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Note

Sedimentation field flow fractionation and gas chromatography–mass spectrometry for characterization of streptococcal cell wall particles

JAMES GILBART, ALVIN F. WELLS, MEE H. HOE and ALVIN FOX*
Department of Microbiology and Immunology, University of South Carolina, School of Medicine, Columbia, SC 29208 (U.S.A.)
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Preparations of streptococcal cell wall particles have been shown to consist of a heterogeneous mixture of polymers of a wide molecular weight range[1]. The peptidoglycan has a backbone of alternating units of N-acetylglucosamine and N-acetylmuramic acid to which are attached highly crosslinked peptide side chains. The polysaccharide consists of a rhamnose backbone with N-acetylglucosamine side chains[1,2]. There is a strong relationship between the chemical and physical composition of large cell wall particles and their biological activity[1]. Furthermore, there is a change in size of this material during biodegradation, which may alter these properties[3]. There is thus a need for a means of fractionation and characterization of mixtures of large cell wall particles.

Sedimentation field flow fractionation (SFFF) is a new technique for the rapid separation and molecular weight estimation of microparticulates and macromolecules. The SFFF system is based on a high-performance liquid chromatograph in which the analytical column is replaced by a modified centrifuge. The principle of SFFF and its advantages over conventional approaches to separation of high-molecular-weight particles have been discussed in detail[4–8]. In addition to bacterial cell walls[9] this method has proved valuable in the separation of particulates including viruses[10] liposomes[11] and nucleic acids[12].

One problem with SFFF analysis which has previously not been considered in depth is the fact that the sample is greatly diluted during the physical separation making subsequent chemical analyses difficult. High selectivity and sensitivity in such a chemical method is required. In previous work we devised a procedure for the analysis of the carbohydrate composition of unfractionated cell wall preparations by gas chromatography[13]. We subsequently modified the method for trace analysis of cell wall debris by gas chromatography–mass spectrometry (GC–MS)[14,15]. In the present study, we demonstrate a combined SFFF/GC–MS approach for the isolation and characterization of cell wall particles.

MATERIALS AND METHODS

Preparation of streptococcal cell wall sonicates

Batches (20 l) of *Streptococcus pyogenes* (ATCC 10389) were harvested and the washed cells sonicated for 90 min and centrifuged for 30 min at 12 000 *g*. The

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pooled supernatants were treated sequentially with hyaluronidase (Sigma, St. Louis, MO, U.S.A.), deoxyribonuclease (Calbiochem, La Jolla, CA, U.S.A.), ribonuclease (Calbiochem), papain (Sigma) and pepsin (Sigma). The buffers were changed between each enzyme treatment by dialysis overnight at 4°C and the digestion itself was performed while dialysing at 37°C for 6 h. The cell walls were collected and washed by repeated centrifugation at 111 000 *g* and extracted three times with chloroform–methanol–water (34:17:10). The water phase was then dialysed against water and lyophilized. The cell walls were re-sonicated and filtered through a series of sterile filters including finally a 0.2-μm cellulose triacetate filter (Type GA metricel; Gelman, Ann Arbor, MI, U.S.A.). This method has been previously described[16].

Sedimentation field flow fractionation

A prototype instrument on loan from Dupont was used in these studies. The instrument consisted of an SFFF rotor housed in a modified RC-5B centrifuge, a Waters U6K injector, an 8800 Model HPLC pump, a variable wavelength detector, an analog-to-digital and control interface and a 9816 computer (Hewlett-Packard, Palo Alto, CA, U.S.A.). The sequence of events during an SFFF analysis were computer controlled as follows. Centrifugation was at 10 642 *g* and the pump rate was 0.2 ml/min. A 200-μl aliquot of cell wall suspension in phosphate-buffered saline was injected and the pump continued to run for 1.5 min to allow sufficient displacement for the sample to reach the rotor channel. The flow of PBS was then stopped for a 5-min relaxation time during which the channel equilibrates at the desired field strength. The pump was then restarted at 1 ml/min and detector signal plot was started. The eluent was monitored at 220 nm. After an interval of 4 min the centrifuge speed was decayed exponentially with a decay constant of 4 min. The total run time was about 45 min. The fractions were manually collected after passing through the detector.

The efficacy of separation and leak-free operation of the SFFF system were tested using a 1:1 mixture of phenol red (Sigma; 5 mg/ml) and dextran blue (Sigma, 50 mg/ml). Since dextran is considerably larger than phenol red, the two dyes should be readily separable based on their size difference. With high concentrations of each the color could be easily visualized when collected. A 200-μl aliquot was injected and run under the same conditions as the cell walls. The phenol red was unretained and most of the blue dextran was retained.

