Journal of Chromatography, 639 (1993) 75–80
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 910

Preparation of DNA polymerase from *Bacillus caldotenax*

Judith A. Burrows and Christopher R. Goward*

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

ABSTRACT

A procedure with four chromatography steps was developed for the purification of DNA polymerase from *Bacillus caldotenax* by using fast protein liquid chromatography. The procedure was suitable for use with process-scale media. Elution profiles obtained from ion-exchange chromatography and triazine–dye affinity chromatography with fast protein liquid chromatography and process-scale media were similar. The enzyme showed stronger interaction, however, with phenyl-Sepharose FF in the scaled-up process than with the phenyl-Superose used in fast protein liquid chromatography. The surprising binding of the DNA polymerase to sulphonated ion-exchange media at pH 7.5 may be explained by the structure of the enzyme.

INTRODUCTION

DNA polymerase is an enzyme required for the replication and repair of DNA. Thermostable DNA polymerases are of particular interest because of their potential application in recombinant DNA techniques such as for the polymerase chain reaction (PCR) [1] and for DNA sequencing [2]. Thermophilic organisms from which DNA polymerase has been purified include *Thermus aquaticus* [3,4], *T. flavus* [5], *T. ruber* [6] and *T. thermophilus* [7–9]. The gene coding for DNA polymerase from *T. aquaticus* (*Taq* polymerase) was cloned and expressed in *E. coli* [10]. *Bacillus* spp. studied include *B. stearothermophilus* [11–13] and *B. caldovelox* [9].

DNA polymerase from *B. caldotenax* has been purified by two successive chromatography steps on DEAE-cellulose followed by separation on phosphocellulose and heparin-Sepharose [9]. Further purification was required to obtain homogenous enzyme by using sucrose gradient centrifugation. *B. caldotenax* is the most thermophilic of the *Bacillus* spp. and is capable of growth at 80°C [14].

The aim of the work described here was to use fast protein liquid chromatography (FPLC) to develop a facile purification procedure for DNA polymerase from *B. caldotenax* suitable to be scaled up to provide protein for characterisation and X-ray crystallography studies of the enzyme.

EXPERIMENTAL

Materials

Deoxynucleoside triphosphates (dNTPs) were from BCL, East Sussex, UK and tritiated thymidine triphosphate from Amersham, Aylesbury, UK. Activated calf thymus DNA, dithiothreitol (DTT), phenylmethanesulphonyl fluoride (PMSF), Nonidet P40, polyoxyethylene sorbitan (Tween 20) and trichloroacetic acid (TCA) were from Sigma, Poole, UK. 3MM chromatography paper was from Whatman Lab Sales, Maidstone, UK. Optiscint Hisafe, Q-Sepharose FF, S-Sepharose FF, phenyl-Sepharose FF and M_r marker proteins were from Pharmacia–LKB, Uppsala, Sweden. Blue-Tris-acryl M was from Life Science Labs., Luton,

* Corresponding author.

0021-9673/93/$06.00

UK. All other reagents were obtained from BDH, Poole, UK.

Equipment

The FPLC system supplied by Pharmacia–LKB, consisted of an LCC-500 Plus liquid chromatography controller, two P500 pumps, a Frac 100 fraction collector, a single path monitor UV-1 optical unit and a Rec 482 recorder. Samples were injected either from a 1-ml sample loop or from a 10-ml Superloop from Pharmacia-LKB. Chromatography columns used in FPLC were obtained prepacked and an HR 5/5 column was packed with Blue-Trisacryl M, according to the manufacturers instructions. Adjustable capacity columns were obtained for the scaled-up process from Amicon, Stonehouse, Gloucestershire, UK, and a 101U pump was supplied by Watson Marlow, Smith and Nephew Pharmaceuticals, Falmouth, UK. A single-path monitor UV-1 optical unit, Rec 482 recorder and a Frac 100 fraction collector were also used.

