The Structure of an Extracellular Mucopolysaccharide Produced by a Marine *Pseudomonas*

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(Received February 17, 1992)

The structure of a highly viscous extracellular mucopolysaccharide produced by a marine bacteria designated strain No. 9-12 which was identified as an oxidase negative *Pseudomonas* was characterized by means of partial hydrolysis, periodate oxidation and selective cleavages of the polysaccharide chain. The polysaccharide has a branched structure with a backbone chain of -GalUA-GlcNA-GalNAc- and a side chain of GalUA-Gal-GalNAc-. The terminal galacturonic acid residue in the side chain was linked to D-alanine via an amide linkage.

In 1977, one of the present authors (K. O.) reported the isolation of an antitumor mucopolysaccharide-producing bacterium from seawater.¹⁾ A characteristic feature of this bacterium was the production of a highly viscous extracellular mucopolysaccharide,²⁾ which was composed of galacturonic acid, galactose, glucosamine, galactosamine, alanine, and pyruvate, together with *N*-acetyl groups.³⁾

In this paper we describe further studies of the structure of this extracellular mucopolysaccharide as well as characterization of the bacterium.

Materials and Methods

Bacterial Taxonomy

Taxonomic studies were carried out according to the methods described by Baumann *et al.*⁴⁾ and by the method previously described.⁵⁾

Analytical Methods

The general methods and methods of isolation and purification of the extracellular mucopolysaccharide from *Pseudomonas* strain No. 9-12 have been described previously.^{2,3,6)}

Electron Microscopy

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

NMR Spectroscopy

The 500 MHz (¹H) and 125 MHz (¹³C) NMR spectra were obtained with a JEOL JNM GSX

500 spectrometer at 75°C.

Selective Cleavages of the Polysaccharide Chain

Methanolysis of the Polysaccharide: Methanolysis of the polysaccharide was performed with an acidic dimethylsulfoxide (DMSO) solution by the method of Takada *et al.*⁷⁾ with some modifications. The polysaccharide (100 mg) was dissolved in DMSO (27 ml), followed by addition of methanol (3 ml) and H₂SO₄ (0.5 ml). After refluxing for 3 days at 70°C, reaction was terminated by neutralization with 6 *N* NH₄OH.

The reaction mixture was freed from methanol, applied to a charcoal-celite (1 : 1) column (2.5 × 15 cm), and eluted in steps with 1 l each of water, 5, 10, and 15% ethanol at a flow rate of 5 ml/h. Fractions were then separated by chromatography on a Bio-Gel P 2 column (2.5 × 90 cm) with water : pyridine : acetic acid (500 : 5 : 2) at a flow rate of 5.5 ml/30 min. Eluates were assayed by the phenol-sulfuric acid method,⁸⁾ and carbohydrate-containing fractions were lyophilized. Oligosaccharides which were obtained by a Bio-Gel P 2 column were further fractionated by HPLC or Amberlite IR-410 resin. HPLC was performed on a Wakopak WBT 130 E column (7.8 × 300 mm) by using RI detector and water as a mobile phase at 60°C and a flow rate of 0.5 ml/min.

Partial Acid Hydrolysis: The polysaccharide (100 mg) was hydrolyzed with 2 *M* TFA (20 ml) for 12 h at 100°C. The reaction mixture was repeatedly evaporated with water under diminished pressure, and passed through a column of Amberlite IR-410 resin (formate form). Acidic sugars

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were eluted from the resin with 10% formic acid, followed by fractionation on a Bio-Gel P 2 column.

Periodate Oxidation

Periodate oxidation and subsequent Smith hydrolysis were conducted as described previously.⁹⁾

N-Deacetylation

N-Deacetylation of the polysaccharide was accomplished with hot alkaline solution and the purified preparation was obtained from DEAE-cellulose column chromatography as described previously.³⁾ The product was partially N-deacetylated and was free of alanine and pyruvate.⁶⁾

Borohydride Reduction of Oligosaccharide

A portion of the oligosaccharide was treated with 5% methanolic hydrogen chloride, and the mixture was neutralized with silver carbonate. The reaction mixture was treated with sodium borohydride in anhydrous methanol to obtain reduction products of uronic acids.

