AFFINITY PURIFICATION OF BACTERIOPHAGE T4 LYSOZYME FREE OF NUCLEASE

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Received 4 January 1982

1. Introduction

Bacteriophage T4 lysozyme (phage *e* gene product) is produced upon phage infection of *Escherichia coli* cells. Both egg white and T4 lysozyme hydrolyse the *N*-acetylmuramyl linkages in bacterial cell walls, but T4 lysozyme is more specific, as it splits only those glycosidic bonds which are next to *N*-acetyl-muramic acid substituted with peptide side chains [1]. The main products of the digestion of *Escherichia coli* B murein with the phage T4 lysozyme are a disaccharide-peptide (GlcNAc–MurNAc–L-Ala–D-Glu–msA_2pm–D-Ala or C6 muropeptide) and a dimer of the C6 muropeptide connected by a D–D peptide bond between D-Ala and msA_2pm (the C3 muropeptide), which were found to be good competitive inhibitors of the enzyme [1].

The most commonly used methods for the purification of bacteriophage T4 lysozyme are those in [2,3]. The first method is based on repeated ion-exchange chromatography on a cationic resin followed by molecular sieving on Sephadex G-75. This method was modified [3] by adding chromatography on chitin as a preliminary purification step.

Bacterial cell lysis with phage T4 lysozyme is advantageous to more commonly used methods of lysis with hen egg white lysozyme because the possibility of the formation of artifactual complexes between DNA and proteins is greatly reduced when the former enzyme is used [4,5]. This is due to the very high specific activity (~250-times greater than that of egg white lysozyme) and lower basicity of phage T4 lysozyme. In the purification method described here, advantage was taken of the affinity inhibition of phage T4 lysozyme by C6 muropeptide. The lysozyme purified by this method fulfills the demands of gentle lysis procedures for highly concentrated enzyme of very high specific activity. The method is faster than those in [2,3] as it eliminates the use of bulky Sephadex columns and the concentration of samples before application to a chromatographic column.

Abbreviations: msA_2pm, meso-diaminopimelic acid; $PhMeSO_2F$, phenylmethylsulphonyl fluoride; rivanol, 6,9-diamino-2-ethoxyacridine lactate; OC, open circular DNA; CCC, covalently closed circular DNA

2. Materials and methods

2.1. *Amberlite chromatography*

A concentrated solution of partly purified enzyme was prepared by rivanol treatment of *E. coli* B lysate (4 litres) produced by infection with T4 phage, concentration of the enzyme on Amberlite CG 50I and column chromatography on Amberlite CG 50I with NaCl (0–0.6 M) gradient elution [2]. We did not attempt to obtain maximum purification on this step, but to minimize loss of the lysozyme. Therefore, we pooled all fractions having activity >20 bacteriolytic units/ml.

2.2. *Preparation of C6 muropeptide*

Escherichia coli B murein (100 mg) prepared as in [6], was digested (37°C, overnight) with 10^4 bacteriolytic units of bacteriophage T4 lysozyme from the Amberlite column dialysed against 0.1 M ammonium acetate (pH 6.5). The digested murein was concentrated and applied to a Bio-Gel P10 column (1.8 × 120 cm). The elution was monitored by thin-layer chromatography on cellulose (*n*-butan-1-ol/acetic acid/water, 4/1/5).

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

2.3. *Preparation of the affinity adsorbent*

Affi-Gel 202 (Bio-Rad Labs.) (1 ml) was diluted with 5 ml of water and the mixture adjusted to pH 4.75 with 0.01 N HCl. 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride EDAC, (50 mg) was added and pH 4.75 was maintained for 2 h. C6 Muropeptide (20 μmol) was then added and the pH was immediately adjusted to 7.0 with 0.1 N NaOH. The reaction mixture, gently stirred on a mechanical stirrer, was maintained at this pH for 6 h, and then the gel was washed free of reactants.