Measurement of DNA polymerase activity

A 50-μl portion of assay mixture (50 m*M* Tris–HCl buffer, pH 7.5, containing 70 m*M* $MgCl_2$, 1 m*M* DTT, 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 25 μM dTTP, 6.25 μg activated calf thymus DNA and 0.5 μCi [^{3}H]dTTP, of specific radioactivity 43 Ci mmol^{-1}). The reaction was stopped by placing the reaction mixture on ice and 50 μl were transferred to a strip (3 cm × 1 cm) of 3MM chromatography paper. DNA was precipitated by a wash in 10% (w/v) TCA for 1 h followed by two further washes for 30 min each in 5% (w/v) TCA and then a final wash in ethanol for 30 min. The chromatography paper strips were dried in air, placed in 4 ml of Optiscint Hi-Safe Scintillant and incorporated radioactivity counted in an LKB Wallac 1215 Rackbeta counter. The definition of one unit of DNA polymerase is that required to catalyse the incorporation of 10 nmol dNTP into acid-insoluble radioactivity in 30 min at 70°C.

Measurement of protein concentration

Protein concentrations were determined by the Folin method of Lowry *et al.* with bovine serum albumin as the standard [15]. The protein content of column eluates was also monitored by A_{280}.

Bacterial cell culture

B. caldotenax [14] obtained from Dr. R.J. Sharp (Division of Biotechnology, PHLS Centre for Applied Microbiology and Research) was grown in a 25-l New Brunswick fermenter in double-strength Lüria broth (30 g l^{-1} tryptone, 10 g l^{-1} yeast extract and 10 g l^{-1} NaCl). The temperature was maintained at 60°C and the pH maintained at 7.2 ± 0.1 by the addition of NaOH or HCl. Cells were harvested after 4 h, while still in the exponential phase of growth (A_{600} 7.2), quick frozen and stored at −20°C. The culture produced 230 g of cell paste.

Lysis of cells

Cells were thawed at 4°C in 50 m*M* Tris–HCl buffer, pH 7.5 containing 1 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* PMSF, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P40 and 0.01% (v/v) Tween 20 (buffer A). The cell suspension was disrupted with an MSE Soniprep 150 at a frequency of 18 MHz for periods of 30 s followed by cooling periods of 1 min in an iced-water chamber. Cell debris was removed by contrifugation for 45 min at 13 000 *g* and at 4°C.

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed under denaturing conditions in a Pharmacia–LKB PhastGel apparatus with PhastGel Gradient 10–15 gels and SDS buffer strips as described by the manufacturer. The protein bands were visualised with silver stain as described by Pharmacia–LKB. Alternatively, slab gels containing 12.5% (w/v) acrylamide were run in an LKB vertical electrophoresis unit under denaturing conditions [16], and the protein bands stained with Coomassie Brilliant Blue R-350.

PREPARATION OF DNA POLYMERASE

Development of a purification procedure by using FPLC

An FPLC system (Pharmacia–LKB) was used

to determine optimum conditions for purification of the enzyme. Buffers were prepared with Milli-Q water (Millipore) and filtered through a 0.22-μm pore-size filtration membrane. Mono Q, phenyl-Superose and Mono S have an average particle size of 10-μm and Blue-Trisacryl M has a particle size of 40 to 80 μm. Chromatographic media were equilibrated, cleaned and stored according to the manufacturers instructions. All separations with FPLC were conducted at ambient temperature (18°C to 25°C). DNA polymerase activity was determined in each of the fractions collected.

Cell-free extract from 2 g of cell paste was applied to an 8-ml Mono Q HR 10/10 column (10 cm × 1 cm I.D.), equilibrated with buffer A at a linear flow-rate of 300 cm h^{-1}. Protein was eluted with a 160-ml linear gradient of 0 to 1 *M* NaCl in buffer A and fractions of 8 ml were collected. Ammonium sulphate was added to the Mono Q eluate to a final concentration of 1 *M*. A 1-ml phenyl-Superose HR 5/5 column (5 cm × 0.5 cm I.D.) was equilibrated with buffer A, containing 1 *M* ammonium sulphate, at a linear flow-rate of 150 cm h^{-1}. A 1-ml portion of sample was applied to the column and protein was eluted with a 20-ml linear gradient of equilibration buffer to buffer A containing no ammonium sulphate and fractions of 1 ml were collected. Active fractions were pooled and dialysed exhaustively against buffer A. A 1-ml HR 5/5 column (5 cm × 0.5 cm I.D.) was packed with Blue-Trisacryl M according to the manufacturers instructions. The column was equilibrated with buffer A and 1 ml of the phenyl-Superose eluate was applied at a linear flow-rate of 300 cm h^{-1}. Protein was eluted with a 20-ml linear gradient of 0 to 1 *M* NaCl in buffer A and fractions of 1 ml were collected. Active fractions were combined and dialysed exhaustively against buffer A. A 1-ml Mono S HR 5/5 column (5 cm × 0.5 cm I.D.) was equilibrated with buffer A and 1 ml of the Blue-Trisacryl eluate was applied to the column at linear flow-rate of 300 cm h^{-1}. Protein was eluted with a 20-ml linear gradient of 0 to 1 *M* NaCl in buffer A and fractions of 1 ml were collected. Active fractions were combined based upon activity and electrophoretic homogeneity and dialysed exhaustively against buffer A.

