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Technique for a rapid and efficient purification of the SHV-1 and PSE-2 β-lactamases

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Abstract

A simple procedure is described which results in an optimised resolution in molecular sieve chromatography. A sample exhibiting a large initial volume (about 20 ml) and conditioned in a buffer of low ionic strength (<20 m*M*) by filtration through a 53-ml G25 molecular sieve column, is adsorbed on a 1.7-ml ion-exchange (SOURCE) column. The proteins are released by a 10-ml pulse of 1 *M* NaCl and the eluate directly injected onto a 120-ml Sephacryl S100-HR column. The very low volume of the eluate ensures optimal conditions and resolution for the molecular sieving process. The method is applied as the polishing step in the purification of the SHV-1 and PSE-2 β-lactamases. It could easily be scaled up for the treatment of larger samples.

Keywords: Purification; Molecular sieve; β-Lactamases

1. Introduction

Molecular sieve chromatography constitutes a powerful method for the purification of proteins [1]. However, for optimal resolution, it requires a sample of relatively low initial volume, not more than 2% of that of the column [2,3]. In consequence, the protein solution must often be concentrated before it can be injected onto the column. In this paper, we describe a very simple ''on-line'' procedure which ensures optimal resolution of the molecular sieving process. After desalting on a Sephadex G-25 column, the protein mixture is adsorbed on a small volume of ion-exchange gel, eluted by a short pulse of 1 *M* NaCl and directly injected onto a 120-ml Sephacryl S100-HR column.

The efficiency of this procedure is demonstrated by its utilisation as the polishing step in the purification of the SHV-1 [4] and the PSE-2 [5] β-lactamases. These enzymes are responsible for the resistance of the producing bacteria to antibiotics of the penicillin family [6]. To understand their catalytic mechanism, it is necessary to produce large amounts of enzymes for kinetic and structural studies. Simple, rapid and efficient purification procedures are thus required to develop new antibiotics which escape the hydrolytic activity of these enzymes.

2. Materials and methods

2.1. Strains and plasmids

The SHV-1 β-lactamase was produced by an *Escherichia coli* K12 strain carrying plasmid p453 [4] conferring resistance to cephalothin. For the production of the PSE-2 enzyme, plasmid pDML 22b conferring resistance to kanamycin and where the β-lactamase gene is under the control of the

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lac-Z promotor was used to transform an *Escherichia coli* BL-21 strain. The producing strain was a gift of E. Delye (Centre for Protein Engineering, Liège, Belgium).

2.2. Antibiotics and enzymes

Cephalothin was from Eli Lilly (Indianapolis, IN, USA), kanamycin was from Merck, Sharp and Dohme Research Labs. (Rahway, NJ, USA), benzylpenicillin was from Rhône-Poulenc (Paris, France), and nitrocefin was purchased from Oxoid (Bastingstoke, UK). Lysozyme was from Belovo (Bastogne, Belgium) and benzonase from Merck (Darmstadt, Germany).

2.3. Culture conditions

2.3.1. SHV-1

Four 1-l Erlenmeyer flasks, each containing 250 ml of Luria–Bertani (LB) medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl) added with 50 μg/ml cephalothin were inoculated with 10 ml of a 12-h preculture and the culture was grown at 37°C under orbital agitation (250 rpm). After 9 h the culture was stopped and the cells separated by centrifugation for 30 min at 6000 *g* and 4°C.

2.3.2. PSE-2

Four 1-l Erlenmeyer flasks, each containing 250 ml of LB medium added with 50 μg/ml kanamycin were inoculated with 10 ml of a 12-h preculture. After 5.5 h of culture at 37°C (250 rpm), isopropylthiogalactoside (IPTG, 1 m*M*, final concentration) was added and the culture grown for an additional 2.5 h. The cells were separated as described above.

2.4. Chromatography gels

All the chromatography gels (SP Sepharose High Performance, QAE Sepharose Fast Flow and Sephacryl S100-HR) were from Amersham Pharmacia Biotech (Uppsala, Sweden). The following pre-packed columns were purchased from the same company: SOURCE 15 S PE 4.6/100 and SOURCE 15 Q PE 4.6/100 containing a cation and an anion exchangers, respectively; Mono Q HR 5/5 containing an anion exchanger and HIPREP Desalting 26/10 containing Sephadex G-25.