Estimation of Molecular Weight

Average molecular weight was determined by HPLC by using GPC on an Asahipak GFA 7 M column (Asahi Chemicals, 7.6 × 500 mm) as described previously.²⁾ A Shodex Standard Kit P-82 (Showa Denko) was used for calibration of molecular size.

Results and Discussion

Figure 1 shows an electron micrograph of the

Fig. 1. Electron micrograph of *Pseudomonas* sp., strain No. 9-12 (×4,000).

Table 1. Characteristics of the strain

Characteristics	Isolate
Indole production	—
Voges Proskauer	—
Oxidase (Kovac's)	—
Gram stain	—
Spores	—
Motility (25°)	+
Glucose OF medium	O
Growth in reduced oxygen	Obligately aerobic
Hydrolysis of	
Gelatin	—
Casein	—
Starch	—
Urea	—
Tween 80	+
DNA	—
Aesculin	—
Lysine decarboxylase	(+)
Arginine dihydrolase	(+)
Ornithine decarboxylase	—
H ₂ S production	(+)
β-galactosidase	—
Nitrate reduction	—
Assimilation of	
D-glucose	+
D-Arabinose	—
L-Arabinose	—
D-Mannose	—
Mannitol	+
N-acetyl-D-glucosamine	—
Maltose	+
Gluconate	+
Caprate	+
Adipate	—
Malate	+
Citrate	+
Phenylacetate	—
Arginine	—
Betaine	—
Lactate	+
Acetate	—
Mol % G + C of DNA	58.3
Growth at	
37°C	+
41°C	—
Growth in	
0% NaCl	—
2% NaCl	+
10% NaCl	+
Sensitivity to	
O/129	—
Penicillin	—
Streptomycin	+
Chloramphenicol	+
Tetracycline	+
Novobiocin	+
Polymyxin	+
Pigment in King's B medium	Greenish yellow

+, Positive reaction; —, negative reaction; (+), weakly positive reaction.

marine bacterium strain No. 9-12. The characteristics of this strain are listed in Table 1. The isolate is a Gram negative straight rod ($0.6-0.8 \times 1.0-1.5 \mu\text{m}$); it is asporogenous, motile by means of a polar flagellum, and obligately aerobic. It grows oxidatively on an OF medium. It does not produce oxidase but produces a greenish yellow pigment on the surface of King's B medium prepared with seawater. Except for the oxidase reaction, these characteristics correspond with those of *Pseudomonas*, according to general bacteriology manuals.¹⁰⁾

Oxidase negative strains of *Pseudomonas* were listed among the aerobic marine eubacteria studied by Baumann *et al.*⁴⁾ However, the present isolate differs from strains studied by them in some important biochemical reactions such as the production of lipase and the assimilation of maltose, acetate, *N*-acetylglucosamine, *L*-arginine, and betaine. We consider that it is probably one of the oxidase-negative *Pseudomonas*, but it does not fit well with any of the described species. The fact that this isolate grows well on a medium prepared with seawater and can grow at 10% NaCl indicates that this isolate is a marine strain.

NMR Spectroscopy

The ^1H and ^{13}C NMR spectra of the polysaccharide revealed methyl signals at δ_{H} 2.06, 2.02, and 1.90, and at δ_{C} 24.6, 24.9, and 25.2, indicating the presence of *N*-acetylated amino sugar res-

idues¹¹⁻¹³⁾ (Fig. 2). The signal at δ_{C} 26.2 disappeared upon depyruvation, thereby suggesting the presence of acetal-linked pyruvic acid.^{14,15)} The signals at δ_{H} 1.45, δ_{C} 20.5, and 53.1 suggest the presence of alanine.¹⁶⁻¹⁸⁾ The signals at δ_{C} 52.5, 54.4, and 56.3 indicate the presence of carbons linked to nitrogen, suggesting the presence of amino sugars.¹⁸⁾ No signals attributable to *O*-acetyl groups were observed in the NMR spectra.¹¹⁾