Scale-up of the purification procedure

The purification scheme developed with FPLC media was scaled-up by a factor of about 25 fold. The FPLC media were replaced with Q-Sepharose FF, phenyl-Sepharose FF and S-Sepharose FF. The particle size of each matrix is 45–165 μm. The matrix for the triazine-dye affinity chromatography step was the same as for FPLC. DNA polymerase activity was determined in each of the fractions collected.

Cell-free extract from 50 g of cell paste was applied to a 200-ml Q-Sepharose FF column (12.5 cm × 4.5 cm I.D.). The column was equilibrated with buffer A at a linear flow-rate of 60 cm h^{-1}. The protein was eluted with a 4-l gradient of 0 to 1 *M* NaCl in buffer A and fractions of 95 ml were collected. Active fractions were combined and ammonium sulphate added to a final concentration of 1 *M*. The eluate was applied to a 25-ml phenyl-Sepharose column (12 cm × 1.6 cm I.D.) equilibrated with buffer A containing 1 *M* ammonium sulphate, at a linear flow-rate of 25 cm h^{-1}. The protein was eluted in a 500-ml linear gradient of 1 *M* to 0 *M* ammonium sulphate in buffer A followed by 100 ml buffer A. Fractions of 10 ml were collected and active enzyme fractions were combined. The active fractions were applied to a 25-ml Blue-Trisacryl M column (12 cm × 1.6 cm I.D.) equilibrated with buffer A at a linear flow-rate of 25 cm h^{-1}. The protein was eluted with a 500-ml linear gradient of 0 to 1 *M* NaCl in buffer A. Fractions of 10 ml were collected, active enzyme fractions were combined and dialysed exhaustively against buffer A. The dialysed active fractions were applied to a 25-ml S-Sepharose FF column (12 cm × 1.6 cm I.D.), equilibrated with buffer A at a linear flow-rate of 25 cm h^{-1}. Protein was eluted in a 500-ml linear gradient of 0 to 1 *M* NaCl in buffer A and fractions of 10 ml were collected. Fractions containing DNA polymerase were combined based on activity and electrophoretic homogeneity and dialysed exhaustively against buffer A.

RESULTS AND DISCUSSION

Elution profiles for DNA polymerase purification on FPLC and on process-scale media are shown in Figs. 1 and 2 and a summary of each

