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Journal of Chromatography A, 740 (1996) 273–278

JOURNAL OF CHROMATOGRAPHY A

Determination of peptidoglycan-associated protein in *Escherichia coli* NCIB 8545 by capillary zone electrophoresis

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Received 8 December 1995; accepted 6 February 1996

Abstract

An efficient reliable and sensitive capillary zone electrophoresis assay for the six major bacterial peptidoglycan-associated proteins of *Escherichia coli* NCIB 8545 is described. The method provides the facility to determine quantitatively the effect of antibacterials on bacterial peptidoglycan-associated protein synthesis and thus to further elucidate the mechanism of antibacterial action of such drugs as the antifolates which recently have been shown to adversely affect peptidoglycan synthesis.

Keywords: *Escherichia coli*; Antibacterial agents; Proteins; Peptidoglycans; Dibromopropamidine isethionate

1. Introduction

Previous publications [1–6] have indicated that the mechanism of synergism of combinations of trimethoprim and sulphonamides is related to mutually increased bacterial uptakes of both antibacterials and this is induced by a mechanism which involves an effect on cell envelope integrity and function and appears to be the result of effects on peptidoglycan and membrane protein synthesis in both Gram-positive and Gram-negative bacteria. Recent work [7] has indicated that an inhibition of the bacterial peptidoglycan biosynthesis is produced by trimethoprim or sulphadiazine alone and in combination and that this additional effect is a fundamental part of the antibacterial action of the antimetabolites. Capillary electrophoresis has been used in this laboratory to determine the muropeptides of bacterial peptidoglycan, 5-uridinediphosphate-N-acetylmuramyl (UDP-MurNAc) and the disaccharide pentapeptide derivative of peptidoglycan [7,8]. The purpose of the present investigation was to develop an assay which would enable the proposed action of sulphonamides and trimethoprim on bacterial peptidoglycan-associated protein synthesis to be further elucidated by quantitatively measuring the effects on protein synthesis.

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2. Experimental

2.1. Materials

Escherichia coli NCIB 8545 was obtained from the National Collection of Industrial Bacteria, Torry Research Station (Aberdeen, UK). Isosensitest broth was obtained from Oxoid (Basingstoke, UK). Unless otherwise stated, all the reagents used were commercial analytical grade and obtained from Fisons (Loughborough, UK). SDS, Coomassie Brilliant

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PII S0021-9673(96)00126-4

Blue, sodium N-lauroyl sarcosinate (Sarkosyl), glycerol and phenymethylsulphonylfluoride (PMSF) were obtained from Sigma (Poole, UK). Dibromopropamidine isethionate was a gift from Rhone-Poulenc Rorer (UK). Glucose and Tris–HCl were obtained from BDH (Poole, UK). Water was glass distilled and then further purified by a Millipore Milli-Q system.

2.2. Electropherograph equipment

The capillary zone electrophoresis (CZE) system used for these experiments was the Model 3850 electropherograph from Isco (USA). The fused-silica capillary was 50 μm I.D. and had a total length of 65 cm and a separation length of 45 cm. The sample was introduced into the column by hydrodynamic loading. Data were collected with a Spectraphysics Model 4270 integrator.

2.3. Preparation of running buffer

The running buffer was 40 m*M* disodium tetraborate and adjusted to the desired pH with either boric acid or sodium hydroxide. A Mettler Delta 320 pH meter calibrated with standard buffer, pH 4.0 and 7.0, was used for pH adjustment. Both buffer concentration and pH were validated for the running buffer without organic modifier.