Carbohydrate analysis by GC–MS

The SFFF fractions were analysed by the alditol acetate procedure and GC–MS. This alditol acetate method has been previously described[14,15]. In brief, the samples were adjusted with 6 *M* sulfuric acid to a 1 *M* final concentration and a final volume of 1.2 ml and hydrolysed under vacuum at 100°C for 3 h. Arabinose (5 μg) and methylglucamine (5 μg; as internal standards for neutral and amino sugars) were added and neutralized with 40% N,N-dioctylmethylamine in chloroform. The aqueous layer of each hydrolysis mixture was extracted on a C_{18} column. The samples were reduced with 50 μl of sodium borohydride (100 μg/ml) at 37°C for 90 min. A 2-ml volume of acetic acid–methanol (1:200, v/v) was added to each sample which were then evaporated to near dryness at 60°C under vacuum, repeated four additional times and were allowed to dry for 3 h after the last evaporation. After cooling, 300 μl of acetic anhydride were added to each vial and the samples were heated at 100°C

for 13–16 h. Water (1 ml) was added to each vial and left for 30 min. Chloroform (1 ml) was added, and the aqueous phases removed and discarded. To each chloroform phase, 0.8 ml of cold ammonium hydroxide (80%, v/v) was added. The mixtures were each poured onto a silica (Chem Elut) column and eluted with 2 ml of chloroform. The chloroform solutions were evaporated to dryness under vacuum and redissolved in about 40 μl of chloroform before analysis.

GC–MS analyses were carried out using a Hewlett-Packard 5970 mass selective detector interfaced via a capillary direct inlet to a HP-5890 gas chromatograph equipped with a capillary inlet system and a 25 m × 0.22 mm I.D. BP10 (OV-1701 bonded phase) fused silica column (SGE, Austin, TX, U.S.A.). Three groups of ions were monitored at the appropriate retention times: first, *m/z* 289.05 and 303.1 for neutral sugars, secondly, *m/z* 318.0 and 403.1 for glucosamine and muramic acid, respectively, and thirdly, *m/z* 327.25 for methylglucamine. The injection was in the splitless mode at 100°C, held for 45 s then programmed at 30°C/min to 230°C and then at 4°C/min to 250°C and 3°C/min to 265°C and held for 4.5 min. The injector temperature was 250°C and the MS interface, 270°C.

Reducing group determination

The relative reducing capacity of the fractions was determined by the method of Thompson and Shockman[17,18] modified to accommodate larger sample volumes. Glucose (Sigma) was used as a standard. The method was linear over a 0–20 μg/ml range. To 2.8 ml of each sample, 0.1 ml of 0.5% (w/v) potassium ferricyanide and 0.1 ml of a sodium carbonate–potassium cyanide mixture (53 g sodium carbonate and 6.5 g potassium cyanide/l double distilled water) were added. The tubes were vortexed and then boiled for 15 min in a water bath. After cooling in ice for 5 min, 2.5 ml of 0.05 *M* sulfuric acid was added to all tubes and vortexed. Finally 1 ml of a solution containing equal volumes of ferric ammonium sulfate (15 g/l 0.025 *M* sulfuric acid), sodium dodecyl sulfate (3 g/l 0.025 *M* sulfuric acid) and polyethylene glycol (0.25 ml/100 ml 0.025 *M* sulfuric acid) was added. The tubes were vortexed and left to stand at room temperature for 15 min. All tubes were then read spectrophotometrically at 700 nm.

RESULTS AND DISCUSSION

In initial experiments suspensions of cell wall sonicates were established to absorb well at 220 nm. This was probably due to the presence of peptide bonds within the peptidoglycan–polysaccharide (PG–PS) structure. The fractionation of the streptococcal cell wall preparation achieved by SFFF is shown in Fig. 1. The peak is very broad, indicating that the sample does not consist of discretely sized polymers but a range of molecular weights from *ca.* 10^6 to 10^{10} with a mode of 10^8. This distribution confirms our previous observations[9]. Seven 200-μl samples of PG–PS were separated by SFFF and 4-ml fractions collected. Each run produced similar fragmentograms and computer-calculated molecular weight values. Corresponding fractions from the seven runs were pooled to provide sufficient volume for multiple analyses. These intervals are plotted on the fragmentogram in Fig. 1.

SFFF is theoretically an absolute method for the determination of molecular weight using post-run calculations based on the variables: retention time, sample density, mobile phase flow-rate and field strength[7]. However, based on our experience there is a need for the development of defined biological molecular weight standards for instrument calibration.

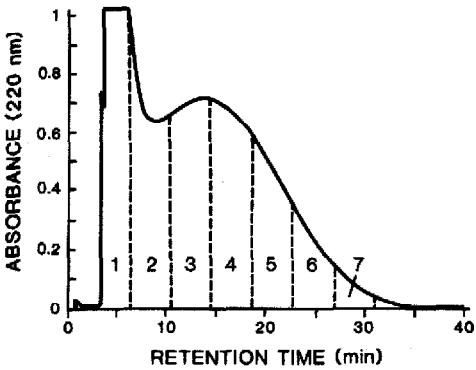

Fig. 1. SFFF fragmentogram of streptococcal cell wall sonicate. The times at which fractions were collected are shown. The average molecular weights for each fraction were: (1) $1.06 \cdot 10^7$; (2) $3.65 \cdot 10^7$; (3) $9.08 \cdot 10^7$; (4) $2.47 \cdot 10^8$; (5) $6.73 \cdot 10^8$; (6) $1.67 \cdot 10^9$; (7) $4.9 \cdot 10^9$.

