors. Their chemical analyses of the antigen revealed that it contained reducing sugar 15–20%, hexosamine 17–22%, protein 0.4–1.7% and acetyl 14.5–15.7%. However, neither a known neutral sugar nor hexosamine has yet been detected in acid hydrolysates of the antigen. Moreover, despite its acidic nature no uronic acid, sialic acid or phosphoric acid was found in it. Then the chemical entity of the antigen has been left unknown.

An enzyme which was able to degrade the antigen was isolated from a culture of a microorganism originated from soil [2]. On the action of the enzyme the K-antigen liberated compounds which was acidic and positive to the Morgan-Elson reaction [3]. This fact suggested that the antigen might contain 2-acetamido-2-deoxy-hexuronic acid. As 2-amino-2-deoxy-hexuronic acids were reported to be very labile to acid treatment [4,5], it was attempted first to detect hexosamines (2-amino-2-deoxy-hexoses) after esterification, reduction and hydrolysis of the antigen, and then to isolate the constituent 2-amino-2-deoxy-hexuronic acids from an acid hydrolysate without esterification and reduction. This communication describes the results of the experiments.

Abbreviations. 2-Amino-2-deoxy-glucose, GlcN; 2-amino-2-deoxy-galactose, GalN; 2-amino-2-deoxy-mannose, ManN; 2-amino-2-deoxy-gulose, GulN; 2-amino-2-deoxy-glucuronic acid, GlcNUA; 2-amino-2-deoxy-mannuronic acid, ManNUA; 2-amino-2-deoxy-guluronic acid, GulNUA; arabinose, Ara; lyxose, Lyx; ribose, Rib; xylose, Xyl.

MATERIALS AND METHODS

K-Antigen

Preparation of K-antigen of *V. parahaemolyticus* A55 (05:K15) was previously described [2].

Sugars

D-Arabinose, D-ribose, D-lyxose, D-xylose, D-galactosamine hydrochloride, D-glucosamine hydrochloride, and D-mannosamine hydrochloride, used as reference substances, were commercial samples. Gulosamine was prepared by hydrolysis of benzyl 2-acetamido-2-deoxy-α-D-gulopyranoside (kindly supplied by Prof. P. Sinaÿ, Universite D'Orleans-Tours, France) with 2.5 M hydrochloric acid at 100 °C for 2 h. 2-Amino-2-deoxy-D-glucuronic acid was a generous gift from Prof. H. Paulsen (Univer-

sität Hamburg, Germany). 2-Amino-2-deoxy-D-mannuronic acid was prepared by acid hydrolysis of *Micrococcus lysodeikticus* acidic polysaccharide, which was kindly provided by Prof. Y. Matsushima of Osaka University [6], according to the method described by Biely and Jeanloz [7]. The mannosaminuronic acid sample showed a characteristic yellow-brown coloration on reaction with ninhydrin at 105 °C [4] and had R_{GlcN} value 0.49 (Mayer, 0.48 [8]) by paper chromatography and M_{GlcNUA} value 1.15 (Biely and Jeanloz, 1.16 [7]) by paper electrophoresis, respectively. 2-Amino-2-deoxy-D-guluronic acid was prepared as follows. Benzyl-2-acetamido-2-deoxy-α-D-gulopyranoside (95 mg) was dissolved in water (20 ml) containing sodium bicarbonate (25.6 mg) and oxidized at 75 °C by oxygen and platinum catalyst (50 mg) according to the method described by Heyns and Beck [9]. After the pH of the reaction mixture reached about 4.4, the catalyst was removed by filtration and centrifugation. The clear solution was concentrated to about 0.2 ml and mixed with ethanol (1.8 ml). The resulted precipitates were centrifuged down, washed with ethanol and dried (115 mg). The infrared data of this material showed the presence of ionized carboxyl: ν_{max}^{KBr} 1410 and 1550 cm^{-1}. The dried material (40 mg) was hydrolyzed by 1 M hydrochloric acid at 100 °C for 1 h and concentrated to dryness over P_2O_5 and NaOH *in vacuo*. The residue was dissolved in water and subjected to paper chromatography using solvent A. A material which appeared in the region of about R_{GlcN} value 0.43 gave yellow-brown spot with ninhydrin at 105 °C. The material in a preparative scale was eluted with water, treated with charcoal, then precipitated with excess of ethanol after concentration. This sample was used as 2-amino-2-deoxy-D-guluronic acid standard. R_{GlcN} and M_{GlcNUA} values of this material were 0.43 and 0.71, respectively.

