Identification of L-iduronic acid as a constituent of the major extracellular polysaccharide produced by Butyrivibrio fibrisolvens strain X6C61

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1. SUMMARY

Butyrivibrio fibrisolvens strain X6C61 produces two extracellular polysaccharides (EPS-I and EPS-II) separable by anion-exchange chromatography. The neutral sugar constituents of EPS-I were identified by gas-liquid chromatography (GLC) as the alditol acetates of rhamnose, mannose, galactose, glucose, and an unidentified component. These results were confirmed using thin-layer chromatography (TLC). Neutral sugar analysis of EPS-II, which eluted from DEAE-Sephadex at 0.4 M NaCl, yielded the alditol acetates of rhamnose, galactose, glucose, and idose. However, idose was not found when hydrolysates of EPS-II were analysed by TLC. Further investigations showed that the iditol hexaacetate detected via GLC was an artifact of the commonly-used procedures for neutral sugar analysis. This compound was instead generated from L-iduronic acid, as shown by GLC-MS studies.

2. INTRODUCTION

Butyrivibrio fibrisolvens is one of the most frequently isolated species of ruminal bacteria [1,2]. There are, at present, a large number of isolates that fit the species description, with correspondingly wide range of reported metabolic activities [3].

Stack [4] has recently reported that many strains of B. fibrisolvens produce EPS containing unusual monosaccharide constituents. For example, B. fibrisolvens strain CF3 produces an EPS which contains L-altrose [5], the first reported occurrence of this hexose in nature. However, analysis of L-altrose-containing EPS by conventional alditol acetate procedures was ambiguous, due to the acid-catalyzed formation of 1,6-anhydroaltrose. Following reduction and acetylation, both altritol hexaacetate and 2,3,4-tri-O-acetyl-1,6-anhydroaltrose were produced. These two compounds yielded GLC peaks coincident with the alditol acetates of mannose and fucose, respectively [5].

Nonetheless, GLC analysis of alditol acetate derivatives remains a useful method for the de-
termination of the neutral sugar composition of polysaccharides [6]. However, uronic acids usually cannot be identified or quantitated by these procedures, and are generally determined by other methods.

During the course of our studies on extracellular polysaccharide (EPS) produced by various strains of B. fibrisolvens, one strain, X6C61, produced an EPS which yielded iditol hexaacetate upon hydrolysis, reduction, and acetylation according to the method of Albersheim et al. [6]. While these data would seem to indicate that the EPS of B. fibrisolvens strain X6C61 contains idose, a more thorough investigation has revealed that it contains L-iduronic acid instead.

3. MATERIALS AND METHODS

3.1. Organism and growth conditions
B. fibrisolvens strain X6C61, used in all studies, was kindly provided by N.O. van Gylswyk, National Chemical Research Laboratory, Pretoria, Republic of South Africa. It was isolated from a roll-tube containing 3% xylan-agar which had been inoculated from the rumen of a sheep fed corn stover. Cultures were grown on the chemically defined medium of Cotta and Hespell [7], as previously described [5].

3.2. Polysaccharide purification
Crude EPS was obtained from culture supernatants as previously described [5]. Crude EPS (50–100 mg) was dissolved in 10–20 ml of 10 mM potassium phosphate buffer pH 7.0, applied to a 2.5 x 8 cm column of DEAE-Sephadex A-25 (Pharmacia, Piscataway, NJ) which had been equilibrated with the same buffer, and eluted with a linear gradient of buffered sodium chloride (0–2.0 M, 800 ml). 10 ml fractions were collected and aliquots of each fraction were analyzed for neutral carbohydrate content via anthrone [8] and for uronic acids via the harmine procedure [9]. Pooled fractions (designated EPS-I and EPS-II) were dialyzed against water at 4°C and lyophilized.

