Characterization of Cell Wall Components of the Alkalophilic Bacillus Strain C-125: Identification of a Polymer Composed of Polyglutamate and Polyglucuronate

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The A2 substance, a structural component of the cell wall of alkalophilic Bacillus strain C-125, has an $M_r$ of 21000 and is composed mainly of glucuronic acid and glutamic acid. Hydrazinolysis of the A2 substance yielded homoglucuronate with an estimated $M_r$ of 4800. The $M_r$ of polyglutamate previously prepared from the A2 substance by deglycosylation was estimated to be 14000. It is therefore suggested that the A2 substance is composed of the two polymers, polyglutamate and polyglucuronate, and the name 'teichuronopeptide' is proposed for this complex.

INTRODUCTION

Polyuronic acids which contain no neutral sugars or amino sugars are found mainly in plants, algae and fungi (Haug et al., 1966; Bartnicki-Garcia & Reyes, 1968; Tsuchihashi et al., 1983), although a polysaccharide resembling alginic acid (a polyuronic acid constituent of brown algae) is produced as an extracellular polysaccharide by certain Pseudomonas spp. and Azotobacter vinelandii (Carlson & Matthews, 1966; Linker & Jones, 1966; Gorin & Spencer, 1966; Skjak-Braek et al., 1986). Polyglucuronic acid is rarely found and has not been previously isolated from bacteria.

Two acidic polymers were isolated as structural components of cell walls of the alkalophilic Bacillus C-125: one was a teichuronic acid composed of galacturonic acid, glucuronic acid and N-acetylfucosamine, while the other contained mainly glucuronic acid and glutamic acid, and was tentatively called the A2 substance (Aono, 1985, Aono & Uramoto, 1986). Its chemical nature is still unclear, although poly-γ-L-glutamic acid was isolated by deglycosylation of the A2 substance (Aono, 1987). This paper reports the isolation of homopolyglucuronic acid from the A2 substance and the possible conclusion that the A2 substance is a complex of polyglutamate and polyglucuronate.

METHODS

Organism, preparation of cell wall and isolation of the A2 substance. The alkalophilic Bacillus strain C-125 was grown and cell walls were prepared from early-stationary-phase cells as described previously (Aono & Horikoshi, 1983). Non-peptidoglycan components were extracted with 5% (w/v) trichloroacetic acid. The non-diffusible materials from the extract were separated by DEAE-cellulose column chromatography into two fractions. The fraction containing predominantly glutamic acid and glucuronic acid was pooled and freeze-dried. The substance in this fraction is referred to as 'the A2 substance' (Aono, 1985).

Hydrazinolysis of the A2 substance. The A2 substance (0-5 mg dry weight) was suspended in 0-2 ml anhydrous hydrazine and heated at 100 °C for various times. After removal of the bulk of the hydrazine by evaporation to dryness under reduced pressure over concentrated H$_2$SO$_4$ at 42 °C, the residue was freed of hydrazine by repeated

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evaporation with toluene (Takasaki et al., 1982). The residues were then dissolved in 0-5 ml 50 mm-acetic acid/NaOH buffer (pH 5-0) and samples (0-3 ml) were loaded on a column (1-5 × 91 cm) of Sephadex G-15 equilibrated with 0-2 m-acetic acid/pyridine buffer (pH 5-0). The column was eluted with the same buffer at a flow rate of 20 ml h⁻¹ and fractions (1 ml) collected. The void elution volume (2 ml) was analysed further.

Preparation of polyglucuronic acid. The A2 substance was suspended in anhydrous hydrazine to a concentration of 15 mg ml⁻¹ and heated at 100 °C for 5 h under a N₂ atmosphere. Hydrazine was removed by rotary evaporation at 40 °C with several additions of toluene (Järnefelt et al., 1982). The residue was dissolved in 5 ml 0-2 m-acetic acid and the pH was adjusted to 5-0 with pyridine. The hydrazinolysis product was fractionated on a column of Sephadex G-15. The fraction eluted at the void volume was recovered and freeze-dried. The residual material was dissolved in 40 ml distilled water, to which 0-6 ml 3 m-acetic acid/pyridine buffer (pH 3-0) was added. The product was separated by DEAE-cellulose column chromatography, and carbazole/H₂SO₄ reaction positive fractions were pooled, dialysed (M, cut-off 3500) exhaustively against running water and freeze-dried. The residue was dissolved in 5 ml 0-2 m-acetic acid/pyridine buffer (pH 5-0) and fractionated on a column (2-5 91 cm) of Sephadex G-50 equilibrated with the same buffer at a flow rate of 25 ml h⁻¹. The fractions containing glucuronic acid were pooled and freeze-dried. The main fraction was rechromatographed on the same column and freeze-dried.