2.5. Chromatography system

All the purification steps were performed with the help of an ÅKTA Explorer 100Air apparatus (Amersham Pharmacia Biotech) at 22°C, but the fraction collector was maintained at 4°C. The XK 16/20 and 16/70 columns were from the same company.

2.6. Enzymatic assays

The activity of the SHV-1 β-lactamase was assayed against 1 m*M* benzylpenicillin in 0.5 ml of 50 m*M* sodium phosphate buffer, pH 7.0, at 22°C. The disappearance of the substrate was monitored at 235 nm. One unit hydrolyses 1 μmol of benzylpenicillin per min under these conditions. The activity of the PSE-2 β-lactamase was tested against 150 μ*M* nitrocefin in the same buffer at 22°C and 482 nm. One unit hydrolyses 1 μmol of nitrocefin per min under these conditions.

Protein concentrations were determined with the help of the BCA kit (Pierce, Rockford, IL, USA). The extinction coefficients determined on this basis were in good agreement with those calculated on the basis of the amino acid composition [7], i.e., 47 100 vs. 47 600 and 32 400 vs. 32 500 M^{-1} cm^{-1} for PSE-2 and SHV-1, respectively.

3. Results

3.1. Purification of the SHV-1 β-lactamase

The cells were suspended in 60 ml of 30 m*M* Tris–HCl buffer, pH 8.0 containing 30% of sucrose. Liberation of the periplasmic content was achieved by addition of lysozyme (100 μg/ml, final concentration) and EDTA (5 m*M* final concentration) to the cooled solution. After a 20 min incubation on ice, the reaction was stopped by addition of an excess of $CaCl_2$ (15 m*M*, final concentration) and the sample was centrifuged for 30 min at 39 000 *g* and 4°C. After overnight dialysis of the supernatant against 5 l of 10 m*M* sodium acetate buffer, pH 5.1 (buffer A), the sample was loaded onto a 25 ml SP Sepharose

High Performance column (XK 16/20) equilibrated with the dialysis buffer. After washing successively with four column volumes of buffer A and two column volumes of 0.1 *M* NaCl in the same buffer, the enzyme was eluted (2 ml/min) with a linear NaCl gradient (0.1–0.25 *M* over six column volumes). The active fractions (12 ml, centred at 0.15 *M* NaCl) were pooled and directly desalted on a HIPREP 26/10 column (53 ml) in buffer A (5 ml/min). The protein-containing fractions were directly loaded onto a small SOURCE 15 S column (1.7 ml) where the enzyme was completely adsorbed. The protein was desorbed with a 10-ml pulse of 1 *M* NaCl in buffer A (1 ml/min) and directly injected onto a Sephacryl S100 HR column (XK 16/70, 120 ml) in series with the SOURCE column and equilibrated with 50 m*M* sodium phosphate buffer, pH 7.0. After elution with the same buffer, the fractions in the major peak (Fig. 1) exhibited a constant specific activity and no trace of contaminating protein was found by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), demonstrating a purity higher than 99%. The purification is summarised in Table 1.

The final specific activity (Table 1) was about 5% higher than that reported in the literature [4] but this might not be significant due to experimental errors.

3.2. Purification of the PSE2 β-lactamase

In this case, attempts to specifically isolate the periplasmic content resulted in very poor yields and, as a consequence, the cells were suspended in 45 ml of 20 m*M* Tris–HCl buffer, pH 8.6 (buffer B) and disrupted with the help of a LH Inceltech Z serial apparatus (Toulouse, France). The crude suspension was treated with Benzonase (1.5 units/ml) for 30 min at 37°C. The supernatant was recovered by a 30-min centrifugation at 39 000 *g* and 4°C. The sample was diluted two-fold with buffer B and loaded onto a 25-ml QAE Sepharose Fast Flow column (XK 16/20) and, after washing, the enzyme was eluted with a linear NaCl gradient (0–0.3 *M* over 10 column volumes) in buffer B. The active fractions (16 ml, centred at 0.1 *M* NaCl) were desalted on the HIPREP 26/10 column and the protein adsorbed on a 1.7-ml SOURCE 15 Q column. As above, the proteins were desorbed by a pulse (10 ml) of 1 *M* NaCl and directly injected onto the S-100 HR column, conditioned in buffer B. The enzyme was eluted with the same buffer and the fractions exhibiting the highest specific activity were pooled. At this stage, an SDS–PAGE analysis indicated that the preparation was still contaminated by several minor impurities. As a final step, the pooled S-100 HR fractions were loaded onto a Mono Q HR 5/5 column in buffer B. The enzyme was eluted (1 ml/min) with a linear NaCl gradient (0–0.15 *M* NaCl over 45 ml). A fraction by fraction analysis by SDS–PAGE indicated the presence of a minor contaminant (less than 2%) in the second half of the enzyme peak. Accordingly, two sets of fractions were pooled and analysed separately (Table 2). The specific activities of both pools were similar within the limits of experimental errors and about 10-fold higher than that reported in a previous publication [5].

