A Glutamic Acid-Containing Mucopolysaccharide from a Marine *Pseudomonas*

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A marine strain of *Pseudomonas* (No. 42), which was originally isolated from seawater, produced an acidic mucopolysaccharide containing glutamic acid when grown on seawater agar medium containing sucrose. The polysaccharide was extracted with 1% phenol solution and purified by the addition of ethanol, followed by precipitation with Cetavlon and DEAE-cellulose chromatography. This polysaccharide preparation was homogeneous in both ultracentrifugal and electrophoretic analyses. This polysaccharide was composed of glucosamine, glucuronic acid, and glutamic acid.

Many microorganisms are known to produce mucopolysaccharides such as sugar moieties of lipopolysaccharides and cell wall polysaccharides. However, there are only a few reports on non-conjugated extracellular mucopolysaccharides produced by microorganisms.¹⁻⁵⁾

In the course of screening for extracellular polysaccharide-producing bacteria from marine environments, we isolated a strain, designated No. 42, which was identified as *Pseudomonas* sp. This bacterium was found to produce an extracellular acidic mucopolysaccharide containing glutamic acid. As an amino acid-containing polysaccharide we have already reported an alanine-containing polysaccharide from a marine pseudomonad.⁶⁾ It should be noted that some lipopolysaccharides also contain lysine and *N*-(2-hydroxyethyl)-D-alanine.^{6,7)}

This paper describes the isolation and characterization of the glutamic acid-containing mucopolysaccharide elaborated by a marine bacterium strain No. 42, together with studies of the identification of the bacterium.

Materials and Methods

Bacterial Strain

A bacterial strain No. 42, was isolated from the seawater of the Seto Inland Sea, Japan. The seawater medium used for bacterial isolation and polysaccharide production contained 30 g of sucrose, 5 g of peptone, 1 g of yeast extract, and 15 g of agar per l of seawater. Taxonomic studies were carried out using the methods de-

scribed previously.⁸⁾

Isolation of a Polysaccharide

An actively growing culture was inoculated in 10 ml of the seawater medium (as above, but without agar) which was incubated for 24 h at 28°C with shaking. This 10 ml culture was then poured onto a seawater agar plate (18×26 cm). After incubation for 3 days at 25°C, mucoid growth was scraped from the agar surface using 1% phenol solution. After stirring the mucoid material vigorously, bacterial cells were removed by filtration through a Whatman GFF filter after addition of Celite 545. To the clear filtrate, 2 volumes of ethanol were added. The precipitated polysaccharide was collected by filtration, dissolved in water, and reprecipitated with 5% cetyltrimethylammonium bromide solution (Cetavlon).⁴⁾ The precipitates were collected by filtration and dissolved in 4 M NaCl solution, to which 2 volumes of ethanol were added. The precipitates thus obtained were dissolved in water and the resulting solution was dialyzed against deionized water, followed by freeze-drying.

Further fractionation and purification were achieved by chromatography on a DEAE-cellulose column (2.3×22.5 cm) using stepwise elution with 0.01 M phosphate buffer (pH 7.0) containing 0–2.0 M NaCl. Fractions were monitored by phenol-sulfuric acid method.⁹⁾ Sugar-containing fractions were combined and dialyzed against deionized water, followed by freeze-drying.

O-Deacetylation of the polysaccharide was

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achieved by treatment with 0.1 M NaOH solution for 4 h at 30°C, followed by dialysis and freeze-drying.

Analytical Methods

Cell morphology was observed by electron microscopy. Negatively stained preparations were examined with a Hitachi HU-12A electron microscope.

Optical rotations were measured with a JASCO J-20C spectropolarimeter. Ultracentrifugal analysis was performed with a Hitachi 282 analytical ultracentrifuge.