Antisera

Anti K15 sera (No. 1 and No. 5) were prepared by immunizing rabbits with formalin-killed cells of *V. parahaemolyticus* A55 as described by Omori *et al.* [1].

Paper Chromatography

Toyo filter paper No. 51 A was used after washing with distilled water for 3 days by the descending technique. Ethyl acetate—pyridine—water—acetic acid (5:5:3:1, v/v/v/v, solvent A), phenol—water (9:1, v/v, solvent B), and *n*-butanol—benzene—pyridine—water (5:1:1.6:1, v/v/v/v, solvent C) were used as solvents. For color development silver nitrate-sodium hydroxide reagent [10] or 0.25% ninhydrin solution in *n*-butanol saturated with water was used. R_{GlcN} value was determined on the chromatograms with solvent A.

Paper Electrophoresis

Paper electrophoresis was carried out with horizontal type electrophoresis apparatus (Fuji-Riken Co. Ltd.) at 40 V/cm for 60 min at 2—8 °C, using 0.1 M sodium molybdate, which was adjusted to pH 5.0 with conc. H_2SO_4, as a buffer [11]. Paper and color reagent were the same as those of paper chromatography.

Ion-Exchange Chromatography

Dowex 50X8, 200—400 mesh (1 cm × 78 cm), and 0.3 M hydrochloric acid were used as described by Gardell [12]. Hexosamine was determined by Elson-Morgan reaction [13].

Infrared-Absorption Spectroscopy

Infrared absorption spectra of KBr tablets of samples were determined by Shimadzu infrared spectrophotometer model IR 27G.

Immunological Technique

Antigen-antibody precipitation reaction was carried out by double-diffusion technique [14].

RESULTS

Esterification and Reduction of K15-Antigen

The procedure was performed according to the method described by Mayer [8] as follows. K15-antigen (45 mg) was dissolved in water (9 ml) and acidified by passing through a column of Amberlite IR120(H) (1.4 g). The solution was treated with 30% ethylene oxide solution (1 ml) at room temperature for 2 days and an additional 1 ml was given every other day three times, until the pH of the solution rose to 5—6. After 8-days treatment the reaction mixture was dialyzed against water and lyophilized (28 mg).

This material (19 mg) was dissolved in water (9 ml) and glycerol (one drop) and sodium borohydride (30 mg) were added to the solution at 0 °C. After being kept at 0 °C for 2 days, the mixture was dialyzed against water and lyophilized (10 mg).

Infrared-Absorption Spectroscopy

Samples of starting, esterified and reduced materials were analyzed by infrared absorption spectroscopy. As shown in Fig. 1, major absorption of ester (at 1740 cm^{-1}), which was markedly increased after esterification, was greatly diminished by borohydride reduction.

