LIPID-LINKED INTERMEDIATES IN THE BIOSYNTHESIS OF XANTHAN GUM

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1. Introduction

Xanthan gum is a complex exopolysaccharide produced by the gram negative bacterium *Xanthomonas campestris*. It is composed of pentasaccharide repeating units with the structure [1]:

mannose-β-1,4-glucuronic acid-β-1,2-mannose-α-1,3
|
-4-glucose-β-1,4-glucose-β-1, –

Pyruvic acid acetal and *O*-acetyl groups are also present in differing proportions [1,2].

Although thousands of tons of xanthan gum are produced in a year, because of its industrial applications [2], nothing has been reported about its biosynthesis. Evidence on the mechanism of the in vitro assembly of this polysaccharide is described in this communication: UDP-Glc, GDP-Man and UDP-GlcUA sequentially donate their sugar moieties to a lipidic acceptor to form a pentasaccharide-P-P-lipid which is subsequently polymerized into xanthan gum.

The role of polyprenyl phosphosugars was first described in bacterial lipopolysaccharide [3] and murein [4] biosynthesis, and later on extended to capsular polysaccharides [5,6], teichoic acids [7,8] and other polysaccharides closely associated to the bacterial cell body [9,10].

This is the first report on the participation of lipid-linked sugars in the biosynthesis of an exopolysaccharide freely liberated into the culture medium.

2. Materials and methods

UDP-[^{14}C]Glc (1976 Ci mol^{-1}) was prepared as in previous papers [11]. [^{14}C]Glc-β-1,4-[^{14}C]Glc (cellobiose); [^{14}C]Man-β-1,3-Glc-β-1,4-Glc (β-Man-cellobiose); [^{14}C]GlcUA-β-1,6-Man-β-1,3-cellobiose (tetrasaccharide X_4); and Glc-β-1,6-Glc-α-1,4-[^{14}C]GlcUA-β-1,6-Man-β-1,3-cellobiose (hexasaccharide X_6), as well as their respective cyclic phosphate esters in position 1,2 of the reducing glucose, were prepared by mild acid or alkaline treatment of the respective prenyl-phosphosugars, obtained with an *Acetobacter xylinum* system as in previous work [11,12] (Couso, R., Ielpi, L., Garcia, R. y Dankert, M., in preparation).

Xanthomonas campestris, strain NRRLB-1459 was grown and harvested as reported [13]. The enzyme preparation consisted of EDTA-treated cells obtained as in previous publications [11].

2.1. *Assay procedure*

The incubations were carried out and processed as in the legend to table 1. Two fractions were obtained: a washed pellet and a supernatant. The washed pellets were extracted three times with (0.1 ml each) chloroform:methanol:water (1:2:0.3) [14]. This extract, which is referred to as the 1203 extract, contains the polyprenyl-phosphosugars.

The supernatants were concentrated by lyophilization and xanthan gum was determined as follows. The lyophilized material from each incubation, together with 225 μg of unlabelled carrier xanthan gum, were dissolved in water (0.1 ml). Absolute ethanol (0.2 ml) and 2% KCl in 70% ethanol (0.2 ml) were added and thoroughly mixed. The precipitate formed was separated by centrifugation, redissolved in 0.1 ml of water and reprecipitated with 0.2 ml of absolute ethanol and 0.2 ml of 2% KCl in 70% ethanol. The procedure was repeated 5–6 times until no radioactivity was detectable in the supernatant. The washed pellet was dissolved in water and one aliquot was counted for radioactivity.

The unlabelled xanthan gum was obtained from the harvested culture media following a similar procedure.

Mild acid hydrolysis was performed at pH 2

(0.01 M HCl) and 100°C for 10 min, as reported [11]. Cyclic phosphoric esters were opened and phosphoric or pyrophosphoric esters were cleaved by treatment at pH 1 (0.1 M HCl) and 100°C for 10 min.

Borohydride reduction and alkaline phosphatase treatments were carried out as described [11]. Polyprenol phosphosugars were treated with phenol and reduced with H_2–Pt as in previous work [15].

Chromatography and electrophoresis were performed in Whatman No. 1 paper under the conditions already described [11]. The following solvents were used: (A) 1.2 M pyridinium acetate buffer pH 6.5; (B) 0.03 M sodium borate pH 9.8; (C) 0.1 M sodium molybdate buffer, pH 5.0; (D) 0.25 M sodium carbonate buffer, pH 9.2; (E) isopropanol:acetic acid:water (27:4:9); (F) 3 M ammonia in 80% (v/v) ethanol; (G) 3 M ammonia in 70% (v/v) ethanol; and (H) ethanol:acetic acid:1 M ammonium acetate (75:26:4). Reducing substances were located with the alkaline silver reagent [16] and ultraviolet-absorbing compounds with a Mineralight lamp. Paper strips were scanned for radioactivity on a Packard radiochromatogram scanner, model 7201.

