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Improved purification and characterization of the OXA-2 β-lactamase

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An improved and scaled-up procedure has been developed for purifying the OXA-2 plasmid-mediated β-lactamase. This has enabled us to improve the characterization of this enzyme, including a revised determination of its amino acid composition and the sequence of the *N*-terminal region of the protein.

The resistance plasmid R46, formerly known as R-1818 (Dale & Smith, 1971) or R-Brighton (Anderson & Datta, 1965) mediates the production of the unusual β-lactamase type known as OXA-2 (Dale & Smith, 1974; Matthew & Hedges, 1976). The principal characteristic feature of this enzyme is its ability to hydrolyse oxacillin at a rate several times faster than that at which it hydrolyses benzylpenicillin. Additional unusual features include its anomalous behaviour in M_r determinations, which led Dale & Smith (1976) to suggest that it is a dimeric enzyme, and its interaction with Cibacron Blue and other anthraquinone dyes (Monaghan *et al.*, 1982), a property more usually associated with nucleotide-binding enzymes.

The purpose of the present investigation was to improve the purification procedure so as to obtain sufficient material for a detailed investigation of the structure and function of this enzyme.

Materials and methods

Bacterial strains and plasmids

The following *Escherichia coli* strain was used as the initial host for cloning: ED8654, *met*, *trpR*, *hsdR*, *supE*, *supF* (Murray & Murray, 1975). The production *E. coli* strain CA265 originated from Dr. B. F. Clark, Laboratory of Molecular Biology, Cambridge, U.K. and was obtained from the Microbial Products and Production Laboratory at the Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts., U.K. The plasmids used were R46 (Meynell & Datta, 1966) and pMB9 (Rodriguez *et al.*, 1976).

Abbrevation used: SDS, sodium dodecyl sulphate.

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DNA isolation

R46 plasmid DNA was routinely isolated by using a sheared-lysate technique adapted from the methods described by Bazaral & Helinski (1968) and Barth & Grinter (1974). Other plasmids (pMB9 and recombinants) were isolated by a cleared-lysate method (Clewell & Helinski, 1969) or by the procedure of Birnboim & Doly (1979).

Preparation and assay of β-lactamase

Small-scale β-lactamase extracts were prepared from 150-ml L-broth cultures as described by Hedges *et al.* (1974). β-Lactamase activity was measured by using the spectrophotometric method of Samuni (1975). The substrate routinely utilized was benzylpencillin at 500 μg/ml. The assay temperature was 30°C and the assay was monitored at 240 nm. One unit of enzyme activity is the amount required to hydrolyse 1 μmol of benzylpenicillin/min at 30°C and at pH 7.4. Protein concentrations were measured by the method of Lowry *et al.* (1951).

DEAE-cellulose treatment

For the batch treatment, DEAE-cellulose (DE52; Whatman) was prepared as recommended by the manufacturers and equilibrated with 25 mM-sodium phosphate buffer, pH 7.4, before use. The required amount was added to the enzyme and the slurry stirred slowly at 4°C with a glass stirrer. After 10 min the ion exchanger was removed by filtration, leaving the enzyme in the supernatant.

For column chromatography the DE52 exchanger was prepared in the same way, except that the buffer used was 10 mM-Tris/HCl, pH 8.9. After applying the sample, the column (150 cm × 5 cm^2) was washed with 500 ml of starting buffer before eluting the enzyme with 225 mM-NaCl in the same buffer.

Biochemical characterization of the purified β-lactamase

The M_r of the purified enzyme was determined by SDS/polyacrylamide-gel electrophoresis (Laemmli & Favre, 1973). The isoelectric point of the protein was measured as described by Matthew (1978) on LKB pre-prepared Ampholine PAG plates, pH 5.5–9.5, mounted on an LKB Multiphor apparatus fitted with a cooling system. The samples were applied to the centre of the gels.

Amino acid analysis was performed on freeze-dried salt-free OXA-2 enzyme samples at a specific activity of 230 units/mg of protein, in a Beckman 120C amino acid analyser, the methodology of Spackman (1963) being used. Tryptophan was determined after hydrolysis with mercaptoethanesulphonic acid as described by Penke *et al.* (1974). Analysis was performed on two independent enzyme samples.