Precipitin Reaction of Esterified and Reduced Materials

The modified samples were tested by the agar diffusion technique. As can be seen in Fig. 2, both

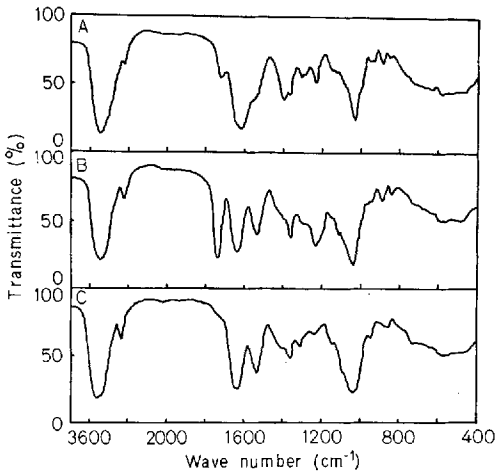

Fig. 1. *Infrared-absorption spectra of K15 antigen (A), its esterified (B) and reduced samples (C).* Spectrophotometry was carried out with about 1 mg samples in 200 mg KBr

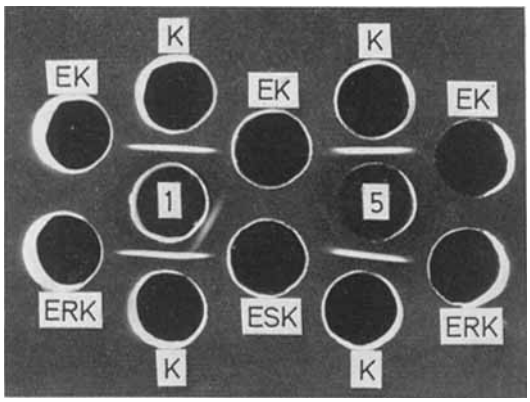

Fig. 2. *Precipitin reaction of K15 antigen and its derivatives with anti K15 sera.* K, EK and ERK are K15 antigen, esterified K15 antigen and reduced K15 antigen, respectively. ESK is NaOH-treated EK. 1 and 5 are rabbit anti K15 sera

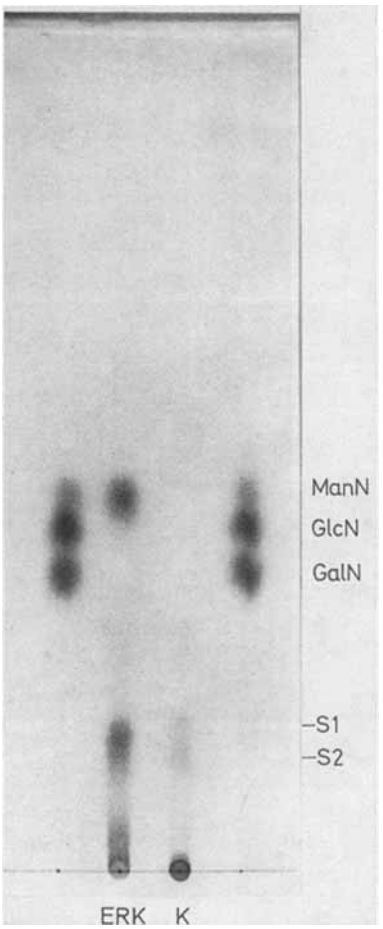

Fig. 3. *Paper chromatogram of hydrolysates of K15 antigen (K) and its reduced sample (ERK).* Chromatography was carried out by multiple ascending technique (3 times) with solvent A. Color was developed by ninhydrin. ManN, GlcN and GalN are references

esterified and reduced materials did not give any precipitin reaction with anti-K15 sera in agar. But on treatment with 0.2 M NaOH of the esterified material the ability to precipitate with the antisera was partly recovered. Direct treatment with borohydride of K15-antigen did not give any effect on the precipitin reaction.

The results obtained by infrared spectroscopy and precipitin reaction suggest that both reactions of esterification and reduction actually occurred.

Hydrolysis of Reduced Material

Reduced material (7 mg) was hydrolyzed with 4 M hydrochloric acid (1.4 ml) at 100 °C for 2 h in a sealed tube. After cooling the reaction mixture was concentrated to dryness over P_2O_5 and NaOH *in vacuo*.

The residue was dissolved in water and subjected to paper chromatography using solvent A. As shown in Fig. 3, a spot with R_F value corresponding to mannosamine was observed, although no spot with such an R_F value was found in the hydrolysate without reduction. A sample corresponding to the spot was obtained by preparative paper chromatography.