Analytical methods. Amino acids were analysed with an automatic amino acid analyser after hydrolysis in 4 M-HCl in sealed tubes at 105 °C for 15 h. L-Glutamic acid was determined with L-glutamate dehydrogenase (Beutler & Michal, 1974). N-terminal amino acid residues were determined by dinitrophenylation using glutamic acid as a reference (Aono, 1987). Amino sugars were analysed with the amino acid analyser after hydrolysis with 4 M-HCl at 100 °C for 5 h. Glucuronic acid was determined by the carbazole/H₂SO₄ reaction (Sprio, 1966). Sodium was determined with an atomic absorption spectrometer (Aono, 1985). M₅ values of the samples were estimated by gel chromatography on two columns of Shodex WS 802.5F (Showa Denko Co.) connected in series, with a high-pressure liquid chromatograph (HPLC) apparatus, as described previously (Aono, 1987). P-5, 10, 20 and 50 used as M₅ references were chemical degradation products of pullulan (Showa Denko Co.).

Avicel cellulose thin-layer chromatography. Ascending Avicel cellulose thin-layer chromatograms were run at room temperature in the following solvents: (A) pyridine/ethyl acetate/water/acetic acid (5:4:3:1, by vol.); (B) ethyl acetate/water/acetic acid (10:6:5, by vol.); (C) n-butanol/pyridine/water (6:4:3, by vol.). The components of the samples were located with an alkaline silver nitrate or ninhydrin spray.

Reduction of the A₂ substance and the polyglucuronic acid preparation. The A₂ substance or the polyglucuronic acid preparation (3 mg) was dissolved in 5 ml distilled water and reduced twice by the following method. To the solution, 0-5 mmol solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added. The pH of the reaction mixture was maintained at 4-6-4-9 with 0-02 M-HCl for 90 min. To the reaction mixture, 2 m-NaBH₄ (10 ml) was added dropwise. The pH of the reaction mixture was maintained at 6-8-7-3 with HCl for 1 h (Taylor & Conrad, 1972). Finally, the pH of the reaction mixture was adjusted to 5-0 with 6 M-HCl to destroy excess NaBH₄. The reduction product was dialysed (M₅, cut-off 3500) against 1 litre of 0-5 M-NaCl, then against distilled water, and analysed by DEAE column chromatography. The sample was loaded on a Shodex IEC DEAE-825 column (0-8 × 7-5 cm, Showa Denko Co.) attached to an HPLC instrument. The column was eluted with 20 mm-ethanolamine/H₂SO₄ buffer (pH 9-0) containing 0-05 m-Na₂SO₄ for 10 min at a flow rate of 1-0 ml min⁻¹, followed by a linear gradient elution from 0-05 to 0-6 m-Na₂SO₄ in the 20 mm-ethanolamine buffer for 27-5 min at the same flow rate. The elution pattern was monitored by absorbance at 210 nm. Fractions (1 ml) were collected and analysed by the phenol/H₂SO₄ reaction (Sprio, 1966), and assayed for L-glutamic acid to confirm that the elution pattern corresponded with the absorbance.

Materials. L-Glutamate dehydrogenase (EC 1.4.1.3) was purchased from Boehringer Mannheim. Anhydrous hydrazine was prepared by distillation of hydrazine hydrate in the presence of excess solid NaOH.

RESULTS

Dissociation of the A₂ substance with hydrazine. The effects of hydrazine on release of glutamic acid residues and on recovery of intact polyglucuronic acid chains were first examined. Recovery of glutamic acid in comparison to that of glucuronic acid was decreased after hydrazine treatment (Table 1). The γ-peptidyl linkage through which glutamic acid residues are joined was easily cleaved with hydrazine treatment, whereas the glycosidic linkage between glucuronic acid residues was comparatively resistant. Prolonged incubation with hydrazine, however, caused a decrease in the recovery of glucuronic acid.