The *N*-terminal amino acid sequence of the OXA-2 enzyme was determined by automatic Edman degradation in a Beckman 890C spinning-cup sequencer. For each sequencing run, 10 mg of freeze-dried salt-free enzyme was dissolved in 400 μl of formic acid and mixed with 100 μl of Polybrene. The sample was added to the spinning cup of the sequencer and dried under vacuum by using a subroutine drying program. Sequencing of the enzyme utilized a dilute Quadrol program. The thiazolinone derivatives were converted into their corresponding phenylthiohydantoin derivatives by using trifluoroacetic acid. The phenylthiohydantoin derivatives were identified by both t.l.c. and h.p.l.c. One-dimensional t.l.c. was performed on silica-gel plates, containing a fluorescent indicator, which were developed with trichloromethane containing 3% (v/v) ethanol. In order to achieve better separation of the slower-moving derivatives, the plates were then dried and re-run with a second solvent, trichloromethane/methanol (9:1, v/v). The phenylthiohydantoin derivatives were detected by long-wave-u.v. illumination and identified by their positions relative to a set of standards run on the same plate. H.p.l.c. was performed on a Du Pont model-830 liquid chromatograph using a column packed with Partisil 5 purged with 50% (v/v) methanol before chromatography. The eluting solvent was acetonitrile/sodium acetate, pH 4.0. The phenylthiohydantoin derivatives were detected by u.v. absorption and identified by their retention times relative to a set of standards.

Results

Insertion of the OXA-2 bla gene into pMB9

One of the difficulties of working with the OXA-2 β-lactamase is the low level of enzyme production compared with that of many other penicillinases. An attempt was therefore made to increase enzyme yields by cloning the OXA-2 gene with a high-copy-number vector.

DNA from the R46 plasmid was subjected to partial digestion with the restriction endonuclease *Eco*RI in low-ionic-strength buffer, which reduces the specificity of the enzyme to the central tetranucleotide AATT (*Eco*RI* conditions; Polisky *et al.*, 1975).

The fragments generated were ligated, without further purification, with *Eco*RI-cleaved pMB9. The ligation mixture was then used to transform *E. coli* strain ED8654, and selection was made for clones that carried both the ampicillin (R46)- and tetracycline (pMB9)-resistance genes. Eleven clones were selected; all of these carried the colicin-immunity gene of pMB9. Eight of these strains produced β-lactamase at a level not significantly different from that specified by the parental plasmid R46; the other three strains, however, did produce elevated levels of the enzyme, between two and three times that of the control strain. One of these strains, carrying a recombinant plasmid designated pSU3, was selected for further analysis. The substrate profile and isoelectric-focusing pattern of the β-lactamase produced by pSU3 were verified to be that of the OXA-2 enzyme as specified by R46 (Holland, 1983). From a series of single and double restriction digests the restriction map of pSU3 DNA illustrated in Fig. 1 was produced. pSU3

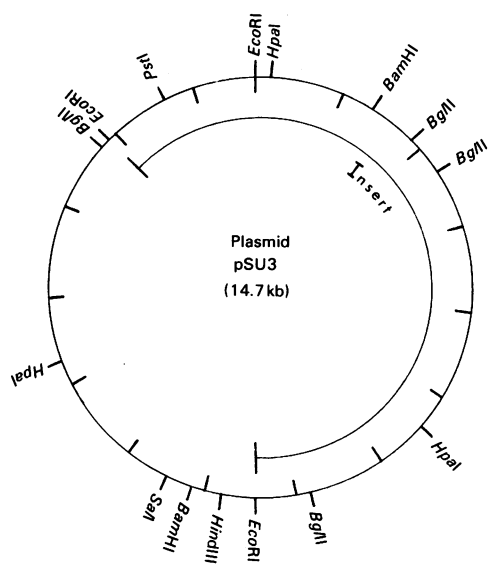

Fig. 1. *Restriction map of plasmid-pSU3 DNA*
The position of restriction-endonuclease cleavage sites are shown for the following enzymes: *Eco*RI, *Hpa*I, *Bam*HI, *Bgl*II, *Hin*dIII and *Sal*I. The inner scale marks are spaced by 1 kb.

consists of a 9.3-kilobase fragment of R46 inserted into the single *Eco*R1 site of pMB9. The DNA fragment from R46 encompasses the OXA-2 *bla* gene and the origin of replication. The fragment terminates in the region of the u.v.-protection gene (Brown & Willetts, 1981).