Electrophoresis was performed on cellulose acetate strips (Sartorius 11200, 57×145 mm) in 0.2 M calcium acetate buffer (pH 7.5) or in 0.05 M sodium borate buffer (pH 9.4). The strips were stained either with 0.1% Alcian Blue in 10% acetic acid⁽¹⁰⁾ or alkaline silver nitrate reagent.⁽¹¹⁾

The ¹H NMR spectrum was obtained with a JEOL JNM GSX 500 spectrometer (¹H 500 MHz) at 31°C. The sample was dissolved in D₂O containing DSS as the internal standard.

Paper chromatography was carried out by the descending method on Whatman No. 1 paper (for analytical use) or Whatman 3MM paper (for preparative use) with ethyl acetate-acetic acid-formic acid-water (18:3:1:4) as the solvent. Sugars were detected either with alkaline silver nitrate, *p*-anisidine hydrochloride, or ninhydrin reagents. Mobilities of sugar components are expressed relative to D-glucose (*R*_{g10}).

Sugars were analyzed with a Hitachi 655 HPLC equipped with an RI detector on a Wakopak WBT 130E column (Wako Pure Chemicals, 7.8×300 mm) using water as a mobile phase at 60°C at a flow rate of 0.5 ml/min. The average molecular weight of the polysaccharide was determined with a Hitachi 655 HPLC equipped with an RI detector on an Asahipak GFA 7 M column (Asahi Chemicals, 7.6×500 mm). For minimizing the association effect of the polysaccharide solution, aqueous 0.1 M NaCl solution was used as a mobile phase at 30°C and at a flow rate of 0.4 ml/min. A Shodex Standard Kit P-82 (Showa Denko, *M*. W. = 0.58×10⁴–85×10⁴) was used for calibration of the molecular size.

Component Analyses

For the analyses of components, the polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 12 h at 100°C in a sealed glass

tube. After evaporation to dryness, the hydrolyzates were analyzed by paper chromatography, electrophoresis and HPLC. For the analyses of amino acids and amino sugars, the polysaccharide was hydrolyzed with 4 N HCl for 12 h at 100°C, followed by concentration to dryness over P₂O₅ and NaOH in vacuo. The residue was analyzed by paper chromatography, electrophoresis, HPLC, and with a Hitachi L-8500 amino acid analyzer.

Sulfate contents were determined with a Shimadzu HIC-6A ion chromatograph using a Shimpack IC-A1 column (4.6×100 mm) and a conductivity detector using 2.5 mM phthalic acid containing 2.4 mM Tris (hydroxymethyl) aminomethane (pH 4.0) at 40°C after hydrolysis of the polysaccharide with 2 M TFA for 12 h at 100°C.

Uronic acid contents were determined by the carbazole-sulfuric acid method.⁽¹²⁾ Protein contents were determined by the method of Lowry *et al.*⁽¹³⁾ Nucleic acid contents were estimated from UV absorption at 260 nm. Pyruvate was determined by ion chromatography as follows: the polysaccharide was hydrolyzed with 0.05 N H₂SO₄ for 1 h at 100°C, and the hydrolyzates were analyzed on a Shimpack IC-101H column (Shimadzu, 7.9×300 mm) at 50°C with 5 mM perchloric acid as a mobile phase using a Shimadzu HIC-6A ion chromatograph equipped with a UV detector (at 210 nm).

Periodate Oxidation and Smith Degradation

A solution of the polysaccharide (50 mg) in deionized water (25 ml) was oxidized with 0.02 M sodium metaperiodate (25 ml) for 3 days at 5°C in the dark. After the excess of periodate was decomposed with ethylene glycol (12.5 ml), the oxidized polymer was reduced with sodium borohydride (250 mg) overnight at room temperature. After the excess of borohydride was decomposed by acidifying with acetic acid, the product was dialyzed against deionized water, followed by freeze-drying (40 mg). Smith hydrolysis of the periodate oxidized and reduced polymer (20 mg) was effected with 0.5 M TFA for 12 h at 25°C to yield the polyol.