Preparation and purification of polyglucuronic acid from the A₂ substance. After hydrazinolysis of the A₂ substance (567 mg dry weight), most of the glucuronic acid found in the starting
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Table 1. Recoveries of glutamic acid and glucuronic acid after hydrazinolysis

The A2 substance was incubated in anhydrous hydrazine at 100 °C. A part of the hydrazinolysis product was fractionated by Sephadex G-15 gel chromatography. Macromolecules eluted at the void volume were assayed for glucuronic acid and glutamic acid. Recoveries (given in parentheses) are calculated taking the amount in the fraction recovered without hydrazinolysis as 100%.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Glucuronic acid (nmol)</th>
<th>Glutamic acid (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>206 (100%)</td>
<td>860 (100%)</td>
</tr>
<tr>
<td>1</td>
<td>209 (101%)</td>
<td>382 (44%)</td>
</tr>
<tr>
<td>2</td>
<td>216 (105%)</td>
<td>161 (19%)</td>
</tr>
<tr>
<td>3</td>
<td>197 (96%)</td>
<td>56 (7%)</td>
</tr>
<tr>
<td>5</td>
<td>162 (79%)</td>
<td>41 (5%)</td>
</tr>
<tr>
<td>7</td>
<td>94 (46%)</td>
<td>21 (2%)</td>
</tr>
<tr>
<td>10</td>
<td>87 (42%)</td>
<td>28 (3%)</td>
</tr>
</tbody>
</table>

material eluted at the void volume of the Sephadex G-15 column (yield: 119 mg dry weight, 486 μmol glucuronic acid, 124 μmol glutamic acid), as shown in Fig. 1. The recovery of glucuronic acid was 90% and that of glutamic acid was 5%. Almost all glutamic acid residues were converted to fragments, which were separated from the glucuronic acid residues. Fraction 40 contained glucuronic acid, L-glutamic acid and N-terminal amino acid residues in a molar ratio of 1:0.022:0.068.

The polymer fractions obtained from two Sephadex G-15 columns were combined (849 μmol glucuronic acid and 194 μmol glutamic acid) and fractionated by DEAE-cellulose column chromatography. Polyglucuronic acid eluted as a single peak together with a small amount of glutamic acid (yield: 175 mg dry weight, 700 μmol glucuronic acid, 49 μmol glutamic acid). A substantial amount of poly- or oligoglucuronic acid was eluted in several broad peaks with the low-pH buffer. The peak fraction contained glucuronic acid and L-glutamic acid in a molar ratio of 1:0.054. The polyglucuronic acid fraction was further purified by Sephadex G-50 column chromatography (Fig. 2). Five pooled fractions were separately freeze-dried for later analysis (Table 2). The peak fraction (no. 55) contained glucuronic acid and L-glutamic acid in a molar ratio of 1:0.064. After rechromatography on the same column, the yield of the main fraction, D, was 74 mg dry weight, containing 382 μmol glucuronic acid and 20 μmol glutamic acid (Table 3).

Chemical composition of the polyglucuronic acid preparation. Fraction D was hydrolysed with various concentrations of HCl at 100 °C and the hydrolysates (20 μg) were analysed by cellulose thin-layer chromatography in solvents A, B and C (data not shown). Alkaline silver nitrate revealed two main spots, corresponding to the authentic lactone and free acid forms of glucuronic acid. Minor spots were found to correspond to galactosamine, some oligomers of glucuronic acid produced in low concentrations of HCl and some degradation products of glucuronic acid produced in high concentrations of HCl. Weak spots revealed with ninhydrin corresponded to authentic galactosamine and glutamic acid standards.

The A2 substance contained predominantly glutamic acid and glucuronic acid, together with small amounts of cell wall peptidoglycan components (Table 3). The main component of fraction D was glucuronic acid, which accounted for about 90% of its dry weight. As fraction D had been prepared partially as the sodium or pyridinium salt, it is concluded that this fraction is composed entirely of polyglucuronic acid, although small amounts of glutamic acid oligomers remained. Hydrazide is presumably present in the preparation and the amount of N-terminal amino acids is probably overestimated. The estimated degree of polymerization of glutamic acids may be two or three.

The polyglucuronic acid preparation was eluted with a broad $M_r$ distribution from the Shodex WS-802.5F columns (Fig. 3). The $M_r$ of the preparation, estimated by comparison with neutral pullulan standards ($M_r$ 2000–15000), was 4800.
Fig. 1. Sephadex G-15 column chromatography of the A2 substance after hydrazinolysis. The A2 substance (567 mg) was incubated in anhydrous hydrazine (36 ml) at 100 °C for 5 h. After removal of the hydrazine, the hydrazinolysis product was loaded on a column (2.6 × 97 cm) of Sephadex G-15 equilibrated with 0.2 M-acetic acid/pyridine (pH 5.0). The column was eluted with the same buffer at a flow rate of 20 ml h⁻¹. Fractions (5 ml) were collected and assayed for glucuronic acid (●) and L-glutamic acid (○). Fractions 38–44 were recovered and pooled.