Enzyme production and purification

In order to facilitate the growth of a large-scale culture, the pSU3 plasmid was introduced into the *E. coli* production strain CA265 by transformation. A 400-litre batch fermentation was carried out by the Microbial Products Development and Production Laboratory at the PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts., U.K. The culture was harvested in the early stationary phase, yielding 13.3 kg of cell paste, which was supplied as 40 litres of broken-cell suspension in a frozen state. The enzyme yield from this procedure corresponds to about 6000 units/litre of culture, compared with 80 units/litre for an *E. coli* K12 strain carrying the parental R46 plasmid.

The purification procedure used is an improved version of that described by Dale & Smith (1971). Aliquots (2.25 litres) of crude material were thawed as required and mixed with DEAE-cellulose as described in the Materials and methods section, using 1 g (wet weight) of DE52 resin to 200 mg of protein. This removed up to 80% of the contaminating protein.

The supernatant from the batch treatment was applied to a column of DEAE-cellulose equilibrated with 10 mM-Tris/HCl buffer, pH 8.9. After washing the column, the enzyme was recovered by a single-step elution with 225 mM-NaCl. This was found to be both more convenient and more effective than the gradient elution described by Dale & Smith (1971). Subsequent Sephadex G-100 gel filtration and $(NH_4)_2SO_4$ precipitation were carried out as in the original procedure, except the column dimensions were increased to 100 cm × 4 cm^2.

Table 1 shows that this method produced, from a 2.25-litre aliquot of the cell extract, 90 mg of enzyme with a specific activity of 230 units/mg of protein. This specific activity is about 50% higher than that previously obtained (Dale & Smith, 1971). The enzyme was shown to be effectively homogeneous by SDS/polyacrylamide-gel electrophoresis.

Biochemical characterization

The purified enzyme yielded a single band on SDS/polyacrylamide-gel electrophoresis, corresponding to an M_r of 32170. The previously reported value of 28000 (Dale & Smith, 1976) was obtained by using the method of Weber & Osborn (1969) with tube gels rather than on slabs. The value reported here is therefore considered to be more reliable. Dale & Smith (1971) reported a value of 44600 on the basis of Sephadex-gel-filtration results; the difference between this and the SDS/polyacrylamide-gel result (together with other evidence) led to the suggestion (Dale & Smith, 1976) that the enzyme was dimeric. However attempts to confirm this by the use of di-imidoester cross-linking agents have proved unsuccessful (Holland, 1983).

There have been several different values quoted for the pI of the enzyme, as obtained by isoelectric focusing, and many workers have commented on the multiple bands obtained by this method. These values have ranged from 7.3 (Matthew & Hedges, 1976) to 8.1 (Matthew *et al.*, 1975). Hedges *et al.* (1974) reported two bands with pI values of 7.45 and 7.7. We consistently found a major band with a pI value of 8.65 with several satellite bands. The number, position and intensity of these satellite bands was markedly influenced by variation in the concentration, purity and loading position of the sample and therefore these probably repreent variants produced by, e.g., deamination of glutamine and asparagine residues. It should be noted that when the OXA-2 enzyme was loaded at the anode end, no enzyme bands were detected.

Table 1. *Purification of the OXA-2 β-lactamase*

Step	Volume	Total enzyme activity (units)	Total protein (mg)	Specific activity (units/mg)
Starting material	2.25 litres	134500	115700	1.16
Batch DEAE-cellulose	2.15 litres	83910	16700	5.02
DEAE-cellulose column	120 ml	43000	1100	39.1
Sephadex G-100	25 ml	21080	123	171
$(NH_4)_2SO_4$	8 ml	20310	89	228

This is presumbly due to the inactivation of the enzymes at the low pH values encountered, since it is known that the OXA-2 β-lactamase is inactivated at pH values below 6 (Dale & Smith, 1971), and this may account for the failure of some groups to detect this enzyme in clinical isolates.