DEAE-cellulose column chromatography was performed in 99% methanol as in [17], except for the ammonium acetate gradient, which was from 0–2.0 M in 99% methanol.

3. Results and discussion

Incubations were carried out as indicated in legend to table 1. In the presence of UDP-[^{14}C]Glc as the only sugar donor, radioactivity was incorporated into material soluble in the 1203 extract (table 1, No. 1). This incorporation was not modified by the presence of unlabeled GDP-Man or UDP-GlcUA in the incubation mixture (table 1, Nos 2 and 3, respectively), but was reduced almost three times when the three nucleotides were incubated all together (table 1, No. 4).

The radioactive material in the 1203 extract was labile to mild acid treatment in all of the cases, but the water soluble products released were different.

The 1203 extract from incubations with UDP-[^{14}C]Glc alone, upon paper chromatography with solvent E, produced a single radioactive compound of R_f = 0.87 and by DEAE-cellulose column chromatography, a sharp peak was eluted at 0.4 M ammonium acetate. The product of mild acid hydrolysis was characterized as cellobiose as judged by its mobility on paper chromatography with solvent E, electrophoresis with buffer B, and, after reduction with sodium borohydrate, electrophoresis with buffer C, where it had the same mobility as cellobiitol.

All these properties are consistent with the presence of a cellobiose-P-P-lipid in the 1203 extract. This was reinforced by paper chromatography in the alkaline solvent F. The compound detected had the same mobility as a standard of cyclic phosphoric ester of cellobiose obtained from an *Acetobacter xylinum* system [11] and, upon paper electrophoresis with buffer A it moved with an R_{UMP} = 0.93 identical with that of the standard. This compound, treated at pH 1 and with alkaline phosphatase, released free cellobiose. The presence of a pyrophosphate bridge was confirmed by phenol treatment and catalytic reduction: in both

Table 1
Effect of GDP-Man and/or UDP-GlcUA on the incorporation of [^{14}C]Glc from UDP-[^{14}C]Glc

Tube No.	Additions	[^{14}C]Glc incorporated into		Tentative structure of lipid-bound oligosaccharide
		polysaccharide (pmol)	1203 extract (pmol)	
1	None	0.8	153	cellobiose
2	GDP-Man	8.0	120	Man-cellobiose
3	UDP-GlcUA	0.8	121	cellobiose
4	GDP-Man + UDP-GlcUA	41.5	51	pentasaccharide

The reaction mixture contained 70 mM Tris–HCl buffer, pH 8.2, 8 mM $MgCl_2$, EDTA-treated cells (400 μg), 15.7 μM UDP-[^{14}C]Glc and, where indicated, 71 μM GDP-Man or 35.7 μM UDP-GlcUA, or both sugar nucleotides. Incubations were carried out at 20°C for 1 h; they were stopped by adding 0.5 ml of 70 mM Tris–HCl buffer containing 5 mM EDTA and spinning down at 6000 × *g* for 5 min. The pellets were resuspended and washed twice with 0.5 ml of 70 ml Tris–HCl buffer. The combined supernates were lyophilized to determine polymer formation as described in section 2. The washed pellets were extracted with chloroform:methanol:water (1:2:0.3) and aliquots were counted for radioactivity

cases cellobiose pyrophosphate was formed (80 and 90% respectively), as shown by paper electrophoresis with buffer D (R_{UMP} = 0.57). Furthermore, this compound produced free cellobiose when treated at pH 1 or with alkaline phosphatase [11]. The lability of the cellobiose-P-P-lipid towards catalytic reduction and phenol treatment indicates, in addition, that the lipidic moiety is very likely of an allylic nature, as would be expected [15].

When the incubations were carried out in the presence of UDP-[^{14}C]Glc and unlabeled UDP-GlcUA (table 1, No. 3) the 1203 extract released only cellobiose by mild acid treatment, as in the previous case; but when GDP-Man was substituted for UDP-GlcUA (table 1, No. 2), a single compound with the mobility of the trisaccharide standard β-mannosyl-cellobiose was detected by paper chromatography with solvent E. The 1203 extract, by paper chromatography with solvent G produced a compound with the mobility (R_f = 0.5) of the cyclic phosphate ester of β-mannosyl cellobiose [12]. Both compounds, upon paper electrophoresis with buffer A showed an R_{UMP} = 0.8, and treated with 0.1 M HCl and alkaline phosphatase, produced the free trisaccharide. Similar results were obtained with 1203 extracts from incubations with GDP-[^{14}C]Man and unlabeled UDP-Glc, confirming that under these conditions, a mannosyl-cellobiose-P-P-prenol had been formed.