We performed GC–MS analyses for carbohydrates to characterize SFFF fractions. In total, 91.6% of the PG–PS injected into the SFFF could be accounted for in the subsequent fraction analysis. Fig. 2 shows comparative chromatograms of alditol acetates from (A) an early eluting SFFF fraction (fraction 1) and (B) a later

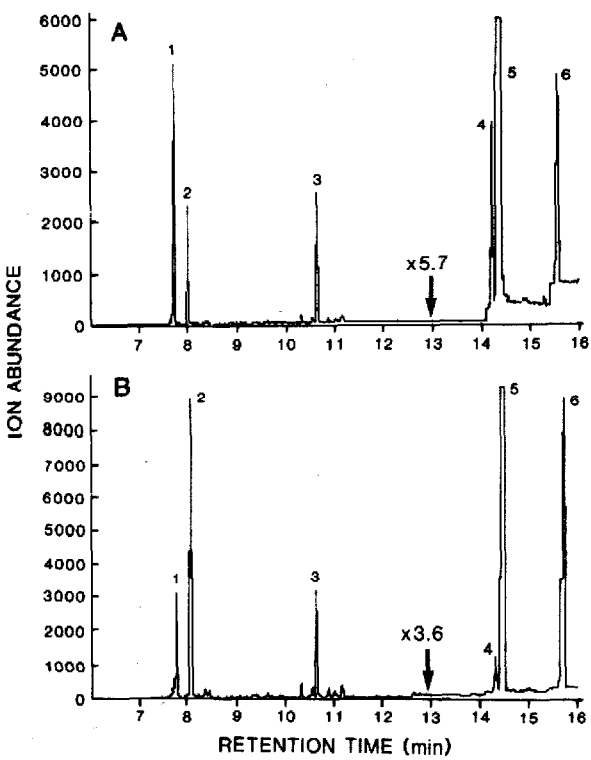

Fig. 2. Selected ion chromatograms of alditol acetates from (A) fraction 1 and (B) fraction 6 derived from SFFF fractionation of streptococcal cell walls. Peak identification: 1 = rhamnose; 2 = arabinose (internal standard); 3 = glucose; 4 = muramic acid; 5 = glucosamine and 6 = methylglucamine (internal standard).

TABLE I

CARBOHYDRATE, REDUCING GROUP CONTENT AND OPTICAL DENSITY MEASUREMENTS OF SAMPLES OBTAINED FROM SFFF

Reducing group content represents the equivalent of glucose per ml. ND = not detected, (reducing group levels in these fractions were too low to be reliably quantitated).

Fraction No.	*Rhamnose (μg/ml)*	*Glucose (μg/ml)*	*Muramic acid (μg/ml)*	*Glucosamine (μg/ml)*	*Total carbohydrate*	*Reducing group (μg/ml)*	*Optical density/ total carbohydrate ratio ($\times 10^{-2}$)*
1	39.1	2.5	10.4	45.0	97.0	25.1	1.0
2	21.7	2.3	6.5	20.4	50.9	16.9	1.2
3	22.2	1.0	5.5	16.7	45.5	9.3	1.5
4	17.0	0.8	4.3	17.5	39.6	7.0	1.7
5	9.9	0.6	3.4	10.2	24.1	3.6	1.8
6	3.8	0.4	1.0	4.0	9.2	ND	2.2
7	0.3	0.9	0.2	0.4	1.7	ND	2.9

eluting fraction (fraction 6), showing less rhamnose, glucose, muramic acid and glucosamine relative to the internal standards, resulting from a decreased concentration of PG–PS towards the end of the SFFF run. Each fraction was analysed in triplicate.

We obtained reducing data on fractions 1–5. Other fractions contained insufficient material for reducing group analysis. The ratio of reducing group to carbohydrate content only decreased two-fold between the lowest (fraction 1) and highest molecular weight fraction (fraction 5) suggesting that the carbohydrate polymer lengths only decreased two-fold. This result was consistent with earlier studies[1].

We also calculated the optical density/carbohydrate ratios for fractions 1–7. As would be expected the ratio gradually increased between the fraction containing the smallest particles and the one containing the largest. Table I summarizes sugar, reducing group and optical density readings for the seven fractions.

The results indicate that SFFF can fractionate mixtures of bacterial cell wall particles on the basis of molecular weight in sufficient quantity for GC–MS analysis. The combination of these two methods allows detailed determination of their physical and chemical composition. The availability of well characterized cell wall particles will be helpful in studying the relationship between their molecular weight and biological activity. Furthermore this combined SFFF/GC–MS approach may have wide utility in the analysis of other mixtures of high molecular weight biological particulate mixtures.

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