Structure of an acidic polysaccharide present in the bacteriolytic complex lysoamidase

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Received 18 May 1995

Abstract The structure of an acidic polysaccharide component of a bacteriolytic complex (lysoamidase), isolated from a bacterium of the genus *Xanthomonas*, was studied. On the basis of sugar analysis and one- and two-dimensional ^1H and ^{13}C NMR spectroscopic study of the initial polysaccharide and its *O*-deacetylated and carboxyl-reduced derivatives, the following structure of the trisaccharide repeating unit of the polysaccharide was established

→3)-β-D-Glc*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→3)-α-L-Gal*p*NAc-(1→
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OAc

where ManNAcA and GalNAcA are 2-acetamido-2-deoxymannuronic acid and 2-acetamido-2-deoxygalacturonic acid, respectively.

Key words: Lysoamidase; Bacteriolytic enzymatic complex; Bacterial polysaccharide; Polysaccharide structure; 2-Acetamido-2-deoxyhexuronic acid; *Xanthomonas*

1. Introduction

Lysoamidase is a bacteriolytic enzymatic preparation from the cultural fluid of a bacterium of the genus *Xanthomonas*, which was elaborated in the Institute of Biochemistry and Physiology of Microorganisms (Pushchino, Moscow Region). Lysoamidase is highly efficient in treatment of external infectious diseases caused by Gram-positive microflora, the therapeutic effect being mainly due to bacteriolytic enzymes [1]. A number of proteinases and phosphatases was also found in the enzymatic preparation [2].

The total amount of proteins in lysoamidase is low (~2% by mass), and the main component of the preparation is an acidic polysaccharide with the molecular mass about 1300 kDa. The activity of lysoamidase is significantly stabilized by interaction of the enzymes with this polysaccharide [3].

We now report the chemical structure of the acidic polysaccharide component of lysoamidase.

2. Experimental

NMR spectra were measured with a Bruker WM-250 (^1H) and a Bruker AM-300 (^{13}C) spectrometer equipped with a BSV-3 generator in D_2O at 70°C (internal standard acetone, δ_H 2.225, δ_C 31.45). Standard Bruker software was used to carry out two-dimensional NMR experiments. A mixing time 0.2 s was used in two-dimensional rotating-frame NOE spectroscopy (ROESY).

Lysoamidase was obtained using a pilot apparatus in the Institute of Biochemistry and Physiology of Microorganisms.

The acidic polysaccharide was isolated from lysoamidase by gel chromatography on Sepharose CL-4B at pH 12 as described earlier [3], precipitated with cetyltrimethylammonium bromide, dissolved in 2 M NaCl, precipitated with ethanol (5 volumes), dissolved in 0.05 M Tris-HCl buffer (pH 8.0), dialyzed against the same buffer, and purified by anion-exchange chromatography on a column of DEAE-Sephadex equilibrated with the same buffer and washed first with water and then with 1 M NaCl.

Partial depolymerization of the polysaccharide was performed by hydrolysis with 0.05 M HCl (15 ml, 100°C, 2 h). Carboxyl reduction was carried out by the published method [4]. *O*-Deacetylation was performed with aqueous 12% ammonia (37°C, 16 h). The modified polysaccharides were isolated by gel filtration on TSK HW-40.

For sugar analysis, the polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (121°C, 2 h), and the hydrolysate was analyzed on an Ostion LG AN B cation-exchange resin using sodium borate buffer (pH 7.24) at 80°C [5] or the standard sodium citrate buffers (pH 3.25, 4.25 and 5.28).

2-Amino-2-deoxy-D-glucose was isolated by hydrolysis of the polysaccharide with 4 M HCl (100°C, 16 h) followed by chromatography on a Chromex UA-8 cation-exchange resin using sodium borate buffer (pH 7.24) at 80°C.

3. Results and discussion

An acidic polysaccharide was isolated from lysoamidase and separated by anion-exchange chromatography from a contaminating minor polysaccharide consisting of rhamnose. The acidic polysaccharide did not contain neutral sugars, uronic acids, or sialic acids. Amino sugar analysis revealed 2-amino-2-deoxyglucose and another component (or components) which has the same retention time as 2-amino-2-deoxygalacturonic acid. The specific optical rotation value, $[\alpha]_D$ + 59° (*c* 0.08, sodium borate buffer, pH 7.24), indicated the D configuration of 2-amino-2-deoxyglucose.

