Journal of Chromatography, 457 (1988) 317–324
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 941

LARGE-SCALE PURIFICATION OF THE CHROMOSOMAL β-LACTAMASE FROM *ENTEROBACTER CLOACAE* P99

CHRISTOPHER R. GOWARD*, GARRY B. STEVENS, PETER M. HAMMOND and MICHAEL D. SCAWEN

Division of Biotechnology, PHLS, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG (U.K.)

(First received April 21st, 1988; revised manuscript received August 1st, 1988)

SUMMARY

Homogeneous β-lactamase (β-lactam hydrolase, E.C. 3.5.2.6) from *Enterobacter cloacae* P99, an enzyme that has an important function in antibiotic resistance, was prepared using a single cation-exchange chromatographic step with CM-Sepharose fast-flow. A 6-g amount of the enzyme was isolated from 5 kg of cell paste, with 84% of the enzyme activity in the cell homogenate being recovered by the single cation-exchange step. The specific activity of the β-lactamase was 587 U/mg protein. The relative molecular mass of the enzyme was determined to be 45 kDa by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and the isoelectric point was 8.95.

INTRODUCTION

The β-lactamases (β-lactam hydrolase, E.C. 3.5.2.6) are a class of enzymes that are of increasing clinical significance because of their role in mediating resistance to β-lactam antibiotics. They are also of biochemical interest because they form a diverse group of enzymes, all of which catalyse the hydrolysis of the β-lactam ring in penicillins and cephalosporins. The enzymes have been classified into three classes, A, B and C, on the basis of their amino acid sequences[1,2].

Many Gram-negative bacteria produce chromosomally encoded β-lactamases; the Class C enzyme from *Enterobacter cloacae* is of particular interest as it is most active against cephalosporins and has a different evolutionary origin from the Class A penicillinases, although sharing a similar active site[3]. In addition, this enzyme has recently been crystallized[4]. The enzyme has been purified by a variety of methods, including chromatography on Sephadex G-50 followed by QAE-Sephadex[5] and chromatography on phenylboronic acid–agarose[6]. We describe here a simple method for the large-scale purification of this enzyme, based on a single chromatographic step using CM-Sepharose fast-flow. The facile and inexpensive nature of this purification makes it suitable for almost any scale of operation, from a laboratory scale to the pilot scale operation described in this paper, to produce several grams of homogeneous enzyme in a few days.

0021-9673/88/$03.50

EXPERIMENTAL

Antibiotics and chemicals

Cephalosporin C, cephaloridine, cephalothin, cefazolin, cephalexin, cefamandole, cefuroxime and [2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino-1-propanesulphonic acid (TAPS) were obtained from Sigma (Poole, U.K.). Nitrocefin was obtained from Glaxo (Greenford, U.K.). All other chemicals were AnalaR grade from BDH (Poole, U.K.).

Culture methods

Enterobacter cloacae P99 was grown in a medium containing yeast extract (10 g/l), casamino acids (10 g/l), glucose (4 g/l), sodium β-glycerophosphate (2.27 g/l), and magnesium sulphate (0.25 g/l) (pH 7.2)[7] in a 400-l fermenter operated as described previously[8]. When the β-lactamase activity in the culture reached a maximum, after 4–6 h of growth, the temperature in the vessel was rapidly reduced to below 10°C and the bacteria were harvested by centrifugation in a Westfalia KA25 centrifuge at a flow-rate of 250 l/h. The cells were washed by suspension in 100 m*M* sodium phosphate buffer (pH 7.0) and harvested by centrifugation as above. The washed cell paste was quick-frozen and stored at −20°C.

Enzyme assay

The β-lactamase activity was determined by a spectrophotometric assay using cephalosporin C as substrate[9]. Cephalosporin C (0.2 m*M*) was dissolved in 50 m*M* TAPS–sodium hydroxide solution (pH 8.5). The reaction was started by the addition of 0.005–0.05 ml of enzyme solution and the decrease in absorbance at 260 nm was followed. The molar absorptivity for the complete hydrolysis of the substrate was determined as 6000 l mol^{-1} cm^{-1} from UV spectra recorded before and after complete hydrolysis. One unit of enzyme is defined as that amount which catalyses the hydrolysis of 1 μmol of cephalosporin C per minute at 30°C and pH 8.5. The activity of the enzyme against other substrates was determined in a similar manner, using 0.1–0.2 m*M* solutions of the reagents.

