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Determination of glycerol in bacterial cell wall teichoic acid by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatography method for the determination of glycerol in teichoic acid was developed. The phosphodiester linkages between repeating glycerol units were hydrolysed by treatment with 5.8 *M* HCl at 100°C for 75 h. The degree of hydrolysis was verified by gas chromatography–mass spectrometry. The hydrolysed samples were applied to an ion-exclusion column and the amount of glycerol determined as the area of the peak at 12.6 min. Using glycerol standards an r^2 value of 0.999, a relative standard deviation of 2.2% and a detection limit of 0.1 μg were obtained.

INTRODUCTION

The cell surface of gram-positive bacteria contains lipoteichoic acid (LTA) and a peptidoglycan–teichoic acid complex (PG–TA) as the major and biologically important molecules. Interest in these molecules is based on their role in the structure and function of the bacterial cell wall [1] and on their potential participation in modifying the course of infection and response to vaccines [2,3].

The basic structure of LTA and TA consists of a long hydrophilic chain of 20–60 repeating phosphoglycerol units where the individual glycerol (Gro) molecules are joined by a phosphodiester linkage [1]. Thus the determination of Gro in these compounds is of crucial importance to structure–function studies. This is usually carried out after cleavage of the phosphodiester bond by treatment with acid or alkaline phosphatase, or both. The liberated Gro is then determined by gas chromatography (GC) [4,5], enzymatically by a glycerol kinase-based assay [6,7], by potentiometric [8] or luminometric [9] methods. A preliminary report has been published describing the determination of Gro in grape juice by high-performance liquid chromatography (HPLC) [10].

The purpose of this study was to develop and validate a simple HPLC-method for the determination of Gro in bacterial cell surface compounds. The goal was to avoid the use of tedious enzyme reactions or toxic HF for the liberation of Gro and the multi-step sample preparations for the liberated Gro. The data obtained demonstrate the feasibility of applying the Gro sample directly after hydrochloric acid treatment to an ion exclusion column coupled to a refractive index (RI) detector using commercial α-phosphoglycerol (P-Gro) and PG-TA of *Bacillus subtilis* to standardize the method.

EXPERIMENTAL

Reagents

All chemicals used were commercially available and of analytical purity. P-Gro and Gro were from

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Sigma (St.Louis, MO, USA) and iodomethane from Fluka (Buchs, Switzerland).

Chemical methods

The hydrolyses were carried out in sealed glass tubes. Inorganic and total phosphate were determined by the spectrophotometric method of Lowry *et al.* [11]. The amount of organic phosphate was calculated from the difference between inorganic and total phosphate. Permethylation was performed by CH_3I in a basic solution of dimethyl sulphoxide after diazomethane treatment according to the method of Ciucanu and Kerek [12].

Gas chromatography–mass spectrometry

Combined GC–mass spectrometry (MS) analyses were performed using a Hewlett-Packard 5890 Series II gas–liquid chromatograph (Avondale, PA, USA) and a Hewlett-Packard 5971A mass selective detector equipped with an HP-5 fused-silica capillary column (25 m × 0.2 mm I.D.). Helium was used as the carrier gas. The initial temperature of 100°C was held for 1 min and then increased at a rate of 7°C/min to the final temperature of 250°C. The injector temperature was 250°C and the ion-source temperature 185°C. Electron impact mass spectra were recorded at 70 eV.

High-performance liquid chromatography

The HPLC analyses were carried out using a Waters Model 510 pump (Milford, MA, USA) with a 20-μl injection loop equipped with a Pharmacia 2142 RI detector (Bromma, Sweden) and a Shimadzu C-R3A integrator (Tokyo, Japan). The ion-exclusion column (Aminex HPX-87H) and the guard column were from Bio-Rad (Richmond, CA, USA). Sulphuric acid (0.004 *M*) in distilled and deionized water was used as the eluent at a flow-rate of 0.6 ml/min.

RESULTS AND DISCUSSION

Optimum conditions for the hydrolysis of glycerol-phosphoester bonds

The liberation of phosphate from P-Gro was first tested in 0.1 *M* HCl. Table I shows that after 3 days of this treatment at 100°C only 50% of the P-Gro was hydrolysed to inorganic phosphate and Gro. Total hydrolysis was achieved only after a prolonged hydrolysis of up to 11 days.

TABLE I

ORGANIC PHOSPHATE DETECTED AFTER PROLONGED HYDROLYSIS OF P-Gro IN 0.1 *M* HCl

P-Gro (1 mg/ml) was hydrolysed in 0.1 *M* HCl at 100°C for various times and organic phosphate (P_{org}) determined as described under Experimental.