2.4. Extraction of peptidoglycan-associated protein

Log-phase cultures were prepared based on the method used previously [3]. The peptidoglycan-associated proteins were extracted as described [9]. A volume of 100 ml of bacterial culture was harvested by centrifugation (15 min, 4000 *g*, 4°C) and washed twice with HEPES buffer (10 m*M*, pH 6.8). The bacteria were resuspended in 10 ml distilled water and broken by sonication (3×60 s with a 30 s cooling period between each burst) at 4°C. Unbroken cells were removed by centrifugation at 5000 *g* for 5 min at 4°C and discarded. A 1-ml volume of 20% (w/v) Sarkosyl was mixed with the supernatant and incubated for 30 min at room temperature. A visible clearing of the envelope suspension indicates solubilisation of the cytoplasmic membrane. The outer-membrane–peptidoglycan complex was sedimented by centrifugation at 40 000 *g* for 45 min at 20°C. The pellet was resuspended in 10 ml of 2% Sarkosyl containing 0.1 m*M* PMSF and the outer membrane complex again recovered by centrifugation. The pellet was resuspended in 40 ml Tris–HCl buffer (pH 7.8, 10 m*M*) containing 2% (w/v) SDS, 10% glycerol and 0.15 *M* NaCl and incubated at 30°C for 1 h. Peptidoglycan-associated protein was sedimented by centrifugation at 100 000 *g* for 45 min at 20°C and was dissolved in water and adjusted to the concentration of 50 mg ml^{-1} by using a modified Lowry assay for the determination of membrane proteins [10]. Bovine serum albumin (from Sigma) was used as the protein standard. The extract was stored at −30°C and rediluted in sodium borate solution just before CZE separation or in sample buffer (see below) before SDS–polyacrylamide gel electrophoresis (SDS–PAGE) separation. The extract was shown to be stable for over 2 months under these conditions.

2.5. SDS–PAGE

SDS–PAGE used a gel system having a 4% stacking gel and a 15% separating gel. A volume of the peptidoglycan-associated protein extract was mixed with a volume of sample buffer containing 0.125 *M* Tris–HCl buffer, pH 6.8, containing 0.04 *M* Na_2EDTA (Sigma), 4% (w/v) SDS, 10% (w/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.1% (w/v) bromophenol blue (Sigma) and boiled for 5 min. The electrophoresis was performed at 8 mA per gel for stacking gel and 15 mA per gel for separating gel to maximise the resolution at the important subtyping areas of the gel. After electrophoresis the separating gel was stained with a Coomassie Brilliant Blue stain for 2 h at room temperature with gentle mixing. The gel was initially destained with 45% ethanol (v/v)–10% acetic acid solution followed by final destaining with 7% acetic acid solution. The following standard proteins (BDH) were used as molecular mass markers: myoglobin (17 200), carbonic anhydrase (30 000), ovalbumin (42 700), albumin (66 250) and ovotransferrin (76 000–78 000).

2.6. Determination of peptidoglycan-associated protein

Peptidoglycan-associated protein extract (0.25 ml) was mixed with 0.5 *M* sodium borate solution (pH 9, 0.2 ml), and internal standard dibromopropamidine isethionate solution 50 μl (20 μg ml^{-1}). Samples were then separated by CZE. The column was given a 1-min flush with 0.1 *M* sodium hydroxide (100 μl), a 1-min flush with methanol (100 μl), then a 1-min flush with running buffer (100 μl) by syringe followed by 5 min running with buffer under high voltage to reach equilibrium. The sample was loaded over 10 s and the electrophoresis was at 20 kV. The column eluent was monitored for UV absorbance at a wavelength of 205 nm.

2.7. Separation of different proteins in CZE

A volume of protein extract was mixed with a volume of sample buffer and incubated at 20°C for 10 min. This mixture was applied as a single broad band to SDS–PAGE carried out as described above. A narrow strip was cut from each side of the gel and stained rapidly with Coomassie Blue to locate the protein bands. The main piece of gel was maintained at 4°C during this process. The appropriate regions of the unstained gel were then cut out and separately crushed with a glass rod and suspended in 1 ml distilled water. Each filtered crushed gel suspension was injected to identify the relative protein by CZE. The protein peak having the same migration time in CZE as the protein separated from the selected gel band section was regarded to be that same protein.

2.8. Quantification

Quantification was performed by comparison of the peak heights of the individual compounds relative to those of the appropriate internal standards.

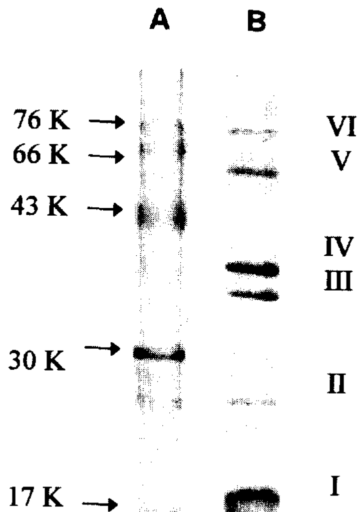

Fig. 1. The peptidoglycan-associated protein profiles of *E. coli* NCTC 8545 on SDS–PAGE: A, molecular mass marker; B, the peptidoglycan-associated proteins; K, kDa.