Results and Discussion

Strain No. 42 is a Gram negative straight rod (0.5–0.6×1.8–2.6 μm) as shown in Fig. 1. It is asporogenous, motile by means of a polar flagellum, and aerobic. It grows on OF medium

Fig. 1. Electron micrograph of bacterial strain No. 42.

oxidatively and produces oxidase. These features together with biochemical characteristics listed in Table 1 were reminiscent of *Pseudomonas* according to general bacteriology manuals.^{14,15)} The biochemical profiles did not fit well with any of the described species.¹⁶⁻¹⁸⁾ Since this isolate grows well on a medium prepared with seawater and can grow at 7% NaCl, we consider this isolate to be a marine strain of *Pseudomonas*.

Preparation of the Polysaccharide

Purification of the polysaccharide by DEAE-cellulose column chromatography showed that most of the carbohydrate was eluted with 0.4 M NaCl solution (Table 2). The polysaccharide preparation thus obtained was used through this study.

The purified polysaccharide preparation was homogeneous in both ultracentrifugal (Fig. 2) and electrophoretic analyses. It was a colorless powder with specific optical rotation $[\alpha]_D^{20} = +5.0^\circ$ (c 0.3, water) and the approximate sedimentation coefficient of 1.8 S. The molecular weight was estimated to be 1.78×10^6 (Fig. 3A).

Component Analysis of the Polysaccharide

The ^1H NMR spectrum of the polysaccharide (Fig. 4) revealed methyl signals of *N*-acetylated groups at δ_H 2.11, 2.08, 2.06, and 2.00, indicating the presence of *N*-acetylated amino sugar residues.¹⁹⁻²¹⁾ The signals attributable to the methyl protons of *O*-acetyl groups were observed

Table 1. Characteristics of the isolate

Characteristics	Isolate
Indole production	—
Oxidase (Kovac's)	+
Gram stain	—
Spores	—
Motility (25°C)	+
Glucose OF medium	O
Lysine decarboxylase	—
Arginine dihydrolase	—
Deoxyribonuclease	+
Catalase	+
Hydrolysis of:	
Gelatin	—
Starch	+
Tween 80	—
Urea	—
Aesculin	—
β -Galactosidase	+
Nitrate reduction	—
Assimilation of:	
Glucose	+
Arabinose	—
Mannose	—
<i>N</i> -acetylglucosamine	—
Maltose	+
Caprate	+
Mannitol	+
Gluconate	+
Malate	+
Citrate	+
Adipate	—
Phenylacetate	—
Growth at:	
25°C	+
37°C	—
Growth in:	
7% NaCl	+
Pigment production in King's B medium	—
Sensitivity to:	
Penicillin	—
Streptomycin	+
Chloramphenicol	+
Tetracycline	—
Novobiocin	—
Polymyxin B	+

+, Positive reaction; —, negative reaction.

at δ_H 2.23 and 2.17.¹⁹⁾ After *O*-deacetylation, the disappearance of these signals was observed. No ^1H signal corresponding to carboxyethylidene substituent was observed.²²⁾

After acid hydrolysis of the polysaccharide an amino sugar ($R_{\text{glc}}=0.77$), uronic acid ($R_{\text{glc}}=0.92$), and an amino acid were observed by paper chromatography. The amino sugar was identified

Table 2. Fractionation of the polysaccharide by DEAE-cellulose column chromatography*

NaCl conc. (M)	Product (mg)
0	0
0.2	3.8
0.3	51.4
0.4	434.5
0.6	69.0
0.8	12.6
1.0	5.0
2.0	3.9
Total	580.2

* The crude polysaccharide preparation (721.3 mg) was applied to DEAE-cellulose column as described in the text.

Fig. 2. The sedimentation pattern of the polysaccharide. Measurement was made in 0.1 M NaCl solution (2.5 mg/ml) at 25°C. Photograph was taken 40 min after the maximum speed (60,000 rpm) was obtained. The direction of sedimentation was from right to left.

as glucosamine by electrophoresis, HPLC (Fig. 5), and amino acid analyzer. The amino acid was identified as glutamic acid by amino acid analyzer. The glutamic acid liberated by acid hydrolysis of the polysaccharide and the authentic L-glutamic acid were destroyed by the enzyme L-glutamic acid dehydrogenase, whereas the authentic D-glutamic acid was unchanged. This indicates that the glutamic acid in the hydrolyzate has the L-absolute configuration.