In addition to 2-amino-2-deoxyglucose, in the hydrolysate of the carboxyl-reduced polysaccharide, mannosamine and galactosamine were identified (the ratios GlcN:ManN:GalN 1:0.35:0.40), which were derived from 2-amino-2-deoxymannuronic acid (ManNA) and 2-amino-2-deoxygalacturonic acid (GalNA), respectively. As the completion of the carboxyl reduction and the hydrolysis was difficult to control, the obtained ratios of the amino sugars did not allow conclusion on the ratios of the monosaccharide components in the initial acidic polysaccharide.

The ^{13}C NMR spectrum of the purified polysaccharide (Table 1) contained two series of signals (A and B) in the ratio ~2:1. The presence in the spectrum of a signal at 21.5 ppm belonging to an *O*-acetyl group suggested that the observed

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SSDI 0014-5793(95)00612-5

Table 1
Data of ^{13}C NMR spectrum of the intact (A) and *O*-deacetylated (B) acidic polysaccharide

Residue		Chemical shift (ppm) for					
		C1	C2	C3	C4	C5	C6
→3)-β-D-Glc*p*NAc	A	102.0	57.6	79.6	69.7	77.0	61.9
	B	102.0	56.4	79.5	69.7	77.0	61.9
→4)-β-D-Man*p*NAcA	A	97.6	53.1	71.2	79.0	77.0	
	B	97.6	53.5	71.1	79.0	77.0	
→3)-α-L-Gal*p*NAcA	A	99.3	49.3	72.9	69.7	69.7	
	B	99.0	48.7	74.8	67.9	72.1	

Chemical shifts for Me of the *N*-acetyl and *O*-acetyl groups are 23–24 and 21.5 ppm, respectively, for CO and COOH groups 174–177 ppm.

heterogeneity of the polysaccharide is most likely associated with nonstoichiometric *O*-acetylation. After *O*-deacetylation with aqueous ammonia, the ^{13}C NMR spectrum of the modified polysaccharide contained only the signals of series B (Table 1).

The ^{13}C NMR spectrum of the nonpurified polysaccharide preparation contained only the signals of series A (Table 1), and the ratio of the intensities of the signals for the *N*-acetyl and *O*-acetyl groups was ~3:1. Therefore, the initial acidic polysaccharide included one *O*-acetyl group per repeating unit, and the heterogeneity of the purified polysaccharide is most likely due to partial *O*-deacetylation during gel chromatography under alkaline conditions.

The ^{13}C NMR spectrum contained signals for three anomeric carbons in the region 98–102 ppm, three carbons bearing nitrogen at 48–57 ppm, one hydroxymethyl group (C6 of 2-amino-2-deoxyglucose) at 61.9 ppm, two carboxyl groups (C6 of 2-amino-2-deoxyhexuronic acids) in the region 174–177 ppm, and three *N*-acetyl groups (CH_3 at 23–24 ppm, CO at 174–177 ppm). These data suggested that the polysaccharide has a trisaccharide repeating unit containing one residue of 2-acetamido-2-deoxyglucose and two residues of 2-acetamido-2-deoxyhex-