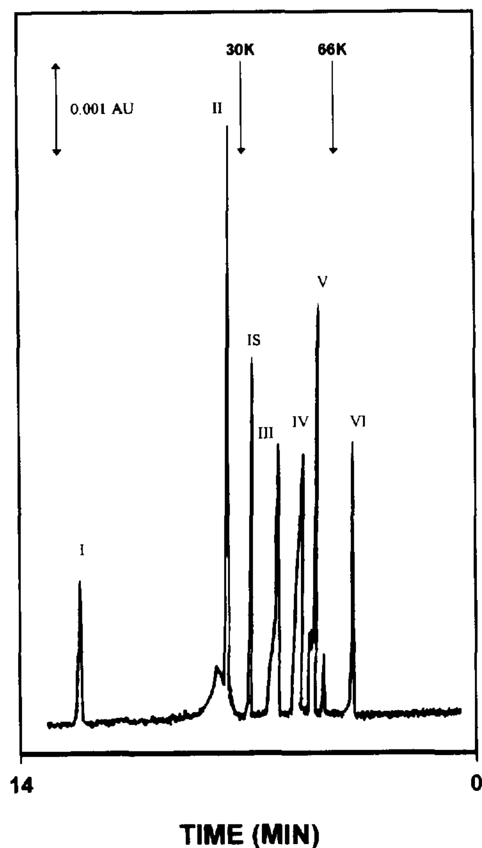

Fig. 2. Electropherogram of the separation by capillary-zone electrophoresis of peptidoglycan-associated proteins with dibromopropamidine isethionate as internal standard. The separation was carried out in a fused-silica capillary, 50 μm I.D., 65 cm in total length and 45 cm separation distance; buffer: 50 m*M* disodium tetraborate, pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV. IS=internal standard; I–VI= protein numbers.

The reproducibility of the assay was determined by carrying out five independent replicate determinations.

3. Results and discussion

The peptidoglycan-associated protein profiles on SDS–PAGE for *E. coli* NCIB 8545 are shown in Fig. 1. This was similar to those reported previously [9]. There are six major bands of the peptidoglycan-associated protein on the SDS–PAGE.

The separation electropherogram by CZE of six peptidoglycan-associated proteins plus dibromopropamidine isethionate as internal standard is shown in Fig. 2. A good separation of the analyte and the internal standard was achieved by the selected electrophoresis conditions within 14 min. The six major proteins which presented on the SDS–PAGE were also determined as relative peaks by CZE.

The factor which limits proper protein separation in CZE is capillary wall adsorption of protein [11]. One method to avoid wall adsorption of proteins in solution is to utilizes the variation of solution pH relative to the isoelectic point of a protein to change its net charge [12]. The effect of pH on the separation was determined at constant buffer concentration. Fig. 3 shows the effect of pH, the range was from pH 6 to 9, on the migration times for the six peptidoglycan associated proteins. From Fig. 3a it can be seen that at pH 6 differences in the mobilities of these six peptidoglycan-associated proteins did not