The ^{13}C NMR spectrum showed anomeric signals at δ 105.4 ($^1J_{\text{CH}}$ 160.2 Hz), 105.2 ($^1J_{\text{CH}}$ 160.2 Hz), 104.4 ($^1J_{\text{CH}}$ 158.7 Hz), 101.4 ($^1J_{\text{CH}}$ 177.0 Hz), 99.1 ($^1J_{\text{CH}}$ 177.0 Hz), and 99.0 ($^1J_{\text{CH}}$ 175.2 Hz), and a C-2 of pyruvate at 110.3,¹⁹⁾ indicating that the polysaccharide has a regular structural feature of hexasaccharide-repeating units. The three of these with $^1J_{\text{CH}}$ values under 170 Hz can be assigned to β -linked glyco configurations and the remaining to α -linked glyco configurations.²⁰⁾ Each repeating unit contains pyruvic acid and the amino sugar residues are *N*-acetylated.

Selective Cleavages of the Polysaccharide

Methanolysis: Cleavages of the glycosidic linkage at the *N*-acetylated hexosamine residue were specifically performed by methanolysis in an acidic DMSO solution.⁷⁾ The methanolysis products were fractionated on charcoal-celite, followed by gel filtration on a Bio-Gel P 2.

Fraction M-1 shown in Fig. 3 was eluted in

Fig. 2. ^1H and ^{13}C NMR spectra of the polysaccharide in D_2O .

Fig. 3. Elution profiles of the methanolizates on a Bio-Gel P2 column after fractionation on a charcoal-celite column with ethanol concentrations of 5, 10, and 15%.

Fractions M-1 (Fr. No. 47-49) in Fig. 3A and M-3 (Fr. No. 46-51) in Fig. 3C which were obtained on a Bio-Gel P2 column were collected and freeze-dried and then isolated by HPLC on a Wakopak WBT 130E column. The peaks with Rt 10.5 and 11.1 min on HPLC were collected to yield purified products of fractions M-1 and M-3, respectively. Fraction M-2 (Fr. No. 42-45) in Fig. 3B was further separated by using Amberlite IR-410 resin (formate form) and the acidic fraction was collected to yield the purified fraction M-2.

the region characteristic of disaccharide on a Bio Gel P2, and further purified by HPLC on a Wakopak WBT 130 E. The peak with Rt 10.5 min was homogeneous in electrophoresis, and gave galacturonic acid and glucosamine on hydrolysis. This also gave galactose and glucosamine in the molar ratio of 1 : 1 on hydrolysis after reduction with sodium borohydride, thus suggesting that this disaccharide is GalUA-GlcNAc.

Eluate with 10% EtOH from a charcoal-celite column gave fraction M-2 in Bio-Gel P2 gel filtration, which corresponded to the region characteristic of trisaccharide (Fig. 3 B). It was further fractionated on Amberlite IR 410 resin (formate form) into acidic and neutral fractions. The acidic fraction was homogeneous in electrophoresis and gave galacturonic acid, galactose, and galactosamine upon hydrolysis. It also gave galactose and galactosamine in the

molar ratio of 2 : 1 on hydrolysis after carboxyl reduction by sodium borohydride, thus suggesting that this trisaccharide is GalUA-Gal-GalNAc.

Eluate with 15% EtOH from the charcoal-celite column gave fraction M-3 in a Bio-Gel P2 gel filtration (Fig. 3 C). It was purified by HPLC on a Wakopak WBT 130 E. The peak with Rt 11.1 min was homogeneous in electrophoresis and gave galactose and galactosamine in the molar ratio of 1 : 1 on hydrolysis, suggesting that this disaccharide is Gal-GalNAc.