Fig. 2. Fractionation of the polyglucuronic acid preparation by Sephadex G-50 column chromatography. The polyglucuronic acid fraction from the DEAE-cellulose column was chromatographed on a Sephadex G-50 column as described in Methods. Fractions (5 ml) were collected and assayed for glucuronic acid (●) and L-glutamic acid (○). The fractions containing glucuronic acid (31–38, 39–45, 46–49, 50–60, 61–70; A, B, C, D and E respectively) were separately recovered (see Table 2).

Table 2. Composition of hydrazinolysis product fractionated by Sephadex G-50 gel chromatography

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Dry wt (mg)</th>
<th>Glucuronate (μmol)</th>
<th>Glutamate (μmol)</th>
<th>Ratio of glutamate to glucuronate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>31–38</td>
<td>7.3</td>
<td>33.9</td>
<td>6.1</td>
</tr>
<tr>
<td>B</td>
<td>39–45</td>
<td>24.4</td>
<td>94.4</td>
<td>10.1</td>
</tr>
<tr>
<td>C</td>
<td>46–49</td>
<td>14.7</td>
<td>60.1</td>
<td>8.7</td>
</tr>
<tr>
<td>D</td>
<td>50–60</td>
<td>87.8</td>
<td>406.1</td>
<td>35.0</td>
</tr>
<tr>
<td>E</td>
<td>61–67</td>
<td>3.6</td>
<td>27.0</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Elution of the reduction products of the A2 substance and polyglucuronic acid from a DEAE column. The A2 substance and the polyglucuronic acid preparation (fraction D) eluted from the Shodex IEC DEAE-825 column at 0.58 and 0.22 M-Na₂SO₄, respectively (Fig. 4). As expected, glucan produced by reduction of the polyglucuronic acid preparation did not absorb to the column. However, the reduction product of the A2 substance eluted at 0.33 M-Na₂SO₄ as a broad peak. Glucose was found together with L-glutamic acid residues in the peak fraction.
Fig. 3. Estimation of the $M_r$ of the polyglucuronic acid preparation by gel chromatography on Shodex WS 802.5F columns. The graph shows elution of (1) the A2 substance (10 μg) and (2) the polyglucuronic acid preparation (10 μg). $M_r$ standards used were pullulans (P-50, P-20, P-10 and P-5), maltoheptaose (M-7) and maltotetraose (M-4).

Fig. 4. Elution of the reduction products of the A2 substance and polyglucuronic acid from a DEAE column. Glucuronic acid residues in the A2 substance and the polyglucuronic acid preparation (fraction D) were reduced with carbodiimide and NaBH₄ twice. The sample was run on a Shodex IEC DEAE-825 column (0.8 x 7.5 cm) as described in Methods. The graph shows the elution of (1) 0.1 mg of the A2 substance, (2) 0.2 mg of the reduction product of the A2 substance, (3) 0.5 mg of the polyglucuronic acid preparation (fraction D) and (4) 0.1 mg of the reduction product of the polyglucuronic acid preparation (fraction D).

Table 3. Chemical composition of the polyglucuronate preparation

The polyglucuronate prepared from the A2 substance by hydrazinolysis was fractionated on a column of Sephadex G-50. Constituents of the main fraction, D (Fig. 2, Table 2), were determined after rechromatography on the same column as described in Methods. Molar ratios (given in parentheses) were calculated taking the content of glucuronic acid as 1.0.

<table>
<thead>
<tr>
<th>Component</th>
<th>A2 substance</th>
<th>Polyglucuronate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucuronic acid</td>
<td>0.96 (1.0)</td>
<td>5.1 (1.0)</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>4.4 (4.6)</td>
<td>0.26 (0.052)</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>0.039 (0.04)</td>
<td>0.043 (0.0084)</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.0086 (0.009)</td>
<td>0.004 (0.0008)</td>
</tr>
<tr>
<td>Fucosamine</td>
<td>0.004 (0.004)</td>
<td>0.004 (0.0008)</td>
</tr>
<tr>
<td>Sodium</td>
<td>4.2 (4.4)</td>
<td>0.67 (0.13)</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0.006 (0.006)</td>
<td>0.0022 (0.0004)</td>
</tr>
<tr>
<td>Diaminopimelic acid</td>
<td>0.002 (0.004)</td>
<td>0.0021 (0.0004)</td>
</tr>
</tbody>
</table>
DISCUSSION