Determination of protein

Protein was determined by using the Coomassie Blue binding method[10], using bovine γ-globulin as standard. Column eluates were monitored at 280 nm.

Cation-exchange chromatography

CM-Sepharose fast-flow was obtained from Pharmacia (Milton Keynes, U.K.). It was suspended in 10 m*M* potassium phosphate buffer (pH 6.0) and packed into a column (16 × 1.6 cm I.D.). It was also slurry-packed into a Whatman Prep 25 column (16 × 45 cm I.D.) at a linear flow-rate of 750 cm/h, as described by the manufacturer (Whatman, Maidstone, U.K.). The column was equilibrated in 10 m*M* potassium phosphate buffer (pH 6.0) at a linear flow-rate of 550 cm/h.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed in a Pharmacia Phast Gel apparatus with gradient gels of

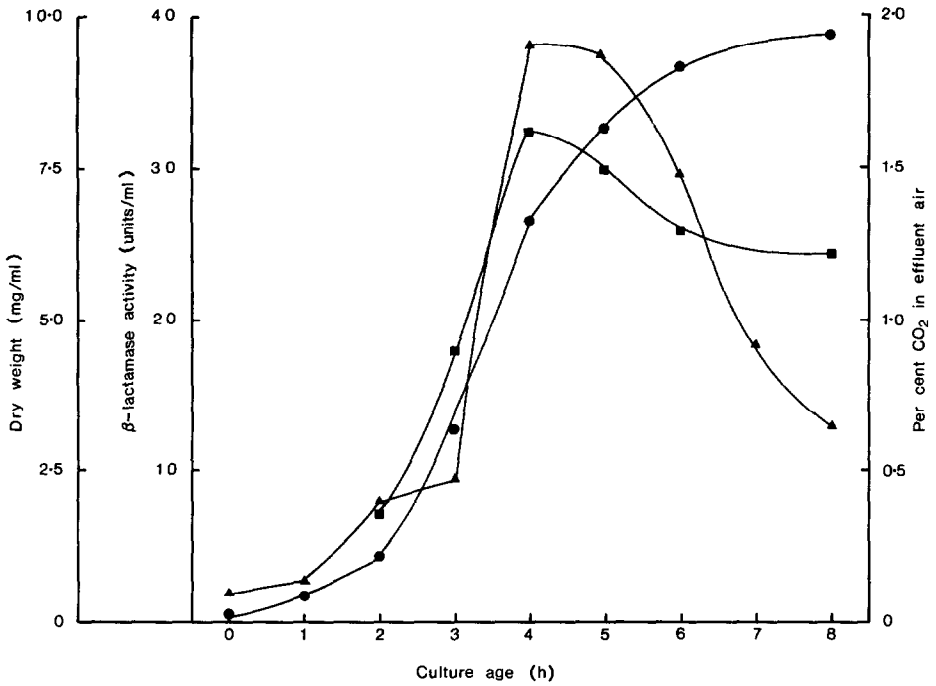

Fig. 1. Growth and β-lactamase production of *Enterobacter cloacae* P99 in 400-l culture. Culture conditions were as described in the text. (●) Dry weight; (■) β-lactamase activity; (▲) CO_2 (%) in the effluent air.

10–15% acrylamide and using low relative molecular mass (M_r) marker proteins from Pharmacia (Milton Keynes, U.K.). The proteins were stained with Coomassie Blue R350 and densitometer scans were used to estimate the M_r.

The isoelectric point (p*I*) was determined by isoelectric focusing on Servalyt precoated polyacrylamide IEF gels (pH 3–10.5) with pH 5–10.5 standards from Pharmacia. Prefocusing was performed for 500 V h and electrofocusing was continued for a further 2000 V h after application of the samples. Proteins were detected with Coomassie Blue R350 and densitometer scans were used to estimate the p*I*.

RESULTS AND DISCUSSION

Growth of the organism

Fig. 1 shows the growth of *Enterobacter cloacae* P99 in a 400-l culture. A typical culture was harvested after 6 h of growth and yielded about 12 kg wet weight of cell paste, containing about 800 U β-lactamase/g of cells (equivalent to 2.9 kg dry cell mass, containing 3310 U β-lactamase/g of dry cells).