Finally, incubations performed in the presence of the three sugar nucleotides yielded smaller amounts of radioactivity in the 1203 extract than the other combinations (table 1, No. 4). DEAE column chromatography of the extract produced a single sharp peak of radioactivity, which was eluted at 0.95 M ammonium acetate. Mild acid hydrolysis of this compound released all the radioactivity as an anionic oligosaccharide which upon paper electrophoresis with buffer A moved slightly less (R_{UMP} = 0.6) than the anionic tetrasaccharide X_4 (R_{UMP} = 0.7) but more than the anionic hexasaccharide X_6. Similarly, by paper chromatography with solvent H the anionic oligosaccharide moved (R_{Glc} = 0.44) less than the tetrasaccharide X_4 (R_{Glc} = 0.53), but more than the hexasaccharide X_6 (R_{Glc} = 0.37). From these results it was tentatively assumed that the compound liberated by mild acid hydrolysis was the pentasaccharide repeating unit. [^{14}C]Man or [^{14}C]GlcUA labelled 'pentasaccharide' could also be obtained, provided the respective radioactive sugar nucleotide donor was present in the incubation mixture.

Paper chromatography with solvent G of the 1203 extract (from tube No. 4, table 1) produced a single substance (R_f = 0.25) which, upon pH 1 and alkaline phosphatase treatments, was shown to be the cyclic phosphate ester of the pentasaccharide, confirming the presence of a pyrophosphate bridge in the undegraded compound.

3.1. *Xanthan gum formation*

The synthesis of polysaccharide was evident only when the three sugar nucleotides were present in the incubation mixture (table 1, No. 4). The decrease in 1203 extractable radioactivity observed in this case is consistent with the pentasaccharide-P-P-lipid being used to form the polymer (polymerization was maximal at 20°C). Gel filtration of the polysaccharide formed showed that it has the same molecular weight (around 4 × 10^6) as the xanthan gum liberated into the culture medium (fig.1).

The precursor–product relationship was demonstrated in a two-step incubation experiment (table 2). The first incubation was performed in the presence of UDP-[^{14}C]Glc alone. The excess nucleotide was removed by washing and the second incubation was carried out with different additions. In the presence of unlabeled GDP-Man and UDP-GlcUA the polysaccharide produced was maximal (table 2, No. 5). No significant amounts of polymer were observed in a control not reincubated (No. 1), or in reincubations with no additions (No. 2), or with UDP-GlcUA (No. 3), or with GDP-Man (No. 4) alone. The high molecular

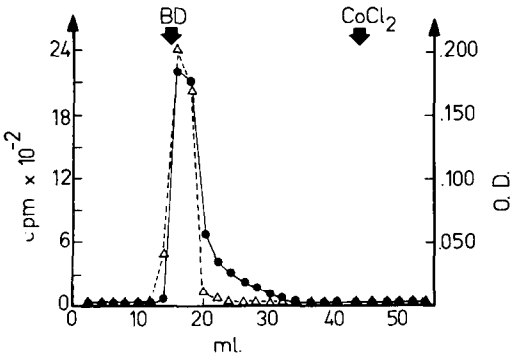

Fig.1. Gel filtration of the polymerization product. ^{14}C-Labelled polysaccharide (8000 cpm) obtained as in table 1, No. 4, was filtered through a Bio-Gel A 5 m column (54 × 1.2 cm) in 50 mM Tris–HCl buffer pH 8.0, at a rate of 0.25 ml min^{-1}. Fractions (2.0 ml each) were collected and divided into two equal aliquots to measure total sugars by the phenol-sulfuric method [18] (△) and radioactivity (●); BD: blue dextran.

Table 2
Polymerization of pentasaccharide-P-P-lipid

Tube No.	Additions in the reincubation	[^{14}C]Glc incorporation into	
		1203 extract (pmol)	polysaccharide (pmol)
1	No reincubation	41	0.2
2	No additions	39	0.6
3	UDP-GlcUA	37	0.6
4	GDP-Man	15.7	3.8
5	UDP-GlcUA + GDP-Man	9.7	12.0

The reaction mixture was as in table 1, Expt 1 but increased 5 fold. The incubation was at 12°C for 30 min, and stopped and the cell pellet washed as in legend to table 1. The washed pellet was resuspended in 70 mM Tris–HCl buffer pH 8.2, 10 mM EDTA and 8 mM $MgCl_2$ and divided into five aliquots. UDP-GlcUA (20 nmol) or GDP-Man (20 nmol) were added where indicated. Reincubation was at 20°C for 30 min and reactions were stopped and processed as in table 1. One aliquot, No. 1, was not reincubated

weight of the polymer formed in No. 5 was confirmed by gel filtration through Bio-Gel A 5 m (not shown).

The proposed structure for the xanthan gum consists of a cellulose backbone (β-1,4-glucose polymer) with trisaccharide branches (mannose-β-1,4-glucuronic acid-β-1,2-mannose-α-1,3-) every two glucose units [1]. It seems of interest to remark that no polymerization occurs unless the pentasaccharide repeating unit is built up (tables 1 and 2). For instance, cellobiose-P-P-lipid accumulates but no cellulose is formed (table 1, No. 1 and table 2, No. 2).

Further studies on the structure of the different lipid intermediates and on the polymerization process are in progress.

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