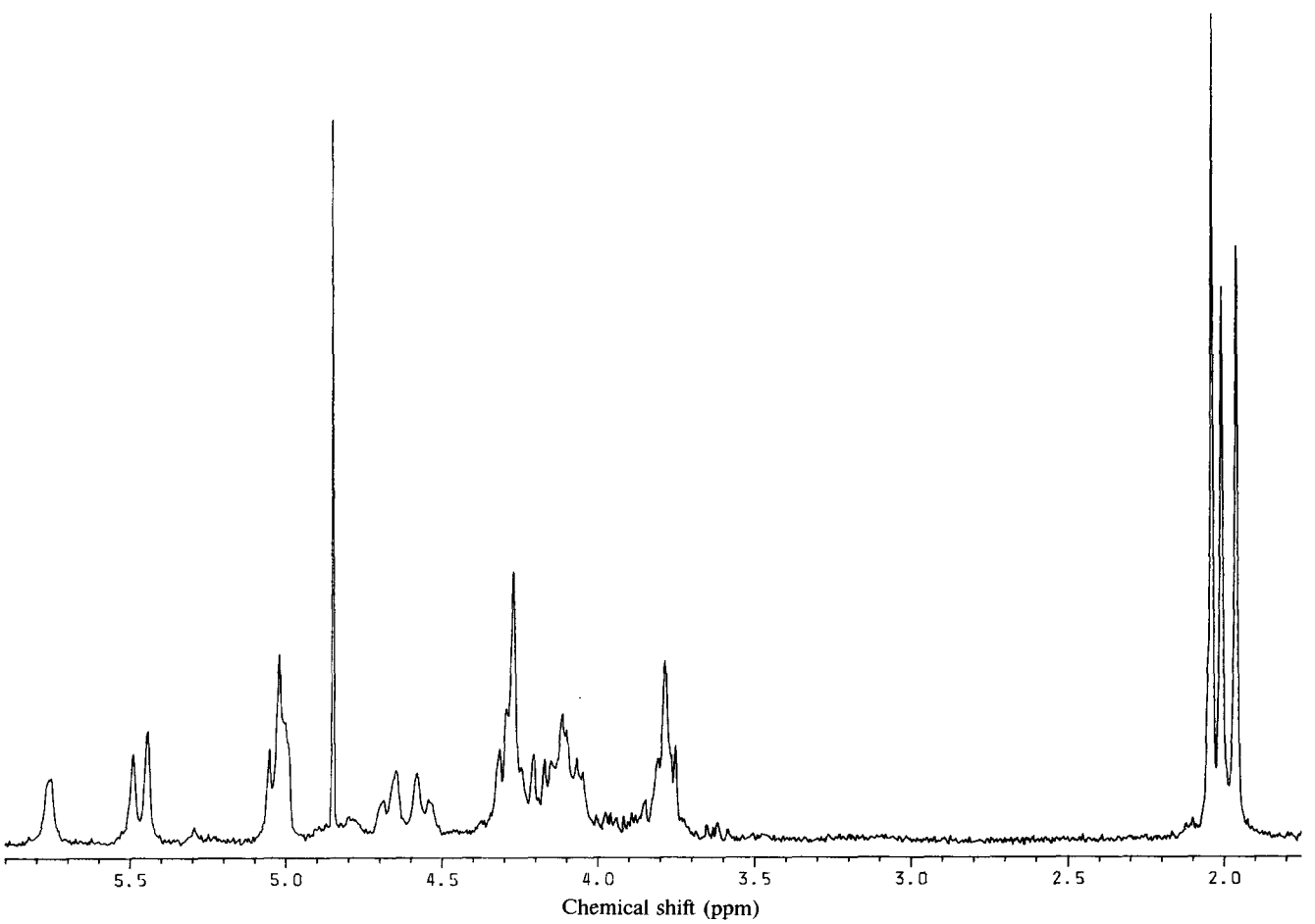

Fig. 1. ^{1}H NMR spectrum of the *O*-deacetylated polysaccharide.

Table 2
Data of ^{1}H NMR spectrum of the *O*-deacetylated polysaccharide

Residue	Chemical shift (coupling constant) (ppm (Hz))						
	H1	H2	H3	H4	H5	H6a	H6b
→3)-β-D-Glc*p*NAc	4.53 ($J_{1,2}$ = 7.5)	3.83 ($J_{2,3}$ = 9.0)	3.73 ($J_{3,4}$ = 9.0)	3.47	3.47 ($J_{5,6b}$ < 2)	3.72 $J_{5,6a}$ = 3.5)	3.90 ($J_{6a,6b}$ = 12)
→4)-β-D-Man*p*NAcA	4.90 ($J_{1,2}$ < 2)	4.48 ($J_{2,3}$ = 3,5)	3.88	3.88 ($J_{4,5}$ = 9.5)	3.74		
→3)-α-L-Gal*p*NAcA	5.12 ($J_{1,2}$ = 3.5)	4.22 ($J_{2,3}$ = 10.0)	4.13 ($J_{3,4}$ = 3.0)	4.51 ($J_{4,5}$ < 2)	4.86		

Chemical shifts for the *N*-acetyl groups are 1.97, 2.01, and 2.04 ppm.

uronic acids. The absence of signals from the region 80–88 ppm indicated that all monosaccharide residues are in the pyranose form [6].

In order to improve the resolution of the ^{1}H NMR spectrum, molecular mass of the polysaccharide was diminished by controlled partial acid hydrolysis. The modified polysaccharide gave a ^{13}C NMR spectrum practically identical to that of the initial polysaccharide and a rather well-resolved ^{1}H NMR spectrum (Fig. 1).