2.4. *Substrates for the estimation of enzymatic activity*

Escherichia coli B freeze-dried cells were prepared according to [2] and murein–lipoprotein as in [6]. [^{3}H]msA_2pm-labelled murein–lipoprotein (0.5 mg murein/ml; 2.6 × 10^6 cpm/mg murein) was kindly given by Dr U. Schwarz (Tübingen). λ-[^{3}H]DNA (3.6 × 10^4 cpm/μg DNA) was prepared as in [7]. The mixture of open-circular and covalently-closed circular pBR322 was prepared according to [8].

2.5. *Analytical methods*

Bacteriophage T4 lysozyme activity was measured by two methods:

Test A: Bacteriolytic activity was estimated by following the decrease of turbidity of the freeze-dried *E. coli* B cells in 50 mM Tris–HCl buffer (pH 7.3) containing 0.03% Triton X-100. This substrate (A_{470} 0.80) (2 ml) was mixed with 100 μl of enzyme solution and the reaction was carried out for 4 min at 37°C. Transmittance was read in 1 cm cuvettes against substrate suspension with 100 μl buffer added. A standard curve was prepared with 1–50 μg/ml of hen egg white lysozyme. As an arbitrary unit, the activity of 1 μg egg white lysozyme (Sigma, grade I) was chosen.

Test B: Quantitative determination of the lysozyme activity was performed by measuring the release of radioactivity from [^{3}H]msA_2pm-labelled murein–lipoprotein suspension as described in [9]. The reaction was carried out in 50 mM Tris–HCl buffer (pH 7.3) containing 0.2% Triton X-100. One unit of activity corresponded to the activity of 1 μg egg white lysozyme (Sigma, grade I).

For the estimation of exonuclease activity, λ-[^{3}H]-DNA digested with *Eco*RI restriction enzyme (prepared according to [10]), phenol-extracted and ethanol-precipitated, was incubated at 37°C in 100 mM Tris–HCl, 10 mM $MgCl_2$ (pH 7.4) with 0, 250 and 500 units of phage T4 lysozyme/μg DNA. After 0, 1, 2 and 4 h, aliquots corresponding to 0.3 μg DNA were removed in triplicate, perchloric acid added to 0.5 M final conc. and the samples were assayed for soluble radioactivity.

3. Results and discussion

The purification of bacteriophage T4 lysozyme is shown in table 1.

3.1. *Affinity chromatography on a C6 muropeptide–Affi-Gel 202 column*

The pooled Amberlite fractions were dialysed against 50 mM Tris–HCl buffer (pH 7.3) containing

Table 1
Purification of bacteriophage T4 lysozyme

Purification step	Vol. (ml)	Total act. (U)[a]	% original activity	Spec. act. (U/μg protein)[a]	Purification factor
Lysate of *E. coli* B	4000	3.8 × 10^5	100	0.2	1
Concentration on Amberlite CG 50I	1500	2.0 × 10^5	53	6.3	31.5
Chromatography on Amberlite CG 50I	375	2.1 × 10^5	55	30.0	150
Affinity chromatography on C6–Affi-Gel 202	22	2.3 × 10^5	60	179.0	895
Chromatography on CM-Sephadex C50	40	1.8 × 10^5	47	130.0	1150

[a] Measured using [^{3}H]msA_2pm murein–lipoprotein as the substrate

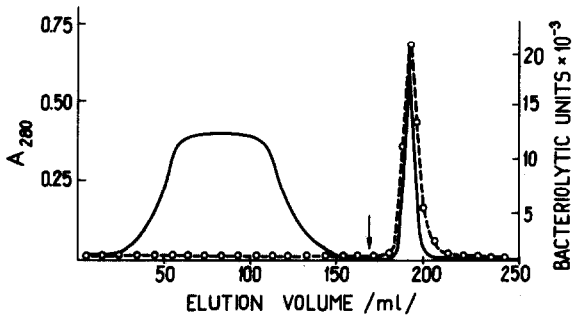

Fig.1. Affinity chromatography of bacteriophage T4 lysozyme on a C6 muropeptide–Affi-Gel 202 column (0.5 × 10 cm). The protein content was monitored by absorbancy reading at 280 nm on a Uvicord III (LKB) and bacteriolytic activity by the decrease of turbidity of *E. coli* B. cells. The arrow indicates the point at which elution was begun with 1 M NaCl: (——) absorbancy at 280 nm; (– – –) bacteriolytic activity.