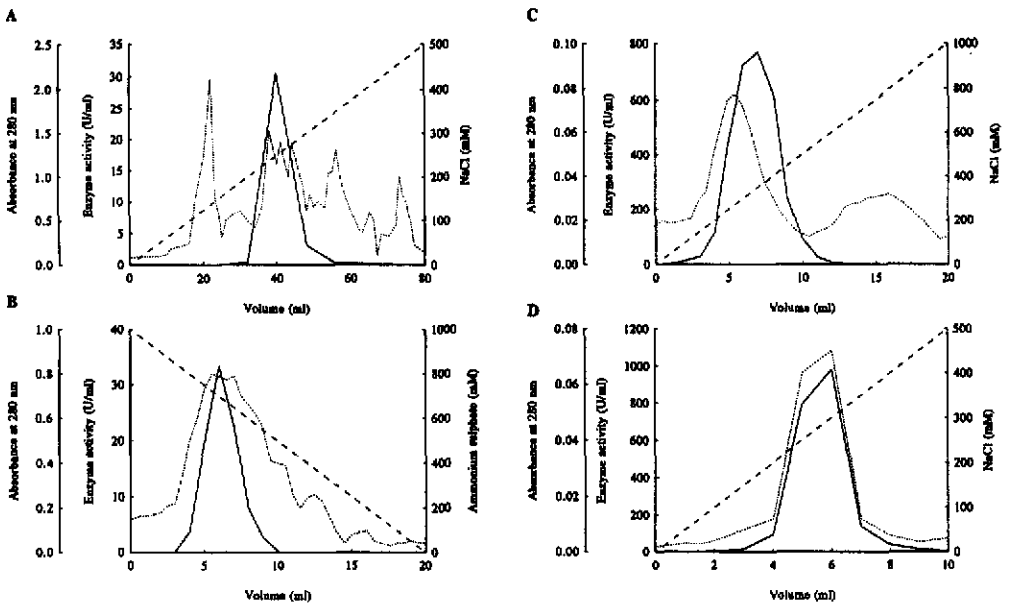

Fig. 1. Chromatography elution profiles for FPLC. The elution profiles of DNA polymerase (——), A_{280} (···) and the salt gradient (– – –) are shown for (A) anion-exchange chromatography on Mono Q, (B) hydrophobic interaction chromatography on phenyl-Superose, (C) triazine-dye affinity chromatography on Blue-Trisacryl M and (D) cation-exchange chromatography on Mono S. The salt gradients shown in the chromatograms for Mono Q and Mono S represent half of the total volumes used in the gradients; the gradients were actually extended from 0 to 1000 m*M* NaCl.

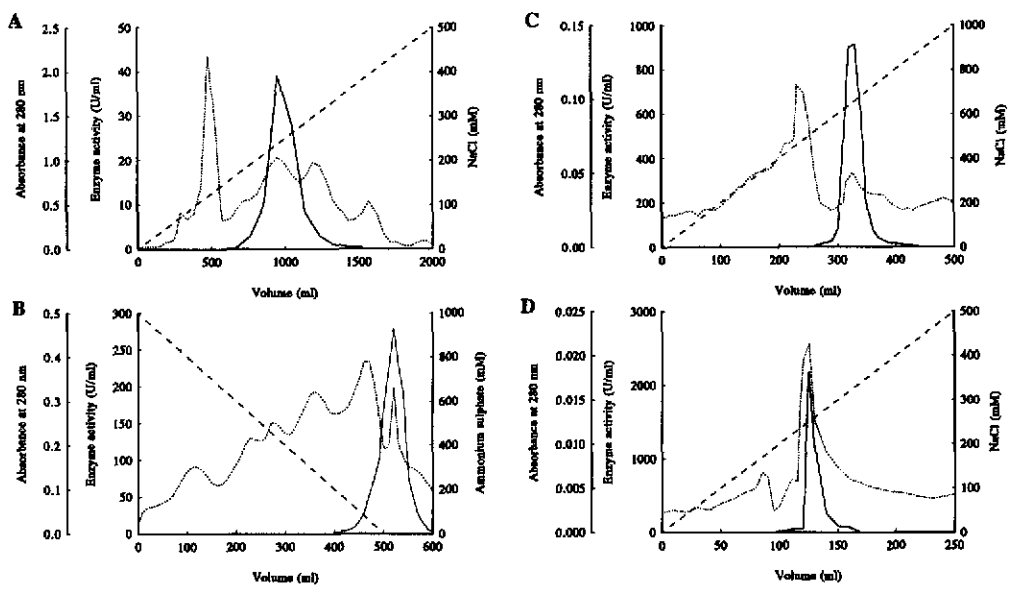

Fig. 2. Chromatography elution profiles for the scaled-up process. The elution profiles of DNA polymerase (——), A_{280} (···) and the salt gradient (– – –) are shown for (A) anion-exchange chromatography with Q-Sepharose FF, (B) hydrophobic interaction chromatography with phenyl-Sepharose FF, (C) triazine-dye affinity chromatography with Blue-Trisacryl M and (D) cation-exchange chromatography with S-Sepharose FF. The salt gradients shown in the chromatograms for Q-Sepharose and S-Sepharose represent half of the total volumes used in the gradients; the gradients were actually extended to 1000 m*M* NaCl.

