Biochem. J. (1989) 263, 309–311 (Printed in Great Britain)

Purification of TEM-1 β-lactamase by immunoaffinity chromatography

Eitan BIBI
Department of Molecular Biology, Hebrew University–Hadassah Medical School, P.O. Box 1172, Jerusalem 91010, Israel

A monoclonal antibody prepared against TEM-1 β-lactamase was found to compete with penicillins and cephalosporins for binding to the enzyme. The purified antibody preparation was linked to Sepharose 4B and used for immunoaffinity-chromatography purification of TEM-1 β-lactamase. Elution with either benzylpenicillin or cloxacillin yielded a highly purified, concentrated and active enzyme preparation.

INTRODUCTION

TEM-1 β-lactamase (EC 3.5.2.6) has attracted considerable attention in recent years, mainly as a model for the study of structure–function relationships in β-lactamases. Included in these studies are many types of mutant protein, either naturally occurring [1,2] or genetically manipulated [3–5].

In published purification procedures, TEM-1 β-lactamase was prepared by non-specific methods such as ion-exchange chromatography, usually involving multiple steps, and resulting in low recovery of active β-lactamase even when high specific activity was achieved [6].

In the present paper I describe the purification of TEM-1 β-lactamase by a method based on the binding characteristics of an inhibitory monoclonal antibody.

MATERIALS AND METHODS

CNBr-activated Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden, Benzylpenicillin was purchased from Teva, Jerusalem, Israel, and cloxacillin from Bristol Italiana (Sud) Sp.A., Italy. DE-52 DEAE-cellulose was obtained from Whatman BioSystems, Maidstone, Kent, U.K., and Aquacide-II from Behring Diagnostics, La Jolla, CA, U.S.A.

TEM-1 β-lactamase from the periplasmic space of *Escherichia coli* (JM109) harbouring plasmid PJN (kindly provided by Dr. J. H. Richards of Caltech, Pasadena, CA, U.S.A.) was collected as described by Fisher *et al.* [6]. The crude enzyme was adsorbed on DEAE-cellulose and eluted by using the procedure of Melling & Scott [7]. The DEAE-cellulose eluate was dialysed against diluted buffer (DPB) and concentrated with Aquacide-II powder. This partially purified preparation had a specific β-lactamase activity of 0.7 unit (μmol/min)/μg.

The β-lactamase activity was determined by the spectrophotometric method [8] with benzylpenicillin as substrate. All spectroscopic measurements were made with a Uvicon 860 (Contron) spectrophotometer, with the temperature maintained at 30 °C.

Standard procedures [9] were used to produce monoclonal antibodies to TEM-1 β-lactamase. The monoclonal antibody B3, used in the present work, was selected on the basis of inhibition of the catalytic activity as determined by direct assays of TEM-1 β-lactamase incubated with culture supernatants of the screened hybridomas. A preparation of monoclonal antibody B3 was purified on a column of DEAE-cellulose [9], dialysed and concentrated with Aquacide-II powder. The antibody content of the preparation was assessed to be 10 mg/ml, by the single-radial-immunodiffusion procedure [9].

Protein was determined as described by Lowry *et al.* [10], with bovine serum albumin as standard, and by the absorption at 280 nm. The homogeneity of preparations was examined by subjecting samples to SDS/polyacrylamide-gel electrophoresis.

Quantitative determination of TEM-1 β-lactamase was also based on the u.v.-absorption spectrum as suggested by Fisher *et al.* [6]. The molar absorption coefficient of TEM-1 β-lactamase at 281 nm is 29000 $M^{-1} \cdot cm^{-1}$.

RESULTS AND DISCUSSION

Monoclonal antibody B3 competes with substrates of β-lactamase for binding to the enzyme and inhibits its activity. This suggested an immunoaffinity procedure for purifying TEM-1 β-lactamase, with the aid of a column of Sepharose 4B to which the antibody was covalently bound.

CNBr-activated Sepharose 4B (1 g, 3.5 ml) was mixed with a solution (1 ml) containing 10 mg of purified monoclonal antibody B3 and incubated for 2 h at 25 °C in an end-over-end mixer. Coupling of the antibody to the resin was monitored by assays of the inhibitory effect of the supernatant on TEM-1 β-lactamase. When coupling was completed, the gel was mixed with 1 M-ethanolamine, pH 9, and incubated for 2 h at 25 °C in order to block remaining free activated sites in the resin. The resin was then equilibrated with DPB, packed in a column (12 mm × 30 mm) and stored at 4 °C.