Time (h)	P_{org} (%)
0	100
1	100
2	97
6	94
24	78
65	50
260	0.1

Increasing the HCl concentration from 0.1 to 2.0 *M* did not influence the rate of hydrolysis (Table II), apparently because the mechanism of hydrolysis at low concentrations is based on an intramolecular rearrangement reaction [13]. An increase in the concentration of acid to above 2.0 *M* increased the rate of hydrolysis, consistent with the change of the reaction to second-order kinetics. Thus after 65 h at 100°C, 87% of P-Gro was hydrolysed in 4.0 *M* HCl and 98% in 5.8 *M* HCl (Table II).

Fig.1 shows the time course of the hydrolysis of P-Gro in 5.8 *M* HCl. About 70% of P-Gro was hydrolysed after 1 day and about 90% after 2 days. The hydrolysis was completed within 75 h.

TABLE II

ORGANIC PHOSPHATE DETECTED AFTER A 65 h HYDROLYSIS OF P-Gro IN HCl

P-Gro (1 mg/ml) was hydrolysed for 65 h at 100°C in HCl of various concentrations and organic phosphate (P_{org}) determined as described under Experimental.

Concentration of HCl (*M*)	P_{org} (%)
0	100
0.1	50
0.5	50
1.0	50
2.0	58
4.0	13
5.8	2.4

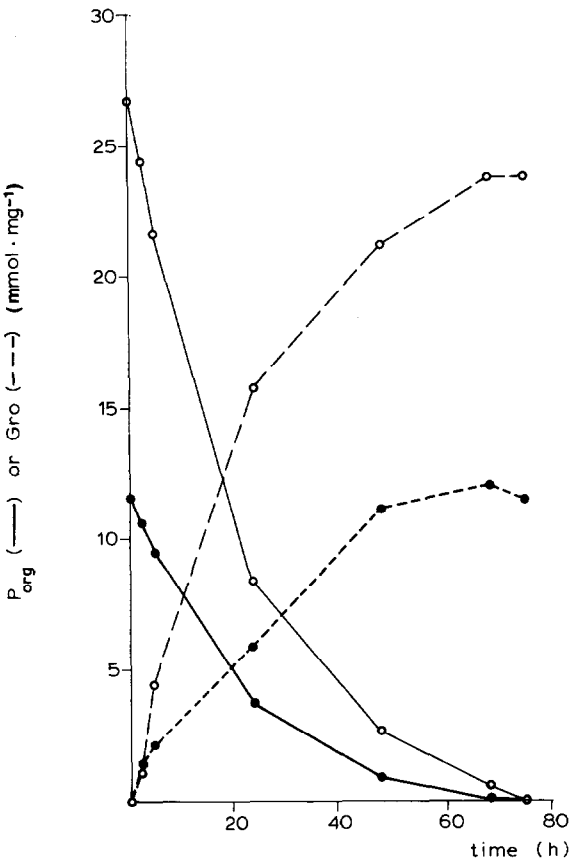

Fig. 1. Kinetics of hydrolysis of P-Gro (○) and PG-TA (●) in 5.8 *M* HCl at 100°C. Solutions (1 mg/ml) of the samples were hydrolysed in HCl and organic phosphate (solid lines) and Gro (dotted lines) were determined at different times as described under Experimental.

The production of Gro by the hydrolysis of P-Gro with HCl was verified by GC. Intact and hydrolysed (5.8 *M* HCl, 100°C, 75 h) samples of P-Gro were permethylated and investigated using GC–MS. Intact, permethylated P-Gro was detected by GC as one major peak (retention time 8.1 min). This peak yielded the electron impact mass spectrum shown in Fig. 2a, showing characteristic fragments of methylated phosphate at *m/z* 109 and 127. In the hydrolysed sample this peak was negligible and instead a prominent peak at 3.7 min appeared. The mass spectrum of this peak (Fig.2b) was indistinguishable from that obtained with authentic Gro, including the molecular ion of permethylated Gro (*m/z* 134).

The results show that the use of HCl is applicable in hydrolysing phosphoglycerol compounds. Both HCl and the commonly used HF [14] demand prolonged treatment, but HF is more expensive, potentially toxic and inconvenient to use.

Determination of glycerol

In the HPLC analysis authentic Gro yielded a single peak with a retention time of 12.6 min. The calibration graph for Gro was obtained by applying Gro standards to the ion exclusion column and comparing the areas of the peaks at 12.6 min. The results followed the linear regression equation $y = 1.087x + 0.580$ ($r^2 = 0.999$), where y is the relative area and x is the amount of glycerol (Fig.3).