Identification of the Product Contained in the Hydrolysate

The sample purified by paper chromatography was subjected to Dowex 50 ion-exchange chromatography. As seen in Fig. 4, the sample was separated into two peaks (P1 and P2), corresponding to mannosamine and gulosamine, respectively. Paper chromatography with solvent A also showed that P1 and P2

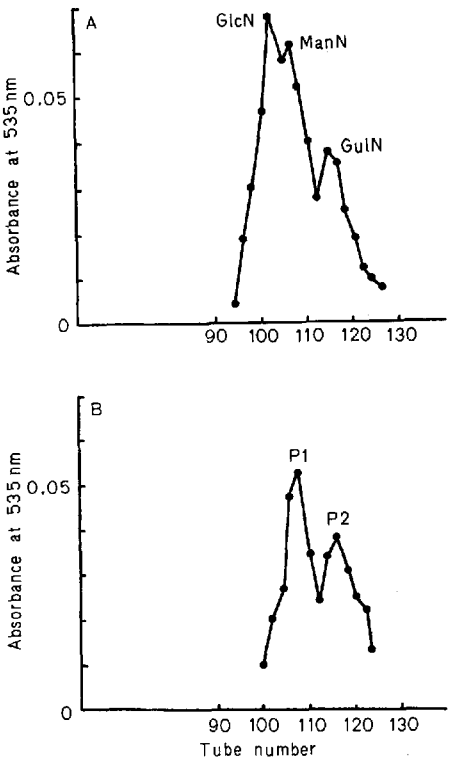

Fig. 4. *Dowex 50 ion-exchange chromatography of sample separated by paper chromatography.* (A) Reference experiment; (B) chromatogram of the sample separated from hydrolysate of reduced K15 antigen

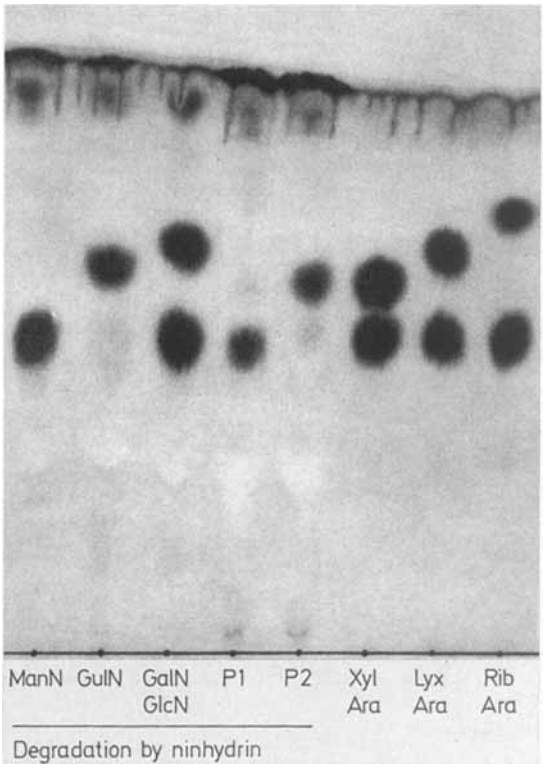

Fig. 5. *Paper chromatogram of ninhydrin-degraded products of fractions P1 and P2.* Paper chromatography was carried out by multiple ascending technique (twice) with solvent C. Color was developed by silver nitrate-sodium hydroxide method

were identical with mannosamine and gulosamine, respectively. P1 and P2 were each degraded by ninhydrin as described by Stoffyn and Jeanloz [15], and the degraded products were examined by paper chromatography using solvents A, B and C. As shown in Fig. 5, P1 and P2 gave arabinose and xylose, respectively. Control experiments with known hexosamines are also given in Fig. 5.