The increased amount of purified enzyme available has enabled a re-examination of the amino acid composition of the protein. The results are reported in Table 2 and are compared with previously obtained (Dale, 1971) values recalculated to a M_r of 32170. Although qualitatively the overall pattern remains similar, there are significant quantitative differences for residues. In part these may reflect the greater purity of the enzyme used in the present study. The wider discrepancies in the values for the number of tryptophan and proline residues are thought to be due to differences in the methods used; for example, the previous report (Dale, 1971) was based solely on spectrophotometric determination of tryptophan. The unusually high content of arginine in particular will partly account for the basic nature of this enzyme; the procedures used do not allow a distinction to be made between glutamate and glutamine nor aspartate and asparagine.

The *N*-terminal amino acid sequence of the OXA-2 enzyme was determined as:

Gln-Glu-Gly-Thr-Leu-Glu-
Arg-Ser-Asp-(Trp)-(Arg)-(Arg).

The identification of a unique *N*-terminal sequence indicates that the two subunits of the enzyme are probably identical. The assignments at positions 10, 11 and 12 are tentative because of the extremely low yields that were encountered. Repeated attempts to extend the sequence were unsuccessful for the same reason. Analysis of the sequencing data indicated that the recovery of *N*-terminal glutamine residues was approximately one-third of the expected value, which implies that the OXA-2 enzyme contains a partially blocked *N*-terminal residue. In view of the assignment of a glutamine residue at the *N*-terminus, the blockage probably results from the partial conversion of glutamine to pyroglutamic acid. Attempts to remove the putative pyroglutamic acid residue with pyroglutamate aminopeptidase in order to extend the length of the region that could be sequenced were unsuccessful. Solid-phase sequencing of the OXA-2 enzyme was also unsuccessful, probably owing to the failure of the protein to couple to the support matrix (*p*-phenylene diisothiocyanate-activated 3-aminopropyl-glass beads).

Discussion

The production of the recombinant plasmid pSU3 resulted in a 3-fold increase in the specific activity of the OXA-2 β-lactamase in cell extracts. This is presumably due to a gene-dosage effect (although the copy number of pSU3 has not been

Table 2. *Amino acid composition of the OXA-2 β-lactamase*

All values were calculated on the basis of an M_r value of 32170. The values previously published by Dale (1971) have been recalculated to this M_r. The mean values from samples A and B have been rounded to the nearest whole number; the M_r of the total as shown is 32473.

Amino acid residue	Number of residues per enzyme molecule			Values from Dale (1971)
	Sample A	Sample B	Mean	
Lysine	14.78	14.92	15	16
Histidine	3.51	3.41	3	3
Arginine	20.6	20.08	20	24
Tryptophan	5.4	5.43	5	11
Cysteine	0.91	0.68	1	0
Aspartate/asparagine	28.22	27.52	28	26
Threonine	10.89	10.95	11	8
Serine	13.18	14.2	14	13
Glutamate/glutamine	26.31	27.04	27	24
Proline	9	7.93	8	2
Glycine	17.41	18.26	18	16
Alanine	24.61	24.10	24	23
Valine	16.51	16.97	17	16
Methionine	5.16	5.20	5	4
Isoleucine	13.46	13.19	13	14
Leucine	19.21	19.32	19	19
Tyrosine	6.27	6.13	6	5
Phenylalanine	11.99	11.46	12	15

determined); this does not, however, account for the fact that most of the clones tested did not produce significantly greater amounts of β-lactamase than the parental strain. Combined with the use of the production strain CA265 and the optimization of fermentation conditions, the total yield of the enzyme was increased 78-fold as compared with a control R46 non-production *E. coli* strain. Improvements in the purification procedure, notably the use of a batch DEAE-cellulose step in conjunction with the use of the pSU3 production strain, have enabled us to produce much larger quantities of highly purified enzyme for biochemical characterization.

From the short *N*-terminal amino acid sequence which has been determined, no clear homology with other classes of β-lactamases (Ambler, 1980) can yet be determined. Preliminary DNA sequence information (D. Godwin, P. Stephenson & J. W. Dale, unpublished work) shows some possible regions of low homology with the class A β-lactamases. Furthermore, the interaction of the OXA-2 enzyme with clavulanic acid exhibits a pattern similar to that shown by the TEM-2 enzyme (Holland, 1983).

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