purification procedure is shown in Tables I and II. In both cases homogenous enzyme of specific activity about 1950 U mg^{-1} (Tables I and II) was obtained after four chromatography steps as shown by SDS-PAGE (Fig. 3). The elution profiles for anionic and catonic exchange are similar with both FPLC media and process-scale media. The enzyme eluted from Mono Q in about 220 m*M* NaCl and from Q-Sepharose in about 240 m*M* NaCl. Enzyme eluted from Mono S and S-Sepharose FF in about 250 m*M* NaCl. The elution profiles obtained after hydrophobic interaction chromatography on phenyl-Superose and phenyl-Sepharose FF are, however, different. The enzyme eluted in about 650 m*M* ammonium sulphate on FPLC and yet at the end of the ammonium sulphate gradient in the scaled-up process. An advantage of this delayed elution was that dialysis of the eluate was not required. Blue-Trisacryl M was used in both procedures for triazine-dye affinity chromatography. The enzyme eluted in about 650 m*M* NaCl on the larger scale compared to about 350 m*M* during FPLC. The difference may be due to the linear flow-rate of 25 cm h^{-1} at which the scaled-up process was run compared with 300 cm h^{-1} on FPLC.

Chromatography was carried out under identical conditions for both cation-exchange and anion-exchange chromatography. The p*I* of 4.9 [17], indicates that the enzyme has a negative surface charge and should only bind to the anion-exchange matrices Mono Q and Q-Sepharose FF. The surface charge of the protein is likely to be negative at pH 7.5 and true cation exchange cannot take place. The enzyme therefore binds to Mono S and S-Sepharose FF by some mechanism other than by cation exchange. A possible mechanism is that a positively charged DNA-binding site of the enzyme inter-

TABLE I

DNA POLYMERASE PURIFICATION ON FPLC MEDIA

Step	Volume (ml)	Protein (mg)	Enzyme (Units)	Specific activity (U mg^{-1})
Cell-free extract	10	90	42[a]	0.5
Mono Q eluate	8	18	245[a]	14
Phenyl-Superose eluate	5	4	1046[a]	262
Blue-Trisacryl M eluate	5	2	2989	1495
Mono S eluate	2	0.9	1772	1970

[a] Apparent DNA polymerase activity is low due to nucleolytic enzyme activity.

TABLE II

DNA POLYMERASE PURIFICATION ON PROCESS-SCALE MEDIA

Step	Volume (ml)	Protein (mg)	Enzyme (U)	Specific activity (U mg^{-1})
Cell-free extract	250	2250	1 056[a]	0.5
Q-Sepharose FF eluate	190	285	6 338[a]	22
Phenyl-Sepharose FF eluate	50	36	10 878[a]	302
Blue-Trisacryl M eluate	50	18	31 047	1725
S-Sepharose FF eluate	20	10	19 136	1914

[a] Apparent DNA polymerase activity is low due to nucleolytic enzyme activity.

