A Polysaccharide Antigen from the Gram-positive Organism *Eubacterium saburreum* Containing Dideoxyhexose as the Immunodominant Sugar

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A highly active surface antigen, reacting by precipitation and complement fixation, has been isolated from *Eubacterium saburreum* strain L32. The antigen is a polysaccharide polymer built up of galactose, ribose and dideoxyhexose. The dideoxyhexose is the immunodominant sugar.

INTRODUCTION

Eubacterium saburreum is an anaerobic Gram-positive micro-organism inhabiting the oral cavity of man. Type-specific cell wall polysaccharide antigens of unusual composition have been isolated from three strains of *E. saburreum*: strain L44 (PS L44) (Hofstad, 1971, 1972; Hofstad & Selvig, 1972), strain L49 (PS L49) (Hofstad, 1975) and strain L452 (PS L452) (Hofstad & Lygre, 1977). PS L44 is a linear polysaccharide composed of partly *O*-acetylated (1→6)-linked β -D-glycero-D-galacto-heptopyranosyl residues (Hoffman *et al.*, 1974), whereas PS L49 is a heteropolymer which, in addition to D-glycero-D-galacto-heptose and *O*-acetyl has 6-deoxy-D-altro-heptose as furanosidic end group (Hoffman *et al.*, 1976). PS L452 is a polysaccharide composed of trisaccharide repeating units of D-galactopyranosyl, D-ribo-furanosyl and D-fucufuranosyl residues, with the latter in end position (Hoffman *et al.* 1977).

The present report deals with the immunochemistry of a polysaccharide polymer isolated from another strain of *E. saburreum*.

METHODS

Organism and cultivation. *Eubacterium saburreum* strain L32 was isolated from human saliva. The organism was cultured in 500 ml screw-capped bottles filled to the top with medium, pH 7.0, containing (g l⁻¹): Tryptone (Difco), 15.0; NaCl, 5.0; KH₂PO₄, 1.5; Na₂HPO₄·2H₂O, 3.5; (NH₄)₂SO₄, 0.5; glucose, 5.0; yeast extract (Difco), 3.0; L-cysteine.HCl, 1.0; haemin, 0.016; menadione 0.0006; and human plasma, 50 ml l⁻¹. After incubation for 2 d at 37 °C, the cells were harvested by centrifugation, washed twice with phosphate-buffered saline and stored at -25 °C.

Extraction and purification of the polymer. The polymer was isolated as previously described (Hofstad, 1975). Frozen or acetone-dried cells were digested overnight at 37 °C with trypsin (Trypsin 1:250, Difco) in 0.02 M-Tris/HCl buffer, pH 7.8, containing 0.05 M-CaCl₂, and then centrifuged (8000g, 30 min). The polysaccharide polymer was purified from the supernatant fluid by gel filtration (Sephadex G-75), ion-exchange chromatography (DEAE-cellulose) and affinity chromatography (Bio Gel HTP).

Acid degradation of the polymer. Samples of the purified polysaccharide were hydrolysed with 0.05 M-H₂SO₄ at 100 °C for 4 h, or at 80 °C for 3 h. After neutralization with Amberlite IRA 410 (HCO₃⁻ form) and evaporation to dryness in a rotary evaporator, the hydrolysed samples were taken up in a small volume of distilled water and applied to a column of Sephadex G-15. The column had been equilibrated and was eluted with distilled water. The elution volumes of trisaccharides, disaccharides and monosaccharides were 1.2 × V₀, 1.3 × V₀ and 1.6 × V₀, respectively. The elution of the degradation products was monitored by differential refractometry.

Paper chromatography. Acid hydrolysis was performed in sealed tubes at 100 °C with 2 M-H₂SO₄ for 4 h or with 0.1 M-HCl for 48 h. After removal of acid with Dowex 1 (formate form) or Amberlite IRA 410 (HCO₃⁻ form), the samples were subjected to circular chromatography on Whatman no. 1 paper with 1-butanol/pyridine/water (6:4:3, by vol.). The dried chromatograms were stained with aniline hydrogen phthalate (Partridge, 1949) or silver nitrate (Partridge, 1946).

Gas-liquid chromatography (g.l.c.). Samples hydrolysed in 0.5 M-H₂SO₄ at 100 °C for 18 h were neutralized with Amberlite IRA 410 (HCO₃⁻ form) and the aldoses were converted to alditol acetates by the method of Sawardeker, Sloneker & Jeanes (1965). These were separated in a Perkin-Elmer 900 chromatograph with a flame-ionization detector, and fitted with a glass column (0.2 × 180 cm) packed with 3 % (w/w) ECNSS-M on Gas-Chrom Q, 100 to 120 mesh. The flow of N₂ gas was 30 ml min⁻¹, and the column, injector and detector temperatures were 180, 200 and 250 °C, respectively. D-Xylose was used as an internal standard.