1 mM EDTA and protease inhibitor (20 μg $PhMeSO_2F$/ml), and applied to a small column of C6–Affi-Gel 202. The results are shown in fig.1. The whole activity was retained on the column in these conditions. The lysozyme was eluted with 1 M NaCl as a narrow peak of very high activity (~180 units/μg protein, 2×10^4 units/ml eluate) and free of nucleolytic activity. The column may be reused many times without appreciable loss of capacity for the lysozyme adsorption.

It was found that the lysozyme is completely adsorbed over pH 6.0–8.0 when the buffer level is ≤0.2 M. The lysozyme eluted from the affinity col-

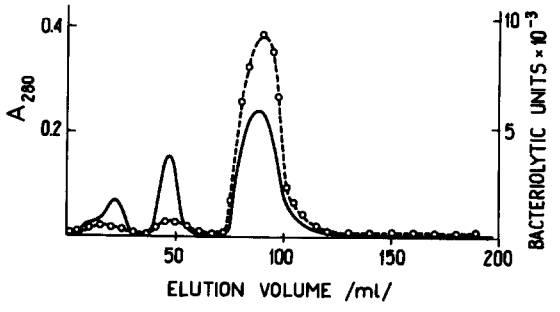

Fig.2. CM-Sephadex C-50 chromatography of bacteriophage T4 lysozyme. The lysozyme solution eluted from the affinity column was dialysed against 50 mM Tris–HCl (pH 7.3) containing 1 mM EDTA and protease inhibitor (20 μg $PhMeSO_2F$/ml), and applied to a CM-Sephadex column (1 × 20 cm). The elution was carried out with a linear gradient (0–1 M) of NaCl: (——) absorbancy at 280 nm; (– – –) bacteriolytic activity.

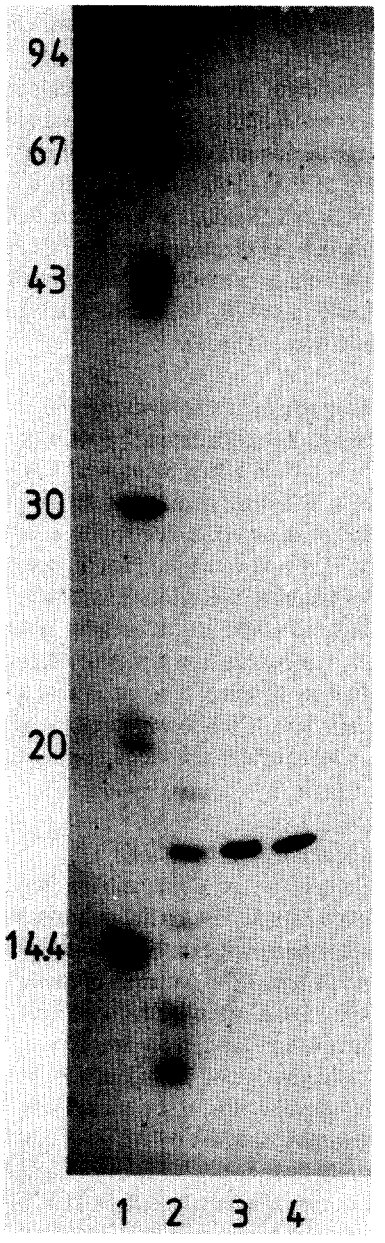

Fig.3. SDS–polyacrylamide gradient gel (14–25%) electrophoresis of bacteriophage T4 lysozyme: (1) protein M_r standards (Pharmacia) from top to bottom, phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), lactalbumin (14 400); (2) T4 lysozyme preparation eluted from the affinity column; (3,4) T4 lysozyme eluted from the CM-Sephadex column.

umn is relatively stable and can be stored at 4°C for a number of days without decrease of activity. The yield of the lysozyme is >100% indicating that some inhibitors are removed during this purification procedure.