Small-scale purification of the enzyme

Frozen cell paste (6 g) was thawed in 33 ml of 100 m*M* potassium phosphate buffer (pH 6.0) at 4°C. About 1.5 mg of deoxyribonuclease was added to the suspension and the bacteria were disrupted by sonication at 20 kHz for six 30-s

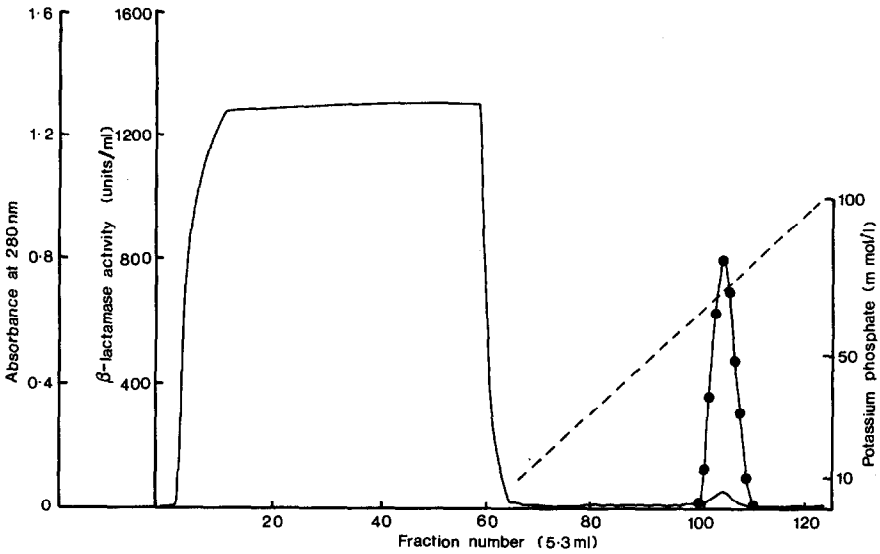

Fig. 2. Small-scale cation-exchange chromatography. The column of CM-Sepharose was 16 × 1.6 cm I.D. and was eluted with a 320-ml linear gradient of 10–100 m*M* potassium phosphate buffer (pH 6.0). Fractions 99–107 were combined. (– – –) Potassium phosphate; (——) protein absorbance at 280 nm; (●) β-lactamase activity (units/ml).

intervals with cooling between each exposure. Cell debris was removed from the homogenate by centrifugation at 21 000 *g* at 8°C for 30 min. The supernatant was diluted to the same conductivity as 10 m*M* potassium phosphate buffer (pH 6.0). The diluted supernatant was applied to the 32-ml column of CM-Sepharose fast-flow (16 × 1.6 cm I.D.), equilibrated in 10 m*M* potassium phosphate buffer (pH 6.0) at a linear flow-rate of 190 cm/h. The column was washed with 55 ml of equilibrating buffer at a linear flow-rate of 190 cm/h and eluted with a 320-ml linear gradient from 10 to 100 m*M* potassium phosphate buffer (pH 6.0) at a linear flow-rate of 100 cm/h. The breakthrough and eluate were collected in fractions of 5.3 ml, as shown in Fig. 2. Fractions containing the enzyme activity were combined and concentrated to 5 ml by ultrafiltration using an Amicon PM 10 membrane. The concentrated enzyme was dialysed against 5 m*M* potassium phosphate buffer (pH 6.0). A summary of this purification is shown in Table I. Similar results were obtained when the enzyme from

TABLE I

PURIFICATION OF *ENTEROBACTER CLOACAE* β-LACTAMASE FROM 6 g OF CELL PASTE

Step	*Volume (ml)*	*Total enzyme (U)*	*Total protein (mg)*	*Specific activity (U/mg)*	*Recovery (%)*
Homogenate	37	4452	374.0	11.9	100
Supernatant	90	4098	136.0	30.2	92
CM-Sepharose fast-flow	48	3786	5.6	676.0	85
Concentrate	5	3590	5.3	677.0	80