3.3. Uronic acid reduction
The uronic acid(s) in EPS-II were reacted with the water-soluble diimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and reduced with either sodium borohydride (NaBH₄) or sodium borodeuteride (NaBD₄) using a modification of the procedure described by Taylor and Conrad [10]. EPS-II (25 mg) was dissolved in 5 ml H₂O and solid EDC (60 mg) was slowly added while the pH was maintained at 4.75 with dilute HCl (10–25 mM). The EDC-activated carboxyl group was reduced with either 2 M NaBH₄ or 2 M NaBD₄ (10 ml) over several hours, while the pH was maintained at 7.0–7.2 with 1–2 M HCl. The reaction mixture was acidified to pH 2 with 12 M HCl, and quickly returned to pH 7 with 10 M NaOH. These preparations were designated as EPS-II-EDC/NaBH₄ (or EPS-II-EDC/NaBD₄) and were dialyzed against water at 4°C and lyophilized.

3.4. Determination of the absolute configuration of iduronic acid
The absolute configuration of the iduronic acid in EPS-II was inferred from the configuration of the idose in EPS-II-EDC/NaBH₄. This was determined by analyzing the acetylated diastereomeric glycosides prepared from (−)-2-octanol and hydrolyzates of EPS-II-EDC/NaBH₄, as described by Leontein et al. [11].

3.5. Miscellaneous techniques
Neutral sugar analyses were done according to Albersheim et al. [6], as previously described [5]. TLC separation of EPS hydrolysates were performed on K5 silica gel plates (Whatman, Inc., Clifton, NJ) using acetonitrile/water (9:1) as the solvent [12]. Carbohydrates were visualized on developed plates using the N-(1-naphthyl) ethylenediamine dihydrochloride (Aldrich Chemical Co., Milwaukee, WI) spray reagent described by Bounias [13]. An idose/1,6-anhydroidose standard for TLC was prepared by heating 5 mg/ml l-idose (Sigma, St. Louis, MO) in 2 M trifluoroacetic acid (TFA) for 1 h at 100°C. Reduction and acetylation of this mixture afforded an iditol hexaacetate/2,3,4-tri-O-acetyl-1,6-anhydroidose standard for GLC and GLC-MS. Electron impact and chemical ionization mass spectra of alditol acetates were obtained as previously described [5]. Total carbohydrate was mea-
Fig. 1. Separation of crude EPS into two components by anion-exchange chromatography on DEAE-Sephadex A-25. O, total carbohydrate; *, uronic acids; •, NaCl gradient.

Table 1

<table>
<thead>
<tr>
<th>Monosaccharide content of various EPS fractions of B. fibrisolvens strain X6C61</th>
</tr>
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<tbody>
<tr>
<td>Polysaccharide preparations were hydrolyzed, reduced, and acetylated. Resulting alditol acetates were analyzed by gas-liquid chromatography/mass spectrometry. EDC-EPS refers to EPS-II-EDC/NaBH₄. Unknown is 4-O-(1-carboxyethyl)-D-galactose; proof of structure to be published elsewhere.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Relative amount (galactose = 1.00)</th>
<th>Total EPS</th>
<th>EPS-I</th>
<th>EPS-II</th>
<th>EDC-EPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,6-Anhydroidose</td>
<td>3.91</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.35</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>4.64</td>
<td>0.85</td>
<td>1.71 a</td>
<td>0.70</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>11.80</td>
<td>trace</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>12.90</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>14.02</td>
<td>0.99</td>
<td>0.67</td>
<td>0.97</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Inositol (internal standard)</td>
<td>15.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Idose</td>
<td>16.15</td>
<td>0.25</td>
<td>-</td>
<td>0.14</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>31.85</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.64</td>
<td></td>
</tr>
</tbody>
</table>

a Sometimes detected as a double peak, see text.
ment. The anhydro form constitutes ~ 90% of the total idose, yet was not detected in EPS-II hydrolysates by TLC or GLC (as 2,3,4-tri-O-acetyl-1,6-anhydroidose) following reduction and acetylation. These results confirm the absence of idose in EPS-II.