Chemical analyses. Protein was measured by the Folin-Ciocalteu phenol method (Lowry *et al.*, 1951). The malonaldehyde-thiobarbituric acid test for 2-deoxy or 3-deoxy sugars was carried out as described by Weissbach & Hurwitz (1959) or with the modification of Cynkin & Ashwell (1960).

Immunoelectrophoresis was performed as described earlier (Hofstad & Selvig, 1972).

Analytical ultracentrifugation. The sedimentation pattern of the purified polymer was examined at 20 °C in a MSE Centriscan 75 ultracentrifuge at a rotor speed of 60000 rev. min⁻¹, using a 10 mm single sector cell. Freeze-dried samples were dissolved in 0.05 M-phosphate buffer, pH 6.8.

Serological methods. Antisera were produced in rabbits by intravenous injection of washed microbial cells (Hofstad, 1969). Treatment of antiserum with 2-mercaptoethanol was performed as described earlier (Hofstad, 1969).

Difco Special Agar Noble (1 %, w/v) in saline was used for gel diffusion tests.

Ring test precipitation was performed with undiluted antiserum, and quantitative precipitation as described by Kabat & Mayer (1961). Undiluted, cleared antiserum (0.2 ml) mixed with an equal volume of antigen was incubated for 1 h at 37 °C followed by 2 d at 4 °C. Precipitated antibody was determined by the Folin-Ciocalteu method (Lowry *et al.*, 1951). In precipitation inhibition experiments, inhibitor in saline (0.1 ml) was mixed with cleared, undiluted antiserum (0.2 ml). After incubation at 37 °C for 30 min, 25 µg antigen in saline (0.2 ml) was added, and the mixture was incubated at 4 °C for 2 d. The methods for inhibition of bacterial agglutination and indirect haemagglutination have been described (Hofstad & Lygre, 1977; Hofstad, 1969). In complement fixation tests (Hofstad, 1969), two 100 % lytic units of complement and two units of amboceptor were used. Serum dilution (0.1 ml), antigen (0.1 ml) and complement (0.2 ml) were incubated overnight at 4 °C, and then 0.2 ml of a 1 % suspension of sensitized sheep erythrocytes was added.

RESULTS

Purification of the polymer

The polysaccharide polymer, referred to hereafter as PS L32, was prepared from two batches of L32 micro-organisms: 2.4 g of acetone-dried cells were digested with 48 mg trypsin in 240 ml buffer, and 20 g of packed, frozen cells were digested with 80 mg trypsin in 400 ml buffer. After centrifugation, the supernatant fluid gave three lines on double diffusion in agar against L32-antiserum: a heavy line produced by PS L32 and two weaker lines due to contaminating antigens.

During the different steps of purification, PS L32 behaved in the same way as the polymers isolated previously, i.e. PS L44, PS L49 and PS L452. Gel filtration through Sephadex G-75 separated PS L32 from low molecular weight substances. The contaminating antigens and ultraviolet light-absorbing material were retained on the DEAE-cellulose column, whereas PS L32 was eluted in the void volume. Chromatography on hydroxylapatite (Bio Gel HTP) separated PS L32 from the last traces of contaminating proteins.

Criteria of purity

When examined by immunoelectrophoresis against L32-antiserum, PS L32 produced a single line of precipitation, located at the cathodic side of the point of application. By analytical ultracentrifugation, PS L32 sedimented as a sharp peak. The uncorrected sedimentation coefficient (*s*₂₀) was 1.23.

Fig. 1. The quantitative precipitation curve of PS L32 with 0.2 ml of undiluted antiserum to L32 micro-organisms.

Chemical composition

Galactose, ribose and a fast-moving component were detected by paper chromatography of hydrolysates made with 0.1 M-HCl. The fast-moving component, which had an R_{rhamnose} of 1.26, gave a red-brown colour with aniline hydrogen phthalate. It was not detected after hydrolysis of PS L32 with 2 M- H_2SO_4 . Amino sugars were not found. On g.l.c. the alditol derivative of the fast-moving sugar showed a retention time relative to xylitol of 0.28. When examined in the malonaldehyde-thiobarbituric acid reaction, PS L32 produced a peak at 532 to 536 nm. Taken together, the chromatographic and colorimetric examinations showed that the unknown sugar is almost certainly a dideoxyhexose. The quantitative composition of PS L32 is shown in Table 1.

Serological properties

The serological activity of PS L32 was examined against antisera to whole L32 micro-organisms. The lowest concentration of PS L32 which gave a positive ring test was $0.98 \mu\text{g ml}^{-1}$. Amounts of PS L32 as low as $0.0039 \mu\text{g}$ gave maximal titres in complement fixation tests. The agglutination of whole L32 microbial cells in L32-antiserum was inhibited by $12.5 \mu\text{g}$ PS L32. PS L32 did not sensitize sheep erythrocytes to agglutination. The quantitative precipitation curve is shown in Fig. 1.