4. Discussion

The method which is described here allows the optimisation of the molecular sieve chromatography step. With both enzymes, the volume of the fractions pooled after the first ion-exchange column is much too large to allow immediate loading onto the Sephacryl column, respectively, 10 and 13% of the total volume of the latter. The insertion of a desalting and a concentration steps just before the molecular sieve column consumes very little time (less than 1 h) and results in optimal resolution during the following step. Indeed, the pulse of concentrated NaCl elutes the proteins from the SOURCE columns in very small volumes (1.5–2 ml), which can be directly injected onto the Sephacryl column. After elution of this column, the width at mid-height of the peaks was 4.5 ml with the SHV-1 enzyme and 6.3 ml for PSE-2, i.e. not more than 5% of the total of the volume column. According to the procedures described here, the SHV-1 enzyme could be prepared in a highly homogeneous form within three days (including the 9-h culture) and the PSE-2 enzyme in 3.5 days, including the 8-h culture. With the latter, an additional ion-exchange step had to be included after the molecular sieve column to remove about 30% of residual impurities. This is probably due to the

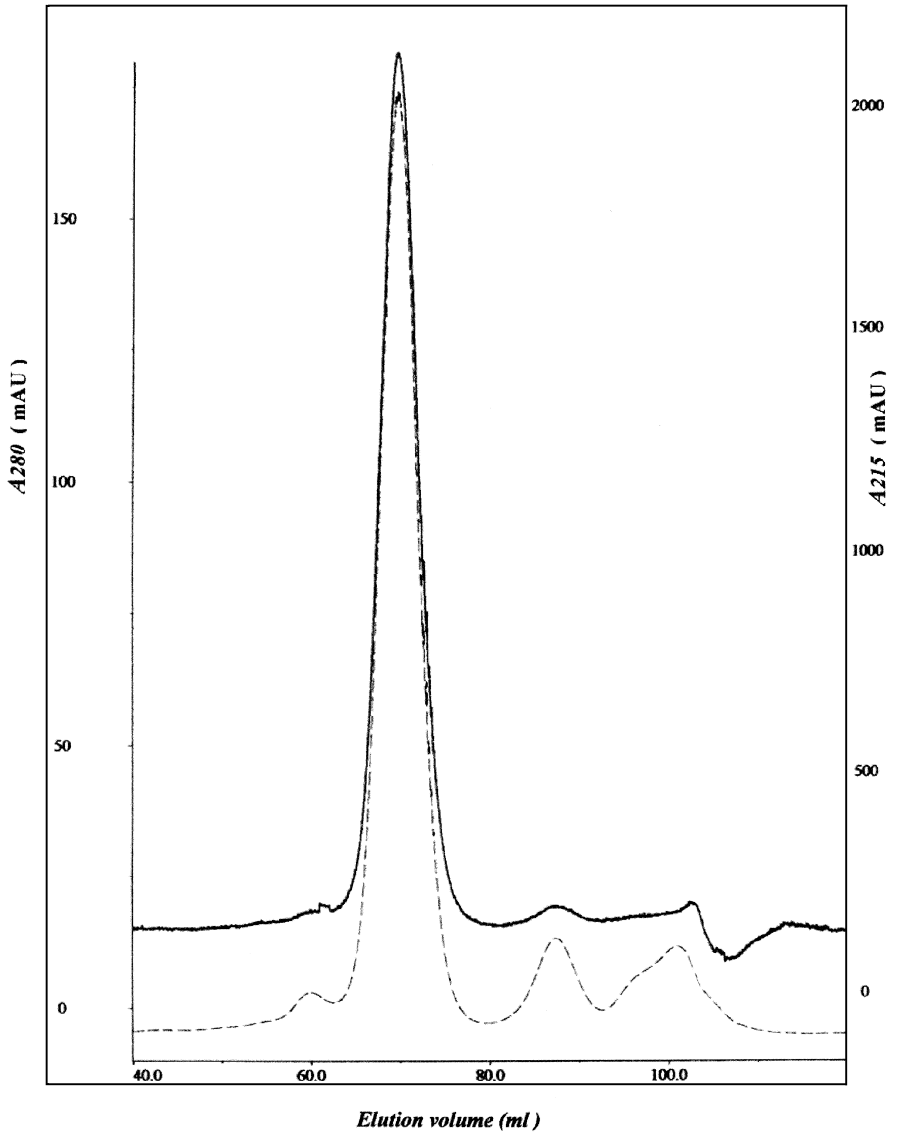

Fig. 1. Protein trace (continuous line, 280 nm; discontinuous line: 215 nm) during elution of the SHV-1 enzyme from the Sephacryl column. The ordinates on the left and right sides indicate the absorbance values at 280 and 215 nm, respectively (AU=absorbance units). The flow-rate was 0.5 ml/min and 2-ml fractions were collected. The void volume was 35 ml and the salt volume 108 ml.

expected fact that the isolation of the periplasmic fraction, which could only be performed with the SHV-1 enzyme yielded a cleaner starting sample than the complete cell disruption method utilised to obtain a good solubilisation of the PSE-2 enzyme.

The procedure could easily be scaled up, since the recommended maximum capacity of the utilised SOURCE columns is about 35 mg of protein and the maximum protein concentration in the peaks from the Sephacryl column was only about 1 mg/ml (see Fig. 1). The utilisation of larger columns which are easily available could certainly allow a 50-fold increase in the purified quantities.