Oligomer shown in the HPLC of the hydrolyzate of the polysaccharide (Fig. 5) was separated on an Amberlite IR 410 resin (formate). The acidic fraction obtained was homogeneous in electrophoresis and gave glucuronic acid and glucosamine on hydrolysis to a molar ratio of 1:1. Thus, the oligomer is an aldobiouronic acid with

Fig. 3. GPC profiles of the polysaccharide. Molecular weights of the polysaccharide were calculated as described in the text. A, native polysaccharide; B, oxidation/reduction product; C, Smith hydrolysis product.

the structure of GlcUA-GlcN. The uronic acid was separated on an Amberlite IR-410 resin (OH⁻ form) from the acid hydrolyzate of the polysaccharide, followed by purification by preparative paper chromatography. Carboxyl reduction of this product gave glucose, indicating the presence of glucuronic acid in the polysaccharide. The glucose derived from glucuronic acid after carboxyl reduction was destroyed by D-glucose oxidase, indicating the D-configuration of the glucuronic acid.

The polysaccharide was hydrolyzed with various concentrations of HCl, and the hydrolyzates were

Fig. 4. ^1H NMR spectrum of the polysaccharide in D_2O .

Fig. 5. Elution profiles of the hydrolyzates on a Wakopak WBT 130E column. The hydrolyzates and the authentic amino sugars were subjected to acetylation with pyridine-acetic anhydride, and then injected to HPLC. The peak with Rt 10.1 was collected to yield the purified product of the oligomer. GlcN, glucosamine; GalN, galactosamine; Oligomer, oligosaccharide.

analyzed for glucosamine and glutamic acid (Fig. 6). The liberation of these components occurred at high acid concentrations of HCl. From the data obtained, hydrolysis with 4 N

Fig. 6. Liberation of glucosamine and glutamic acid on hydrolysis of the polysaccharide. Hydrolysis was carried out with various HCl concentrations for 12 h at 100°C . Components were expressed as μmol per 10 mg of the polysaccharide. GlcN, glucosamine; Glu, glutamic acid.

HCl for 12 h at 100°C was adopted to estimate contents of glucosamine and glutamic acid.

From our investigations, it was indicated that the viscous substance produced by a marine *Pseudomonas* strain No. 42 was composed of glucosamine, glucuronic acid and glutamic acid as the constituent, suggesting a structure analogous to that of hyaluronic acid²⁾ containing glutamic acid. Hyaluronic acid was first isolated from bovine vitreous humour and later from the human umbilical cord and other connective tissues.²⁾ Bacterial formation of hyaluronic acid was reported in cultures of *Streptococcus pyogenes*,¹⁾ *Pseudomonas aeruginosa*,²⁾ and other streptococcal strains.²³⁾ However, no amino acid-containing hyaluronic acid has been reported. These results suggest that the component of the present polysaccharide differs markedly from that of the alanine-containing mucopolysaccharide elaborated by marine *Pseudomonas*.⁴⁾

This polysaccharide preparation did not contain nucleic acids, proteins, pyruvic acids, or sulfates.

Periodate Oxidation

After being treated with periodate followed by NaBH_4 , the polysaccharide had a molecular weight of 4.68×10^4 (Fig. 3B). This differed greatly from that of the intact polysaccharide. The polymeric material remained after the Smith hydrolysis of the polysaccharide was found to be homogeneous according to electrophoresis, and its molecular weight was estimated to be 2.06×10^4 (Fig. 3C). This indicates that the polysaccharide was oxidized by periodate but the main chain of the polysaccharide was resistant

to the periodate.

Further studies of the structure of this polysaccharide are in progress using the techniques of methylation, partial hydrolysis, and NMR spectrometry.

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