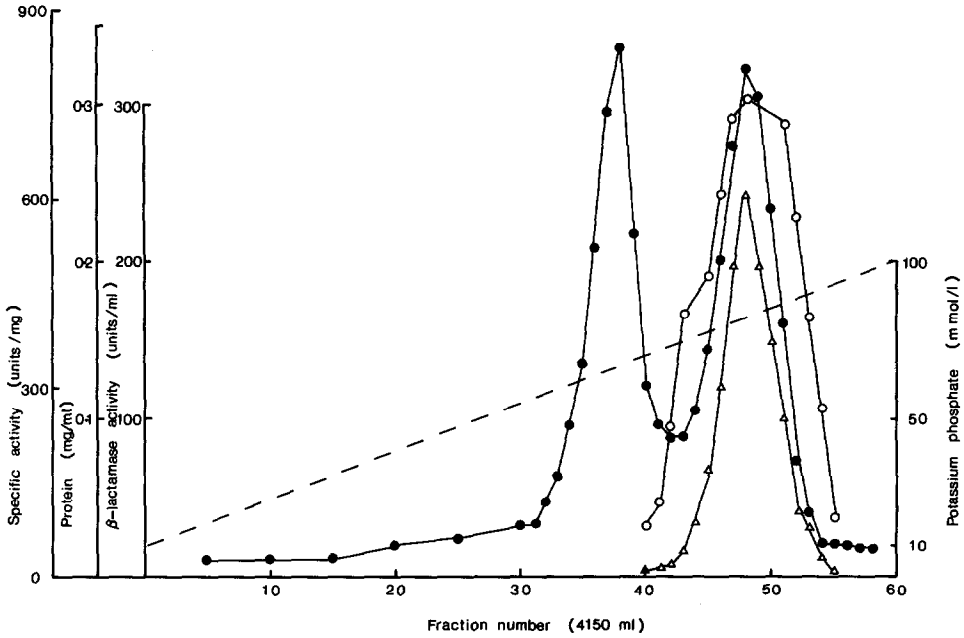

Fig. 3. Large-scale cation-exchange chromatography. The column of CM-Sepharose fast-flow was 16 × 45 cm I.D. and was eluted with a 240-l linear gradient of 10–100 m*M* potassium phosphate buffer (pH 6.0). Fractions 44–52 were combined. (---) Potassium phosphate; (●) protein (mg/ml); (△) β-lactamase activity (units/ml); (○) specific activity (units/mg).

100 g of cell paste was prepared using a 500-ml column of CM-Sepharose fast-flow, so the process was scaled-up to prepare the enzyme from kilogram amounts of cell paste.

Large-scale purification of the enzyme

A 5-kg amount of frozen cell paste was suspended in 20 l of 100 m*M* potassium phosphate buffer (pH 6.0) and allowed to thaw overnight at 4°C. About 100 mg of deoxyribonuclease were added to the suspension and the bacteria were disrupted by a single passage through a Manton–Gaulin homogenizer at 55 MPa. The homogenate was centrifuged at 17000 *g* at 8°C and a flow-rate of 50 l/h in a Sharples AS-26

TABLE II

PURIFICATION OF *ENTEROBACTER CLOACAE* β-LACTAMASE FROM 5 kg OF CELL PASTE

Step	*Volume (l)*	*Total enzyme (MU)*	*Total protein (g)*	*Specific activity (U/mg)*	*Recovery (%)*
Homogenate	23.50	4.13	226.0	18.3	100
Supernatant	250.00	3.84	138.0	27.8	93
CM-Sepharose fast-flow	38.60	3.46	6.0	577.0	84
Concentrate	1.52	3.11	5.3	587.0	75

continuous-flow centrifuge. The supernatant was diluted to the same conductivity as 10 m*M* potassium phosphate buffer (pH 6.0). The diluted supernatant was applied to the 25-l column of CM-Sepharose fast-flow (16 × 45 cm I.D.), equilibrated in 10 m*M* potassium phosphate buffer (pH 6.0) at a linear flow-rate of 190 cm/h. The column was washed with 40 l of equilibrating buffer at a linear flow-rate of 190 cm/h and eluted with a 250-l linear gradient from 10 to 100 m*M* potassium phosphate (pH 6.0) at a linear flow-rate of 100 cm/h. The eluate was collected in fractions of 4.15 l, as shown in Fig. 3. The fractions containing the enzyme activity were combined and concentrated to 1520 ml by ultrafiltration using Amicon PM 10 membranes. The concentrated enzyme was dialysed against 5 m*M* potassium phosphate buffer (pH 6.0) before being freeze-dried for storage. A summary of this purification is shown in Table II.