The ^{1}H NMR spectrum of the partially depolymerized and *O*-deacetylated polysaccharide was assigned using two-dimensional correlation spectroscopy (COSY) and selective spin decoupling (Table 2). The coupling constant values $J_{1,2}$ 7.5 and 3.5 Hz, respectively, showed that GlcNAc is β-linked and GalNAcA α-linked. The β configuration of ManNAcA followed from the $^1J_{C1,H1}$ coupling constant values, determined from the coupled ^{13}C NMR spectrum, which showed that only one of the monosaccharide residues (GalNAcA) is α-linked ($^1J_{C1,H1}$ 172 Hz) and two other monosaccharides are β-linked ($^1J_{C1,H1}$ 162 and 164 Hz) [7].

In the ROESY spectrum of the *O*-deacetylated polysaccharide, correlation peaks H1/H3 and H1/H5 were observed for GlcNAc at δ 4.53/3.73 and 4.53/3.47 and for ManNAcA δ 4.90/3.88 and 4.90/3.74, respectively, which confirmed the β configuration of these monosaccharide residues, while a correlation peak H1/H2 for GalNAcA at δ 5.12/4.22 was consistent with its α configuration.

Correlation peaks H1 GlcNAc/H4 ManNAcA at δ 4.53/3.88 and H1 GalNAcA/H3 GlcNAc at δ 5.12/3.73 in the ROESY spectrum pointed to the presence of disaccharide fragments GLc*p*NAc-(1→4)-Man*p*NAcA and Gal*p*NAc-(1→3)-Glc*p*NAc. Two correlation peaks, with H3 and H4 of GalNAcA, respectively, were revealed for H1 of ManNAcA at δ 4.90/4.13 and 4.90/4.51. This suggested the presence of a disaccharide fragment Man*p*NAcA→Gal*p*NAcA, position of substitution of GalNAcA remaining uncertain.

The ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide was assigned using an H-detected homonuclear multiquantum coherence (HMQC) experiment (Table 1, series B). The obtained data confirmed the structures of the monosaccharide residues (in particular, location of the amino group at C2 of the three amino sugars) as well as the configurations and the positions of the glycosidic linkages. A significant downfield displacement (by ~6 ppm) of the signal for C3 of GalNAcA, as compared with its position in the nonsubstituted GalNAc [6,8], showed that GalNAcA in the polysaccharide is glycosylated at position 3.

Analysis of the effects of glycosylation in the ^{13}C NMR spectrum allowed determination of absolute configurations of ManNAcA and GalNAcA. Thus, a relatively large by the absolute value negative β-effect of glycosylation on C3 of ManNAcA (−2.2 ppm) pointed to the same absolute configuration of GlcNAc and ManNAcA, while a relatively small positive α-effect on C1 of ManNAcA (3.4 ppm) indicated different absolute configurations of ManNAcA and GalNAcA [8]. Therefore, with the D configuration of GlcNAc, ManNAcA is D and GalNAcA is L.

Comparison of the chemical shifts in the ^{13}C NMR spectra of the initial and *O*-deacteylated polysaccharide (Table 1, series A and B, respectively) showed that *O*-acetylation shifted mostly the signals of GalNAcA. A positive effect on C4 (1.8 ppm) and negative effects on C3 and C5 (−1.9 and −2.4 ppm, respectively) of GalNAcA suggested the location of the *O*-acetyl group at position 4 [9].

Therefore, the acidic polysaccharide present in the lysoamidase preparation is built up of the trisaccharide repeating units with the following structure

→3)-β-D-Glc*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→3)-α-L-Gal*p*NAc-(1→
4
|
OAc

While 2-acetamido-2-deoxyglucose is a widespread component of bacterial polysaccharides, 2-acetamido-2-deoxyhexuronic acids present in the studied polysaccharide occur less often. They have been identified in the Vi-antigen (GalNAcA), common enterobacterial antigen (ManNAcA), O-antigens, capsular polysaccharides, and teichoic acids of bacteria [10–12].

The studied polysaccharide is characterized by high content of the carboxyl groups and the *N*- and *O*-acetyl groups, which may be important for interaction with and stabilization of the lysoamidase enzymes. Further study is necessary to reveal the character of this interaction.

Acknowledgements: This work was supported by Grant 93-04-06-118 from the Russian Foundation in Fundamental Sciences.

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