The fact that the reduced material contained both mannosamine and gulosamine suggests that the original antigen contained both mannosaminuronic and gulosaminuronic acids.

Isolation of Hexosaminuronic Acids from Hydrolysate of K15-Antigen

K15-antigen (50 mg) was hydrolyzed by 4 M hydrochloric acid at 100 °C for 2 h. The reaction mixture was concentrated to dryness over P_2O_5 and NaOH *in vacuo*. The residue was dissolved in water and subjected to paper chromatography with solvent A. Two spots, which corresponded to S1 and S2 in Fig. 3, were observed around the area with R_{GlcN} value 0.45→0.50. Since these spots partly overlapped, in the preparative paper chromatography paper was cut into upper, middle and lower parts and the materials were eluted with water. The material from the middle part was paper chromatographed again, two compounds, A and B, were obtained. Both A and B appeared first as yellow-brown spots on ninhydrin coloration at 105 °C and gradually turned to violet. R_{GlcN}, $M_{mannitol}$ and M_{GlcNUA} values of A and B are given in Table 1.

Table 1. *Characterization of compounds A and B by paper chromatography and electrophoresis*

Compound	R_{GlcN}	$M_{mannitol}$	M_{GlcNUA}
A	0.49	0.78	1.15
ManNUA	0.49	0.78	1.15
ManNUA (lit.)	0.48[a]	0.78[b]	1.16[c]
B	0.43	0.48	0.71
GulNUA	0.43	0.47	0.71

[a] Given by Mayer [8].
[b] Given by Mayer and Westphal [16].
[c] Given by Biely and Jeanloz [7].

Titration of Carboxyl Group

The acid form of the antigen (19.136 mg) was titrated with 54.5 mM NaOH and was shown to consume 80.7 μmol NaOH, indicating that content of *N*-acetyl-hexosaminuronic acid residue was about 91.5%.

DISCUSSION

Polysaccharides containing hexosaminuronic acid as microbial products have been isolated. Thus, glucosaminuronic acid was found in protective antigen of *Staphylococcus aureus* [5], type specific substance of *Haemophylus influenzae* type d [17], capsular polysaccharide of *Achromobacter georgiopolitanum* [18] and extracellular polysaccharide of black yeast NRRL Y-6272 [19], galactosaminuronic acid in Vi antigen of Enterobacteriaceae [20] and mannosaminuronic acid in acidic cell wall polysaccharide of *Micrococcus lysodeikticus* [4] and in K7 antigen of *Escherichia coli* [8].

In the present study capsular polysaccharide of *V. parahaemolyticus* A55 has been shown to contain mannosaminuronic acid and gulosaminuronic acid. To our knowledge this occurrence of gulosaminuronic acid is the first reported. From the result of titration, the K15-antigen seems to be composed almost solely of *N*-acetyl derivatives of these two hexosaminuronic acids. However, it is not clear at present whether both of the two sugars are in the same polysaccharide molecule or not.

Since it was established that the K15-antigen mainly contained *N*-acetyl-hexosaminuronic acid, it is assumed that the enzyme which was able to split the antigen and liberated acidic substances positive to Morgan-Elson may be an enzyme or enzymes capable of hydrolyzing glycosidic linkages of *N*-acetyl-hexosaminuronic acid. For this reason this enzyme must be different from Vi antigen-degrading enzyme [21] which was shown to have a pectate lyase activity [22].

An attempt was made to examine a cross-reaction between the K15-antigen an *N*-acetyl-mannosaminuronic-acid-containing polysaccharide of *M. lysodeikticus*. It was shown that the K15-antigen did not cross-react with rabbit anti *M. lysodeikticus* sera [23] and the polysaccharide of *M. lysodeikticus* did not cross-react with rabbit anti K15 sera either (M. Torii, unpublished). It was also shown that the K15 antigen-degrading enzyme [2] did not hydrolyze the *M. lysodeikticus* polysaccharide at all (K. Kuroda and S. Hase, personal communication).

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