Partial hydrolysis: As shown in Fig. 4, the acidic fraction of partial hydrolyzates gave 3 major peaks in gel filtration on Bio-Gel P2, of which fraction A-1 was homogeneous both in electrophoresis and GPC on Asahipak GFA 7 M. Its molecular weight was estimated to be 440. Hydrolysis of A-1 with 2 M TFA for 12 h at 100°C gave alanine, galacturonic acid, ga-

Fig. 4. Elution profile of the partial acid hydrolyzate on a Bio-Gel P2 column.

lactose, and a disaccharide. The disaccharide was purified by preparative paper chromatography ($R_{f10}=0.66$). The purified material gave galacturonic acid and galactose on hydrolysis. Thus, the disaccharide is an aldobiouronic acid, GalUA-Gal.

As described before, alanine is covalently attached to the carboxyl group of the uronic acid residue in this polysaccharide chain.³⁾ Thus, it is suggested that A-1 has the following structure:

Periodate Oxidation

The partially *N*-deacetylated polysaccharide was oxidized by periodate as described previous-

ly.⁶⁾ The major polymeric material (M.W.= 5.0×10^4) which remained after Smith hydrolysis was composed of equimolar amounts of galacturonic acid, glucosamine, and galactosamine.

The ^1H NMR spectrum of this product showed anomeric signals at δ 5.42 (1 H, unresolved) and 5.34 (2 H, unresolved) (Fig. 5). Similarly, the ^{13}C NMR spectrum showed three anomeric signals at δ 104.1, 103.1, and 101.0.

Signals at δ_{H} 2.01 and 1.94, and at δ_{C} 23.3 and 23.0 indicate the presence of *N*-acetyl groups.¹¹⁻¹³⁾ Signals at δ_{C} 54.9 and 52.0 indicate the presence of the carbons linked to nitrogen, probably amino groups of amino sugars.¹³⁾ Signals at δ_{C} 176.0, 175.3, and 175.1 are attributable to carbonyl groups, assuming one carboxyl and two acetyl groups.

These data suggest that the main chain of the polysaccharide which remained after Smith hydrolysis has a regular structural feature of trisaccharide repeating units, involving two amino sugars which are *N*-acetylated.

Combined results indicate that the product formed after the Smith hydrolysis has the following structure: -GalUA-GlcNAc-GalNAc-.

This polymer was resistant to the second periodate oxidation, suggesting that the galacturonic acid residue has a C-3 linkage, whereas the amino sugar residues have C-3 or C-4 linkages.

The product released from the main chain of the polysaccharide by the Smith hydrolysis gave galactose on hydrolysis, indicating that the galactose residue is resistant to periodate oxidation.

Fig. 5. ^1H and ^{13}C NMR spectra of the Smith degradation product of the partially *N*-deacetylated polysaccharide in D_2O .

Combined results with periodate oxidation and the selective cleavages of the polysaccharide by methanolysis and partial hydrolysis show that the polysaccharide has a branched structure containing a backbone chain of -GalUA-GlcNAc-GalNAc- and a side chain of GalUA-Gal-GalNAc-. The terminal galacturonic acid in the side chain linked a D-alanine by an amide linkage.⁶⁾

Further studies of the structure of this polysaccharide, including the linkage position of pyruvic acid to the sugar moiety, are in progress by using the techniques of methylation and NMR spectroscopy.

Acknowledgments

We thank Dr. T. Yoshikumi of the Kureha Chemical Co. for the NMR measurements. We are also grateful to Dr. E. Miki and Dr. T. Tokuda of Kagawa University for operating the electron microscope.