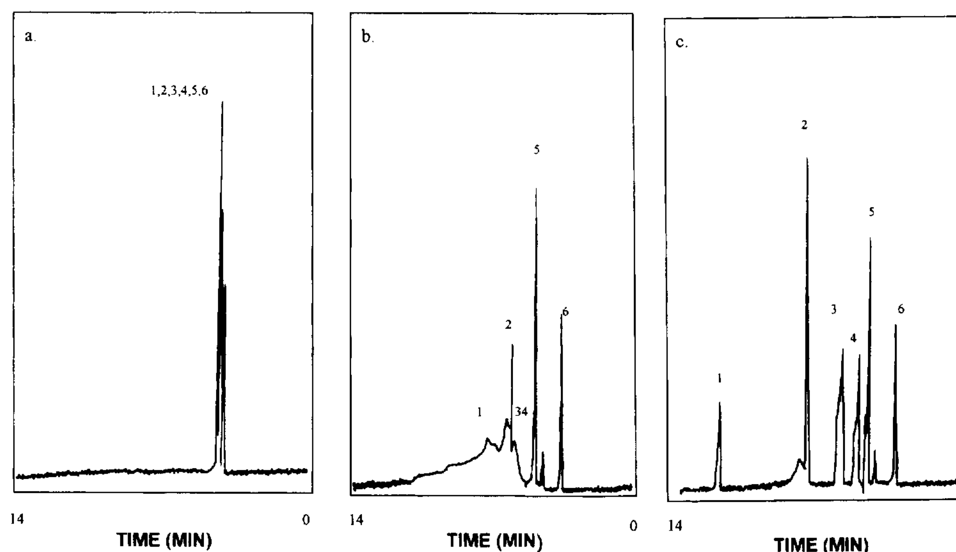

Fig. 3. Electropherogram of the effect of buffer pH on separation of peptidoglycan-associated proteins. The separation was carried out in a fused-silica capillary, 50 μm I.D., 65 cm in total length and 45 cm separation distance; buffer: 50 m*M* disodium tetraborate; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV. I–VI=protein numbers. a, pH 6.0; b, pH 8.0; c, pH 9.0.

Table 1
Reproducibility of migration times of six major peptidoglycan associated proteins

Proteins	*n*	Mean value of migration time (min)	Standard deviation	Relative standard deviation (%)
Protein I	5	12.37	0.401	3.24
Protein II	5	7.46	0.128	1.72
Protein III	5	6.05	0.076	1.26
Protein IV	5	5.42	0.051	0.94
Protein V	5	4.86	0.032	0.66
Protein VI	5	3.84	0.014	0.36

Table 2
Analytical characteristics of the assay method

Proteins	Internal standard	r^2-Value for calibration line	Recovery (%)	Relative S.D. (%, n=5)	Detection limit as a fraction of the original amount[a]
Protein I	DBPI	0.994	99.2	2.46	1/500
Protein II	DBPI	0.983	99.6	1.82	1/500
Protein III	DBPI	0.992	99.6	3.25	1/500
Protein IV	DBPI	0.986	99.4	1.64	1/500
Protein V	DBPI	0.964	99.1	2.86	1/500
Protein VI	DBPI	0.978	99.1	3.02	1/500

[a] The original quantity (50 mg ml^{-1}) extracted from the bacterial cells.

exist. As the pH was raised, mobility differences became greater and partial separation was achieved at pH 8.0 (Fig. 3b). An optimal separation of these proteins was achieved at or above pH 9.0 (Fig. 3c).

The peptidoglycan-associated proteins are usually identified and defined in terms of their mobility on SDS–PAGE [13–15]. The SDS–PAGE technique is simple and inexpensive to implement; However, it is tedious, labour-intensive, time-consuming and semi-quantitative. The CZE separation described in this paper is of high efficiency, gives fast accurate quantitation and the instrument used is simple to operate and experimental conditions are easy to control. The determination and quantitation can be completed in one procedure. The disadvantage of the CZE technique is that it is difficult to prepare material separated by the instrument for use in the identification of each peak. However, this can be resolved by CZE combined with SDS–PAGE. All the proteins present on the SDS–PAGE were separated by CZE at a corresponding migration time by dissolving and detecting the relative SDS–PAGE band.

The reproducibility of migration times for the six peaks from the peptidoglycan-associated proteins was evaluated by CZE and the results are give in Table 1. Relative standard deviations were 3.24% or less. Small deviations in migration times which are encountered in CZE separations can be due to variations in the electro-osmotic flow from run to run and to temperature fluctuations [16]. Calibration was determined by spiking the relative concentrations of the internal standard and samples over the range 2–10 mg ml^{-1}. The use of the internal standard served to eliminate any error incurred due to incomplete injection of the total sample available. Peak heights were measured and the peak-height ratio calculated with reference to the internal standard. The correlation coefficients r^2 for the six proteins calibration regression lines show a good linearity. Detection limits at the detector sensitivity (0.01 AU), under the conditions of this assay were 1/500 of the original amount extracted for peptidoglycan-associated proteins at 205 nm taking a signal-to-noise ratio of 3 as adequate. The precision of the assay was assessed by carrying out five replicate extractions and injections. At a detection wavelength of 205 nm relative standard deviations (R.S.D.) were between 1.67 and 5%. Table 2 lists the constants of the respective linear regression lines and the analytical characteristics of the method.

4. Conclusions

The assay described here provides a method for separating and quantifying peptidoglycan-associated proteins of bacterial cell envelopes. It provides the potential to investigate quantitatively the effect on the cell envelope resulting from the inhibition of the biosynthesis of peptidoglycan-associated proteins and relating this effect to the mechanism of action of the antibacterials responsible.

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