The reproducibility was determined by five independent injections of 20 μg of glycerol. The areas varied from 21.382 to 22.833 and a relative standard deviation of 2.2% was obtained.

The detection limit for glycerol was 0.1 μg, corresponding to a signal-to-noise ratio of 2. As an injection volume of 20 μl was used this is equal to a concentration of 5 μg/ml. This is better than those reported for the potentiometric (10 μg/ml) [8], enzymatic (10 μg/ml) [15] and preliminary HPLC (25 μg/ml) [10] methods. On the other hand, it is clearly less good than the sensitivity of a bioluminescent method (0.3 μg/ml) [9]. When investigating cell surface compounds, however, such extreme sensitivities are rarely required. The correlation was linear up to 100 μg. Above this level tailing of the peaks reduced the accuracy.

Study of PG-TA of the cell wall of Bacillus subtilis

PG-TA was purified from *Bacillus subtilis* and shown in chemical analysis to be composed of essentially pure teichoic acid and peptidoglycan covalently linked to each other [16]. PG-TA (1 mg/ml) was hydrolysed for various times in 5.8 *M* HCl (100°C). The hydrolysis of phosphoester bonds was found to show essentially similar kinetics as the hydrolysis of P-Gro (Fig.1). A typical chromatogram of an HPLC analysis of hydrolysed PG-TA is shown in Fig.4. The glycerol peak at 12.6 min resolved well from other peaks which represent amino sugars and amino acids generated from PG-TA. Fig.1 shows a good correlation between the amount of organic phosphate before hydrolysis and the amount of glycerol after hydrolysis. Decomposition