References

- 1) K. Okutani: A viscous antitumor substance obtained from a marine bacterium No. 9-12. *Nippon Suisan Gakkaishi*, **43**, 323-328 (1977).
- 2) S. Tandavanitj, E. Miki, and K. Okutani: The flow properties of an extracellular mucopolysaccharide produced by a marine strain of *Pseudomonas*. *Nippon Suisan Gakkaishi*, **56**, 1865-1869 (1990).
- 3) S. Tandavanitj, S. Ishida, and K. Okutani: Isolation and characterization of an extracellular mucopolysaccharide produced by a marine strain of *Pseudomonas*. *Nippon Suisan Gakkaishi*, **55**, 2015-2019 (1989).
- 4) L. Baumann, P. Baumann, M. Mandel, and R. D. Allen: Taxonomy of aerobic marine eubacteria. *J. Bacteriol.*, **110**, 402-429 (1972).
- 5) K. Okutani: Taxonomical studies of a polysaccharide-producing bacterium from sea cucumber, *Stichopus japonicus* (SELENKA). *Tech. Bull. Fac. Agr. Kagawa Univ.*, **36**, 135-140 (1985).
- 6) S. Tandavanitj and K. Okutani: Partial structure of an extracellular mucopolysaccharide produced by a marine bacterium. *Nippon Suisan Gakkaishi*, **56**, 2103-2107 (1990).
- 7) H. Takada, Y. Araki, and E. Ito: Structure of polygalactosamine produced by *Aspergillus parasiticus*. *J. Biochem.*, **89**, 1265-1274 (1981).
- 8) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith: Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**, 350-356 (1956).
- 9) S. Tandavanitj and K. Okutani: The structural investigation of a sulfated polysaccharide produced by a strain of marine *Pseudomonas*. *Nippon Suisan Gakkaishi*, **55**, 1845-1849 (1989).
- 10) D. J. Brenner: in "Bergey's Manual of Systematic Bacteriology" (ed. by N. R. Krieg and J. G. Holt) Vol. 1, Williams and Wilkins, London, 1984, pp. 140-406.
- 11) Y. A. Knirel, V. V. Dashunin, A. S. Shashkov, N. K. Kochetkov, B. A. Dmitriev, and I. L. Hofman: Somatic antigens of *Shigella*: structure of the O-specific polysaccharide chain of the *Shigella dysenteriae* type 7 lipopolysaccharide. *Carbohydr. Res.*, **179**, 51-60 (1988).
- 12) P. B. Helander, L. Kenne, B. Lindberg, K. Peterson, and R. Unger: Structural studies of two capsular polysaccharides elaborated by different strains of *Haemophilis influenzae* type e. *Carbohydr. Res.*, **88**, 77-84 (1981).
- 13) E. Katzenellenbogen, I. Ekiel, and E. Romanowska: The structure of the O-specific polysaccharide chain from *Citrobacter* O23 lipopolysaccharide. *Carbohydr. Res.*, **179**, 349-357 (1988).
- 14) P. J. Garegg, P. E. Jansson, B. Lindberg, F. Lindh, J. Lönngren, I. Kvarnstrom, and W. Nimich: Configuration of the acetal carbon atom of pyruvic acid acetals in some bacterial polysaccharides. *Carbohydr. Res.*, **78**, 127-132 (1980).
- 15) D. Oxley and S. G. Wilkinson: Structural studies of an acidic galactomannan from the reference strain for *Serratia marcescens* serogroup O4. *Carbohydr. Res.*, **179**, 341-348 (1988).
- 16) C. J. Pouchert: The Aldrich library of NMR spectra (ed. 2, Vol. 1), Aldrich Chemical, Milwaukee, 1983, pp. 485-486.
- 17) E. Pretsch, T. Clerc, J. Seibl, and W. Simmon: Tabulation of spectral data of organic compounds (translated into Japanese by K. Nakanish), Kodansha, Tokyo, 1982, pp. 95-97.
- 18) F. P. Tsui, R. Schneerson, R. A. Boykins, A. B. Karpas, and W. Egan: Structural and immunological studies of the *Haemophilis influenzae* type d capsular polysaccharide. *Carbohydr. Res.*, **97**, 293-306 (1981).
- 19) S. F. Osman and W. F. Fett: The structure of the acidic exopolysaccharide of *Pseudomonas marginalis* strains PF-05-2 and PM-LB-1. *Carbohydr. Res.*, **199**, 77-82 (1990).
- 20) E. Katzenellenbogen, I. Ekiel, and E. Romanowska: The structure of the O-specific polysaccharide chain from *Citrobacter* O23-lipopolysaccharide. *Carbohydr. Res.*, **179**, 349-357 (1988).