EPS-II contained uronic acid component(s), as determined by the harmine procedure [9]. The carboxyl group(s) of EPS-II was reduced with EDC and NaBH₄ (or NaBD₄), converting the uronosyl constituent(s) to its corresponding neutral sugar(s). GLC analysis of the alditol acetates from reduced EPS-II (EPS-II-EDC/NaBH₄) gave peaks coincident with 2,3,4-tri-O-acetyl-1,6-anhydroidose and iditol hexaacetate in a 9 : 1 ratio, in addition to the alditol acetates of rhamnose, galactose, and glucose. Also, TLC analysis of acid-hydrolyzed EPS-II-EDC/NaBH₄ yielded spots coincident with both idose and 1,6-anhydroidose, rhamnose, galactose, and glucose. GLC-MS analysis of the alditol acetates prepared from EPS-II-EDC/NaBH₄ was used to confirm the identities of both idose and 1,6-anhydroidose. The c.i. mass spectrum of the former compound (retention time = 16.15 min) had an M+1 peak at m/z 435, and its e.i. mass spectrum was identical to a library spectrum of a hexitol hexaacetate (data not shown). Similarly, the c.i. mass spectrum of the latter compound (retention time = 3.91 min) had an M+1 peak at m/z 289, and its e.i. mass spectrum was identical to a library spectrum of a 2,3,4-tri-O-acetyl-1,6-anhydrohexose. Both the retention times and mass spectra of these two compounds were identical to those obtained from similarly-derivatized standard L-idose.

GLC-MS investigations of the alditol acetates prepared from EPS-II-EDC/NaBD₄ showed a net gain of 2 a.m.u. in the M+1 peak of both 2,3,4-tri-O-acetyl-1,6-anhydroidose and iditol hexaacetate (data not shown). No deuterium was incorporated in the peaks corresponding to rhamnose, galactose, or glucose. These data conclusively show that iduronic acid, not idose, is a constituent of EPS-II.

The absolute configuration of the iduronic acid was deduced by GLC analysis of the acetylated diastereomeric glycosides prepared from (−)-2-octanol and hydrolysates of EPS-II-EDC/NaBH₄. Application of the method described by Leontein et al. [11] was complicated by the failure of 1,6-anhydroidose to form glycosides with (−)-2-octanol. Only the idose, representing about 10% of the total amount of idose/1,6-anhydroidose in the acid hydrolysates, formed glycosides with (−)-2-octanol, which, following acetylation, yielded GLC peaks coincident with those from similarly-treated L-idose (data not shown). The acetylated diastereomeric glycosides prepared from D-idose yielded peaks with retention times different from those obtained from EPS-II-EDC/NaBH₄. Therefore, the idose in these preparations must have the L-configuration, and, since no change in configuration occurs during carboxyl reduction, the iduronid acid in the original polysaccharide must also have the L-configuration.

These results conclusively show that L-iduronic acid is a constituent of the major EPS from B. fibrisolvens strain X6C61. The iditol hexaacetate detected in EPS-II using the method of Albersheim et al. [6] must have come from the reduction of iduronolactone, which was probably formed from iduronic acid following acid hydrolysis. Our results illustrate the potential for error when a single method, in this case the Albersheim procedure [6], is relied upon to analyze samples more complex than those for which they were originally intended.

L-Iduronic acid is a common component of several mammalian connective tissue polysaccharides, but the only prokaryotic polysaccharide previously reported to contain iduronic acid is the ‘type-specific’ polysaccharide of Clostridium perfringens strain Hobbs 10 [15]. Tsuchihashi et al. [16] have recently reported that L-iduronic acid is a constituent of the glycuronans produced by several different species of fungi. In all of these reports, and in the present case as well, the iduronic acid found has had the L-configuration.

Of nearly 35 strains of B. fibrisolvens which have been screened for polysaccharide production, only strain X6C61 contains iduronic acid [4]. This could thus represent a useful biochemical marker for following this strain or any recombinant strains derived from X6C61 in the rumen or other environments.
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REFERENCES