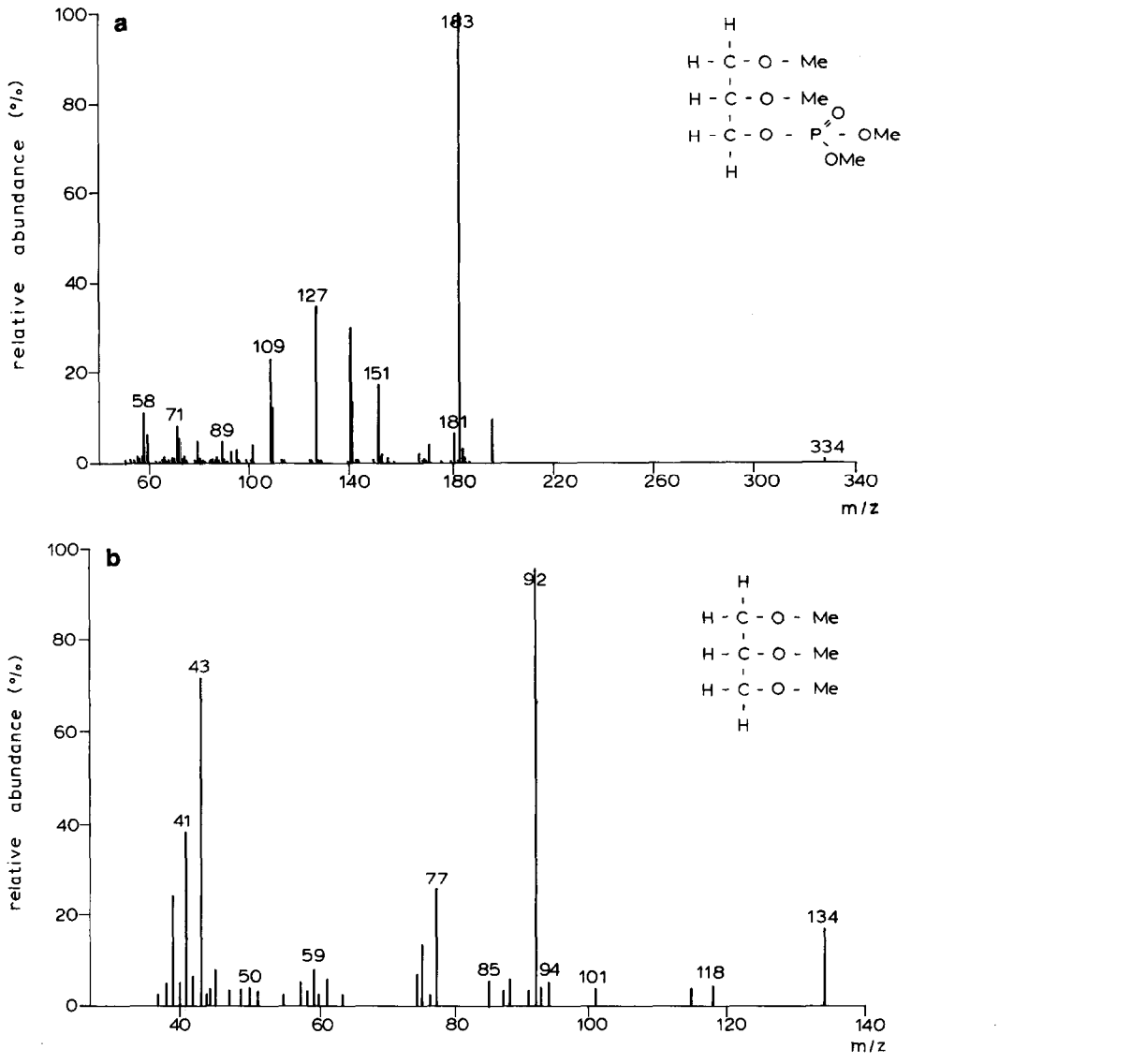

Fig. 2. Electron impact GC–MS spectra of P-Gro. P-Gro was hydrolysed in 5.8 *M* HCl for 75 h at 100°C and determined by GC–MS as described under Experimental before (a) and after (b) hydrolysis. Me = Methyl.

of Gro in HCl did not occur as the area of the peak at 12.6 min was the same before and after treating Gro for 75 h in 5.8 *M* HCl at 100°C.

The described method using ion-exclusion HPLC and a RI detector is fast and accurate for the determination of glycerol. The correlation coefficient (0.999) of the calibration graph indicates a high reproducibility of the method. Chromatography immediately after the hydrolysis, without extra sample preparation steps, eliminates the tedious and time-consuming steps of the enzymatic [15] and bioluminescent [9] assays and diminishes the errors. Especially with GC, sample preparation can be problematic because of the substantial volatility of glycerol [17]. According to experience in this laboratory, one evaporation to dryness decreases the amount of

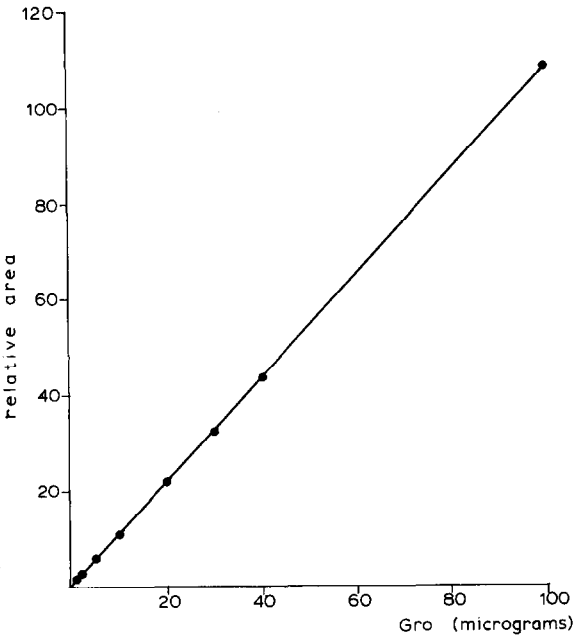

Fig. 3. Calibration graph for the determination of Gro by HPLC. Samples (20 μl; 1–100 μg) of Gro were applied to the HPX-87H ion-exclusion column and the effluent monitored by an RI detector. The area of the peak at 12.6 min was plotted against the amount of Gro in the sample.

peracetylated glycerol to 30% of the original amount.

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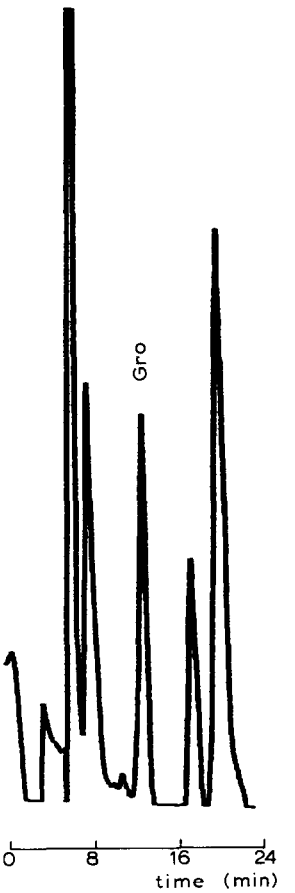

Fig. 4. Typical example of the determination of Gro in purified PG-TA. PG-TA (1 mg/ml) was hydrolysed in 5.8 *M* HCl for 75 h at 100°C and applied to the HPX-87H ion-exclusion column. The amount of Gro in the sample (2.5 μg in 20 μl) was determined by comparing the area of the peak at 12.6 min with the calibration graph.

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