Partially purified TEM-1 β-lactamase (23 mg of protein in 2 ml of DPB) was applied to the chromatography column, pre-equilibrated at room temperature with DPB. The column was initially washed with DPB at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected and protein content was measured continuously. When there was no further protein in the washing buffer, the column was

Abbreviations used: TEM-1 β-lactamase, plasmid-pBR322-encoded β-lactamase; DPB, diluted phosphate buffer (5mM-sodium phosphate buffer, pH 8).

eluted with a 50 mg/ml solution of benzylpenicillin or cloxacillin at a flow rate of 3 ml/min. The eluted 2 ml fractions were collected in tubes each containing 0.2 ml of 1 M-sodium phosphate buffer, pH 8, and each was dialysed for 24 h against three changes of 1000 ml of DPB, to remove the eluting β-lactam and its breakdown products. The activity and protein content of each fraction were then determined. Portions (10 μl) of each fraction were subjected to reductive SDS/polyacrylamide-gel electrophoresis.

A high flow rate turned out to be important in preventing the eluted enzyme from re-binding to the column when benzylpenicillin, which is rapidly hydrolysed, was used as the eluent. The product of hydrolysis, penicilloic acid, had no effect on the dissociation of TEM-1 β-lactamase from the immunoaffinity column. With cloxacillin as the eluent a high flow rate was useful in preventing prolonged exposure of the enzyme to cloxacillin and eventual irreversible inactivation induced by this type A substrate [11].

As shown in Figs. 1 and 2, most of the purified enzyme was collected in a single fraction. High specific activity (Table 1) was obtained with either benzylpenicillin or cloxacillin. Indeed, cloxacillin, which has a high affinity for TEM-1 β-lactamase (K_m 13 μM [12]), was found to be a very effective eluent even at low concentrations (below

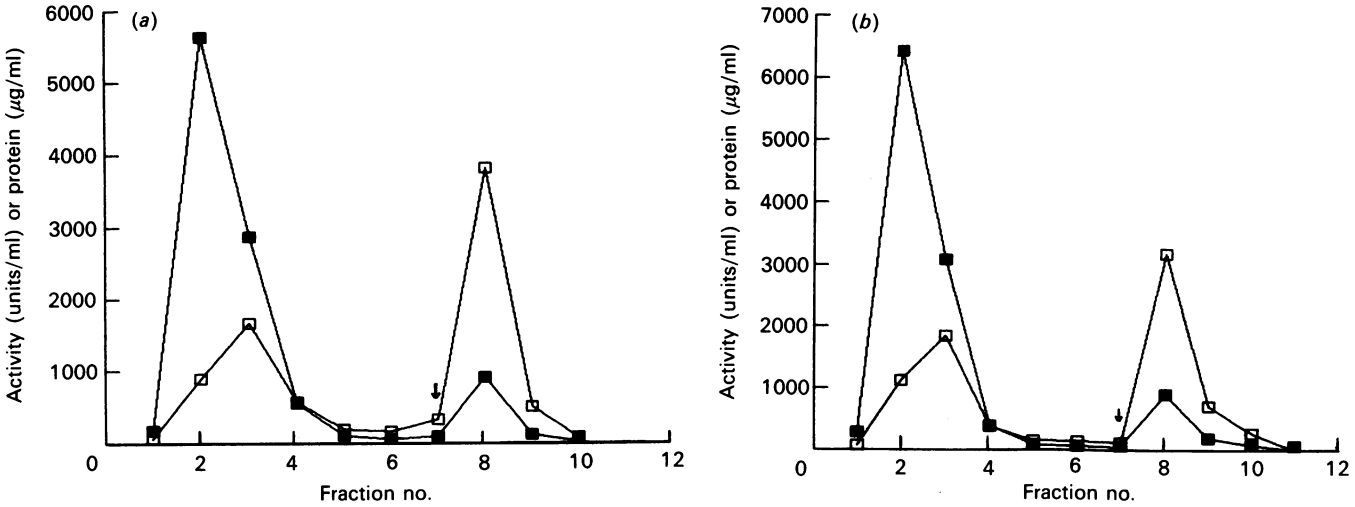

Fig. 1. Affinity chromatography of TEM-1 β-lactamase: (*a*) elution with benzylpenicillin; (*b*) elution with cloxacillin

Fractions (2 ml) were collected into tubes containing 0.2 ml of 1 M-phosphate buffer, pH 8. After dialysis, the fractions were monitored for enzyme activity (□) and protein (■). At fraction 7 (indicated by an arrow) the eluent was changed from DPB to (*a*) benzylpenicillin (50 mg/ml) or (*b*) to cloxacillin (50 mg/ml). The specific activities (units/μg of protein) of the respective eluates were as follows:

Fraction no....	7	8	9	10
(*a*)	3.6	4.2	4.3	3.7
(*b*)	3.1	3.5	3.8	3.4

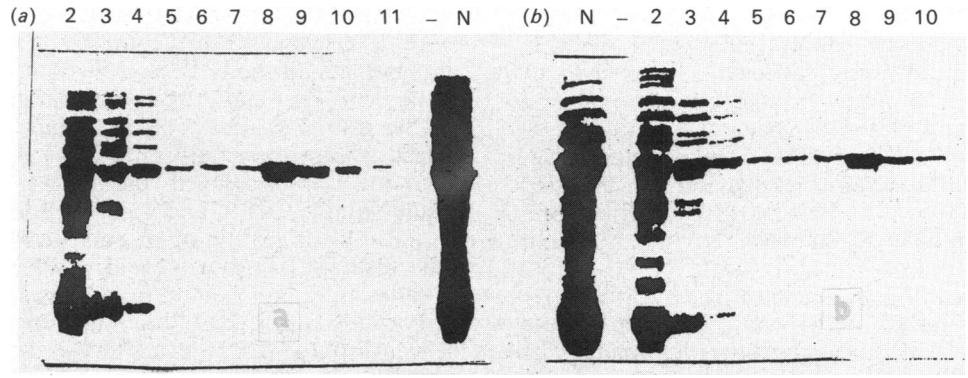

Fig. 2. SDS/polyacrylamide-gel electrophoresis of TEM-1 β-lactamase fractions eluted from the affinity column with (*a*) benzylpenicillin or (*b*) cloxacillin

A 10 μl sample of each fraction was applied to a 17% polyacrylamide gel (N refers to the non-fractionated preparation). After electrophoresis the gels were stained with Coomassie Blue. Amounts of protein (μg) in each well were as follows:

Lane...	2	3	4	5	6	7	8	9	10	11	N
(*a*)	57	29	10	3	3	3	11	6	4	2	230
(*b*)	64	31	6	3	3	4	10	5	3	–	230

Table 1. Comparison of published purification results for TEM-1 β-lactamase

Method	Specific activity (units/μg)		Recovery (%)
	Crude preparation*	Purified preparation	
Melling & Scott [7]	–	3.08	73
Fisher *et al.* [6]	0.98	4.00	83
Present work	0.7	4.19	98.5†

* After the first stage of the purification process.
† Fractions eluted with benzylpenicillin.

5 mM). To ensure saturation of specific binding sites, the column was loaded with a large excess of the TEM-1 β-lactamase preparation. That excess is seen in the first peaks of Figs. 1(*a*) and 1(*b*).

An essential feature of the present immunoaffinity procedure is the rapid, virtually instantaneous, liberation of the enzyme from the column by the added substrate. Monoclonal antibodies that are so readily displaced appear to be rare, probably because conventional selection procedures favour the detection of antibodies firmly immobilized on antigen-coated surfaces.

Received 7 June 1989/28 July 1989; accepted 7 August 1989

I thank Professor N. Citri for many helpful discussions and Mr. K. Abraham for technical assistance.

REFERENCES

1. Sougakoff, W., Goussard, S. & Courvalin, P. (1988) FEMS Microbiol. Lett. **56**, 343–348
2. Sirot, D., Sirot, J., Labia, R., Morand, A., Courvalin, P., Darfeuille-Michaud, A., Perroux, R. & Cluzel, R. (1987) J. Antimicrob. Chemother. **20**, 323–334
3. Schultz, S. C., Dalbadie-McFarland, G., Neitzel, J. J. & Richards, J. H. (1987) Proteins **2**, 290–297
4. Fitts, R., Reuveny, Z., Amsterdam, J. V., Mulholland, J. & Botstein, D. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8540–8543
5. Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C. & Schultz, P. G. (1989) Science **244**, 182–188
6. Fisher, J., Belasko, J. G., Ghosla, S. & Knowles, J. R. (1980) Biochemistry **19**, 2895–2901
7. Melling, J. & Scott, G. K. (1972) Biochem. J. **130**, 55–62
8. Samuni, A. (1975) Anal. Biochem. **63**, 17–26
9. Hudson, L. & Hay, F. C. (1980) Practical Immunology, 2nd edn., pp. 188–213, Blackwell Scientific Publications, Oxford
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
11. Citri, N., Samuni, A. & Zyk, N. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 1048–1052
12. Labia, R., Barthelemy, M., Fabre, C., Guionie, M. & Peduzzi, J. (1979) in Beta-Lactamases (Hamilton-Miller, J. M. T. & Smith, J. T., eds.), pp. 429–442, Academic Press, London