Similarly, the method presents clear advantages for samples containing very dilute proteins, present in low total quantities. Finally, it is quite rapid and

Table 1
Purification of the SHV-1 β-lactamase[a]

Step	Volume (ml)	Total proteins (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg of protein)	Purification factor	Recovered activity (%)
Disrupted cells[b]		2000[b]	16 000	8	–	100
Isolation of periplasm and dialysis	105	40	12 300	300	38	76
SP-Sepharose	12	4.1	8400	2000	250	53
Desalting	20	3.2	6700	2000	250	41
Molecular sieve	14	2.7	6060	2250	280	38

[a] The errors (SD) on the protein concentrations are ±5% up to the dialysis step and ±1% for the last three steps. The errors on the enzyme activity measurements are ±5%.
[b] For allowing a comparison with the PSE-2 enzyme, the total protein content of the cells was estimated.

Table 2
Purification of the PSE-2 β-lactamase (the errors are as in Table 1)

Step	Volume (ml)	Total proteins (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg of protein)	Purification factor	Recovered activity (%)
Disrupted cells	45	1600	5440	3.4	–	100
Benzonase treatment and centrifugation	90	150	4100	27	8	75
QAE-Sepharose	16	21	2800	130	38	51
Desalting	20	20	2600	130	38	48
Molecular sieve	10	9	1540	170	50	28
Mono-Q						
Fraction 1	5	2.20	560	255	74	26
Fraction 2	8	3.6	880	245	72	26

avoids dialysis/concentration steps for unstable proteins.

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References

[1] P. Andrews, Biochem. J. 96 (1965) 595–606.
[2] R.K. Scopes (Ed.), Protein Purification: Principles and Practice, Springer-Verlag, New York, 1994.
[3] J.-M. Frère, C. Gerday, Les Méthodes de Purification et d'Analyse des Protéines, Masson, Paris, 1981.
[4] M. Barthélémy, J. Péduzzi, C. Verchère-Beaur, H. Ben Yaghlane, R. Labia, Ann. Inst. Pasteur/Microbiol. 137B (1986) 19–27.
[5] P. Ledent, X. Raquet, B. Joris, J. Van Beeumen, J.-M. Frère, Biochem. J. 292 (1993) 555–562.
[6] A. Matagne, A. Dubus, M. Galleni, J.-M. Frère, Nat. Prod. Rep. 16 (1999) 1–19.
[7] R. Cantor, P.R. Schimmel, in: Biophysical Chemistry, W.H. Freeman, 1980, p. 380.