A purification process has been described that uses phenylboronic acid–

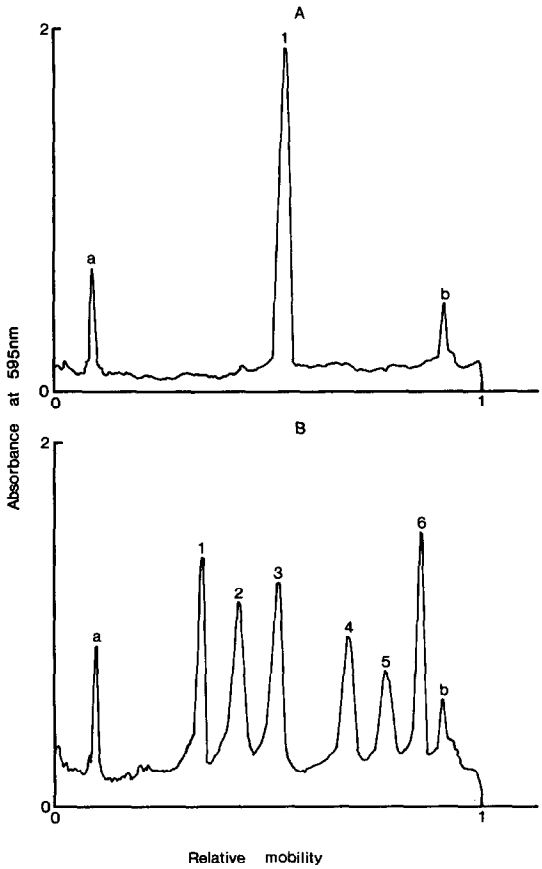

Fig. 4. Densitometer scans following SDS-PAGE. The gel was scanned with a Joyce-Loebl Chromoscan 3. (A) 1 = β-lactamase (M_r 45 kDa). (B) Calibration proteins: 1 = phosphorylase b (M_r 94 kDa), 2 = bovine serum albumin (67 kDa), 3 = ovalbumin (43 kDa), 4 = carbonic anhydrase (30 kDa), 5 = soybean trypsin inhibitor (20.1 kDa) and 6 = α-lactalbumin (14.4 kDa). The gel boundary is designated peak a and the dye marker peak b.

agarose[6], which is a good method for use on the small scale (20-ml matrix), but it is impractical for large-scale applications. A procedure with two chromatographic steps has been described that uses gel filtration followed by application to a 500-ml column of an anion-exchange matrix, QAE-Sephadex, which binds contaminating protein but does not adsorb the enzyme[5]. The specific activity of the purified enzyme (134 U/mg) was considerably lower than that obtained using phenylboronic acid–agarose (750 U/mg). We have developed a procedure for the large-scale purification of the enzyme that is both rapid and economical. The high degree of purification achieved with CM-Sepharose fast-flow takes advantage of the high p*I* of the enzyme; at pH 6.0 most of the proteins in the cell free extract do not bind to the cation-exchange matrix (Fig. 2). CM-Sepharose fast-flow is used instead of the phenylboronic acid–agarose, which would have been prohibitively expensive for the preparation of the enzyme on the large scale. The procedure described allows the economical purification of β-lactamase from kilogram amounts of *Enterobacter cloacae* P99 cells. The specific activities of the