Treatment of L32-antiserum with 2-mercaptoethanol destroyed the antibody activity against PS L32.

Degradation experiments

Chromatography on Sephadex G-15 of the products resulting from acid hydrolysis of PS L32 with 0.05 M- H_2SO_4 for 3 h at 80°C yielded three fractions. The majority of the degraded material was eluted with the void volume (fraction I), and smaller amounts at elution volumes of $1.2 \times V_0$ (fraction II) and $1.7 \times V_0$ (fraction III). Fraction I was freeze-dried, and samples of it were examined for neutral sugars by g.l.c. and for activity as an inhibitor in precipitation inhibition experiments. The remaining two fractions were each divided in two equal parts; one was subjected to g.l.c. and the other tested for activity as an inhibitor. Table 2 shows the results.

Chromatography on Sephadex G-15 of PS L32 degraded by hydrolysis in 0.05 M- H_2SO_4 for 4 h at 100°C yielded three fractions, the elution volumes of which were $1.2 \times V_0$ (fraction I), $1.3 \times V_0$ (fraction II) and $1.7 \times V_0$ (fraction III). The last fraction contained the majority of the degraded material. The fractions were again examined by g.l.c. for neutral sugars, and for their capacity to inhibit the precipitation of PS L32 in L32-antiserum. The results are listed in Table 2.

Table 1. *Chemical composition of the polysaccharide polymer PS L32**

Organisms	Yield (mg)	Protein (%)	Galactose		Ribose		Dideoxyhexose	
			(% dry wt)	(mol %)	(% dry wt)	(mol %)	(% dry wt)	(mol %)
Batch 1 Acetone-dried, 2.4 g	45	4.5	41.8	41.9	25.8	31.0	21.9	27.1
Batch 2 Packed, wet, 20 g	40	—	36.6	34.7	24.8	28.3	31.0	37.0

* Results have not been corrected for water uptake during hydrolysis.

Table 2. *Sugar composition and antibody neutralizing capacity of the polysaccharide polymer PS L32 degraded with acid and chromatographed on Sephadex G-15*

Treatment of PS L32	Fraction	Molar ratios			Inhibition of precipitation*		
		Galactose	Ribose	Dideoxyhexose	(mg inhibitor)†	(% inhibition)	
0.05 M-H ₂ SO ₄ 80 °C, 3 h	I	1.3	1	0.4	0.01 ‡		21
	II	1.2	1	0.2	0.1		22
	III	1.4	1	14.0	0.07		16
0.05 M-H ₂ SO ₄ 100 °C, 4 h	I	0.7	1	ND	0.02		3
	II	1.3	1	ND	0.8		5
	III	0.8	1	1.3	1.0		20

* 25 µg PS L32 in L32-antisera. † Calculated from g.l.c. ‡ Dry weight of freeze-dried material.
ND, Not detected.

DISCUSSION

The present study provides further evidence for the presence in *Eubacterium saburreum* of highly active, type-specific surface polysaccharide antigens of unusual chemical composition. Most likely, the PS L32 polymer is built up of repeating trisaccharide units of galactose, ribose and dideoxyhexose. Structural studies have indicated that the dideoxyhexose is tyvelose (3,6-dideoxy-D-mannose) (J. Hoffman, personal communication). The structure of PS L32 may be analogous to that of PS L452 of strain L452, which contains disaccharides of galactose and ribose, to which are linked D-fucofuranosyl residues (Hoffman *et al.*, 1977).

To the best of our knowledge the present report is the first demonstration of the occurrence of dideoxyhexose in a Gram-positive micro-organism. Dideoxyhexoses have previously been found as immunodominant sugars of the cell wall lipopolysaccharide (O-antigen) of some *Salmonella* species, *Escherichia coli* and a few other bacterial species (Cummins, 1973).

Fractions I and II obtained by gel filtration of the degradation products after treatment of PS L32 with H₂SO₄ for 4 h at 100 °C, and which did not contain dideoxyhexose, were not able to inhibit the precipitation of PS L32 in L32-antiserum. In contrast, all other fractions, containing dideoxyhexose, exhibited some antibody-neutralizing capacity. This clearly indicates that the dideoxyhexose is the immunodominant sugar of PS L32.

The sensitivity to 2-mercaptoethanol of the specific precipitating and complement binding antibodies in L32-antiserum indicates that they were IgM antibodies. In contrast, rabbit antisera against the *E. saburreum* strains L44, L49 and L452 contained low molecular weight antibodies against the specific polysaccharides isolated from the same strains (Hofstad, 1972, 1975; Hofstad & Lygre, 1977).

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