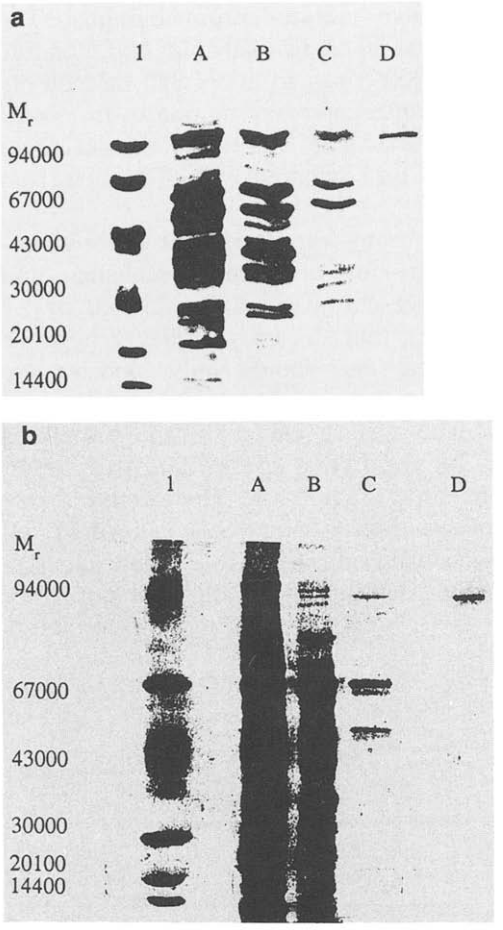

Fig. 3. SDS-PAGE of eluates from FPLC. (a) Mono Q (A), phenyl-Superose (B), Blue-Trisacryl M (C) and Mono S (D) eluates were separated on PhastGel Gradient 10–15 gels and with SDS buffer strips (Pharmacia-LKB). (b) Q-Sepharose FF (A), phenyl-Sepharose FF (B), Blue-Trisacryl M (C) and S-Sepharose FF (D) eluates were separated on a 12.5% (w/v) acrylamide gel in the presence of SDS. The following standards (1) were used: phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100) and α-lactalbumin (M_r 14 400).

acts with the charged sulphonate groups of Mono S and S-Sepharose FF. X-ray crystallography studies of the Klenow fragment of *E. coli* DNA polymerase I [18] have shown that a 400 amino-acid polypeptide forms a deep cleft and is thought to accommodate double-stranded DNA. The enzyme has a negative surface charge and is acidic, but a positive charge was detected in the cleft at physiological pH [19]. The enzyme from *B. caldotenax* is also acidic [17] and may have a similar cleft involved in binding to DNA. This putative positively-charged cleft may be shown by X-ray crystallography studies currently in progress for the enzyme from *B. caldotenax*.

The procedure developed by using FPLC was scaled-up further [17] to provide protein for X-ray crystallography studies and for enzyme characterisation.

ACKNOWLEDGEMENTS

We thank Professor T. Atkinson and Dr. M.D. Scawen for critical comments in the preparation of the manuscript.

REFERENCES

1 R.K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich and N. Arnheim, *Science*, 230 (1985) 1350.
2 F. Sanger, A.R. Coulson, B.G. Barrell, A.J.H. Smith and B.A. Roe, *J. Mol. Biol.*, 143 (1980) 161.
3 A. Chien, D.B. Edgar and J.M. Trela, *J. Bacteriol.*, 127 (1976) 1550.
4 A.S. Kaledin, A.G. Slyusarenko and S.I. Gorodetskii, S.I. *Biokhimiya*, 45 (1980) 644.
5 A.S. Kaledin, A.G. Slyusarenko and S.I. Gorodetskii, *Biokhimiya*, 46 (1981) 1576.
6 A.S. Kaledin, A.G. Slyusarenko and S.I. Gorodetskii, *Biokhimiya*, 47 (1982) 1785.
7 C. Rüttiman, M. Cotorás, J. Zaldívar and R. Vicuña, *Eur. J. Biochem.*, 149 (1985) 41.
8 N. Carballeira, M. Nazabal, J. Brito and O. Garcia, *Biotechniques*, 9 (1990) 276.
9 E. Sellman, K-L. Schröder, I-M. Knoblich and P. Westermann, *J. Bacteriol.*, 174 (1992) 4350.
10 F.C. Lawyer, S. Stoffel, R.K. Saiki, K. Myambo, R. Drummond and D.H. Gelfand, *J. Biol. Chem.*, 264 (1989) 6427.
11 J. Stenesh and B.A. Roe, *Biochim. Biophys. Acta.*, 272 (1972) 156.
12 O.K. Kaboev, L.A. Luchkina, A.T. Akhmedov and M.L. Bekker, *J. Bacteriol.*, 145 (1981) 21.
13 S.Y. Ye and G.F. Hong, *Sci. Sin.*, 30 (1987) 503.
14 W. Heinen, *Arch. Mikrobiol.*, 76 (1971) 2.
15 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
16 U.K. Laemmli, *Nature (London)*, 227 (1970) 680.
17 J.A. Burrows and C.R. Goward, *Biochem. J.*, 287 (1992) 971.
18 C.M. Joyce and T.A. Steitz, in D.L. Oxender and C.F. Fox (Editors), *Protein Engineering*, Alan R. Liss, New York, 1987, Ch. 20, p. 227.
19 J. Warwicker, D.L. Ollis, F.M. Richards and T.M. Steitz, *J. Mol. Biol.*, 186 (1985) 645.