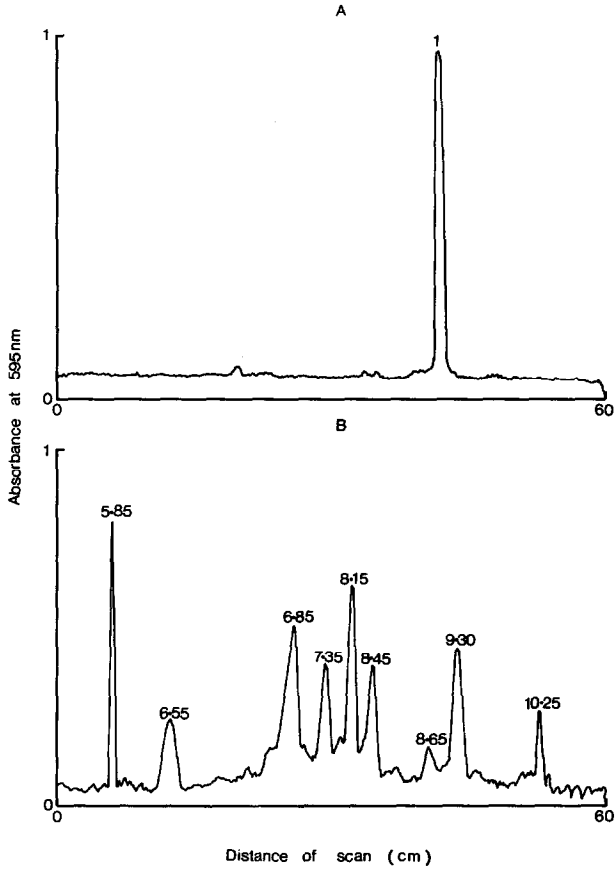

Fig. 5. Densitometer scans following isoelectric focusing. The gel was scanned with a Joyce-Loebl Chromoscan 3. (A) 1 = β-Lactamase (p*I* 8.95). (B) Calibration proteins: bovine carbonic anhydrase b (p*I* 5.85), human carbonic anhydrase b (6.55), horse myoglobin (6.85), horse myoglobin (7.35), lentil lectin (8.15), lentil lectin (8.45), lentil lectin (8.65), trypsinogen (9.30) and cytochrome c (10.25).

TABLE III

RELATIVE $V_{max.}$ OF THE β-LACTAMASE WITH DIFFERENT SUBSTRATES

Rates relative to cephalosporin C = 100%.

Substrate	*Relative $V_{max.}$ (%)*	*Substrate*	*Relative $V_{max.}$ (%)*
Nitrocefin	170.0	Cefazolin	24.7
Cephalothin	105.7	Cephalexin	13.1
Cephalosporin C	100.0	Cefamandole	1.8
Cephaloridine	54.7	Cefuroxime	0.12

β-lactamase, 677 and 587 U/mg, were lower than the 750 U/mg protein reported by Cartwright and Waley[6], but are considerably higher than the 134 U/mg protein described by Ross and Boulton[5].

The β-lactamase was shown to be homogeneous, giving a single protein band of M_r 45000 on SDS-PAGE (Fig. 4). The M_r for *E. cloacae* P99 β-lactamase has previously been reported as 49000 ± 3000[5]. Further evidence of purity is shown by the densitometer scan of an isoelectric focusing gel and the p*I* of the β-lactamase was determined to be 8.95 (Fig. 5). The enzyme from *E. cloacae* P99 has previously been reported to have a p*I* of 7.9[5], 8.0[11] and 8.25[6]. The enzyme was shown to be active against a series of antibiotics, particularly against the antibiotic-like substrate nitrocefin, as shown in Table III.

REFERENCES

1 R. P. Ambler, *Philos. Trans. R. Soc. London, Ser. B*, 289 (1980) 321–331.
2 B. Jaurin and T. Grundström, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 4897–4901.
3 B. Joris, J. Dusart, J.-M. Frère, J. Van Beeuman, E. L. Emanuel, S. Petursson, J. Gagnon and S. G. Waley, *Biochem. J.*, 223 (1984) 271–274.
4 P. Charlier, O. Dideberg, J.-M. Frère, P. C. Moews and J. R. Knox, *J. Mol. Biol.*, 171 (1983) 237–238.
5 G. W. Ross and M. G. Boulton, *Biochim. Biophys. Acta*, 309 (1973) 430–439.
6 S. J. Cartwright and S. G. Waley, *Biochem. J.*, 221 (1984) 505–512.
7 R. P. Novick, *Biochem. J.*, 83 (1962) 229–235.
8 J. Melling and G. K. Scott, *Biochem. J.*, 130 (1972) 55–62.
9 C. H. O'Callaghan, P. W. Muggleton and G. W. Ross, *Antimicrob. Agents Chemother.*, 1 (1969) 57–63.
10 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
11 M. Matthew, A. M. Harris, M. J. Marshall and G. W. Ross, *J. Gen. Microbiol.*, 88 (1975) 169–178.