The A2 substance has not previously been separated chromatographically into two fractions containing only polyglutamic acid or polyglucuronic acid (Aono, 1985). Following reduction with carbodiimide, the reduction product absorbed to the DEAE column and eluted at lower salt concentration than the non-reduced A2 substance. Also, glucose derived from glucuronic acid residues co-eluted with glutamic acid residues (Fig. 4). These results support the possibility that the A2 substance is a copolymer of glutamic acid and glucuronic acid, and not simply a mixture of polyglutamic acid and polyglucuronic acid.

Poly-γ-L-glutamic acid was previously isolated from the A2 substance by deglycosylation with trifluoromethanesulphonic acid (Aono, 1987). The A2 substance was concluded to consist of polyglutamic acid either bound to a polyglucuronan chain or with many (oligo)glucuronate substituents. Attempts to prepare a putative repeating unit composed of residues of glucuronic acid and glutamic acid from partial acid hydrolysates of the A2 substance under various conditions were unsuccessful. This suggests that the polyglutamic acid is substituted with a few glucuronic acid residues or through a linkage which is easily cleaved by acid hydrolysis. A method to prepare N-glycosidically linked oligosaccharide moieties from glycoproteins with hydrazine was therefore applied to the A2 substance to examine the polyglucuronate chain region.

Optimum conditions for removal of glutamic acid residues were first established. The polyglucuronic acid chain was not completely stable during hydrazinolysis of the A2 substance (Table 1). The A2 substance was therefore treated with hydrazine for a shorter time than that usually used for glycoproteins (Järnefelt et al., 1982; Takasaki et al., 1982). Therefore, some of the glutamic acid residues remained in the hydrazinolysis product (Table 3). Hydrazinolysis may also partially cleave the linkages between the glucuronic acid residues, although almost all of the glucuronic acid was recovered as a high-$M_r$ component (Figs 1 and 2). The size heterogeneity of the hydrazinolysis product (Fig. 2) is therefore probably due to the effect of hydrazinolysis, although the size of the glucuronic acid component may be intrinsically polydisperse.

The $M_r$ of the sodium salt of the A2 substance was estimated to be 21 000 from the peak eluted from the Shodex WS 802.5F gel columns, using pullulans and dextrans as $M_r$ references (Fig. 3). The $M_r$ of the polyglutamate prepared previously from the A2 substance was distributed between 7800 and 24 000 with a peak at 14 000 in the same system. The $M_r$ of the polyglucuronate preparation was 4800 in this system, which should comprise on average 26 glucuronate residues, 1-3 glutamic acid residues and 0-2 galactosamine residue, based on the analysis in Table 3. The degree of polymerization of glutamic acid residues remaining in the polyglucuronate preparation should be low, although accurate determination was not possible. The glutamic acid component may contain an average of 110 residues on the basis of the analyses shown in Table 3 and the $M_r$ estimation of the A2 substance of 21 000. In addition, the glucuronic acid component should be composed, on average, of 24 residues. This value agrees with the number of glucuronate residues estimated for the polyglucuronate preparation and indicates that glucuronic acid residues must form only one structural unit in the A2 substance.

The ability to prepare two different homopolymers from the A2 substance suggests that the A2 substance is a complex composed of polyglutamate and polyglucuronate. If the A2 substance is a mixture of the two polymers, each of the polymer preparations should be free from components of the other polymer which was chemically depolymerized through chromatographic separation. It is tentatively suggested that the polyglucuronate acid which contains about 25 residues may be linked to muramic acid or glucosamine of the peptidoglycan (Table 3), and that the polyglutamic acid which contains about 110 residues may be linked to the polyglucuronate acid through galactosamine residues (Aono, 1987). However, the linkage between the polyglucuronate and polyglutamate components of the A2 substance is still unclear. Both the A2 substance and the polyglucuronic acid preparation produced glycolaldehyde and erythronic acid upon periodate oxidation. This preliminary result suggests that the glucuronic acid residues are joined through a 1,4-linkage. More detailed structural analysis is under way. A complex similar to the A2 substance may also exist in the group 2 strains of alkalophilic bacilli, because large amounts of uronic acids and acidic amino acids are found in their cell walls (Aono & Horikoshi, 1983). It is suggested that the A2 complex be called 'teichuronopeptide'.
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