Even very carefully selected fractions from the separation on Amberlite CG 50I contain ≥20 electrophoretic protein bands (sometimes of very low intensity) of M_r 25 000–100 000, apart from a number of proteins of $M_r < 25\,000$. On the other hand, SDS–polyacrylamide gel electrophoresis of the eluate from the affinity column reveals the presence of only 4–6 protein bands, all of $M_r < 20\,000$; the lysozyme band being the strongest (fig.3).

3.2. *CM-Sephadex C-50 chromatography*

The pooled fractions from the affinity column were further purified on a column of CM–Sephadex C-50 (fig.2). The lysozyme leaves the column as the last protein peak and it is electrophoretically homogenous (fig.3). Two remaining protein peaks contain low but measurable bacteriolytic activity. We have not investigated whether this activity is associated with other bacteriolytic enzymes or with the lysozyme bound by physical forces to other proteins. However, in view of the lower stability of purified lysozyme, we consider the second possibility more likely.

3.3. *Exo- and endonuclease tests*

To obtain a lysozyme preparation free of nucleolytic activity with an Amberlite column, one must collect a very narrow peak. Even then, prolonged incubation (4 h at 37°C) of such preparations with λ or pBR322 DNA often leads to nucleolytic digestion. On the other hand, nucleases have no affinity for C6 muropeptide and all of our preparations were completely devoid of nucleolytic activity after chromatography on the C6–Affi-Gel column. Thus, in the exonuclease test <0.5% of radioactivity (a criterion for exonuclease presence [10]) was released from *Eco*RI fragments of λ-[^{3}H]DNA after incubation with 500 bacteriolytic units of the lysozyme for 4 h at 37°C. To test the lysozyme for endonuclease activity, pBR322 DNA was incubated with 500 bacteriolytic units of the enzyme and then electrophoresed. Incubation for up to 4 h caused no conversion of open or covalently closed circles to linear DNA, indicating the absence of endonucleases in the enzyme preparations (fig.4).

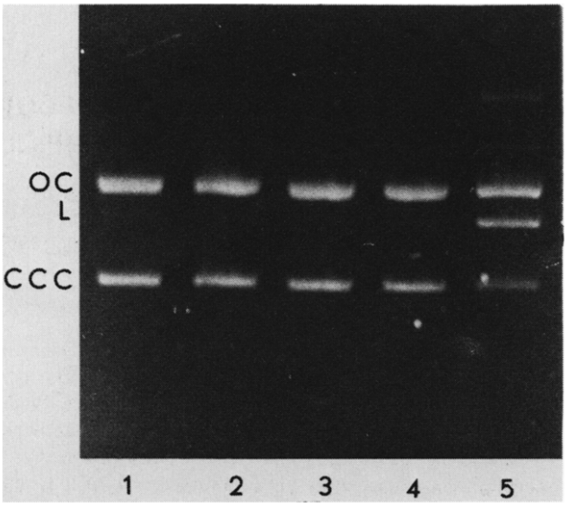

Fig.4. Agarose (1%) gel electrophoresis of pBR322 DNA incubated with bacteriophage T4 lysozyme. Each reaction mixture contained 1 μg DNA (about equal proportions of open circle and supercoil forms), 500 units of T4 lysozyme eluted from the affinity column and was incubated at 37°C for 1 h (slot 2), 2 h (slot 3), and 4 h (slot 4): slot (1), pBR322 DNA without the lysozyme added; slot (5), partial digestion with *Eco*RI endonuclease (all 3 DNA forms present).

Acknowledgements

The authors thank Dr U. Schwarz for the generous gift of ^{3}H-labelled murein–lipoprotein and Dr M. Żylicz for many helpful discussions. This work was supported by Polish Academy of Sciences under project 09.7.

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