Journal of Chromatography, 585 (1991) 153–159
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 502

Preparative high-yield electroelution of proteins after separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and its application to analysis of amino acid sequences and to raise antibodies

Toshitaka Ohhashi, Chie Moritani, Hiromi Andoh, Sachiko Satoh and Shinji Ohmori

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1, Okayama 700 (Japan)

Friedrich Lottspeich

Gene Center of the Max-Planck-Institute for Biochemistry, Am Klopferspitz 18a, 8033 Martinsried (Germany)

Mikiko Ikeda*

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1, Okayama 700 (Japan)

(First received February 27th, 1991; revised manuscript received May 24th, 1991)

ABSTRACT

A method for the preparative high-yield electroelution of proteins from sodium dodecyl sulphate (SDS) polyacrylamide gel strips was established. The method consisted of SDS–polyacrylamide gel electrophoresis, detection of proteins with sodium acetate and electrophoretic elution at 200 V for 3 h by utilizing a horizontal flat-bed gel electrophoresis apparatus. Standard proteins with molecular masses of 14–66 kilodalton (cytochrome *c*, aldolase, ovalbumin and bovine serum albumin) were recovered with an average yield of 73.6 ± 2.3%. A membrane-bound protein, rat skeletal muscle Ca^{2+}-ATPase (100 kilodalton) was also well recovered (over 60%). This method was applicable to the purification of proteins required for N-terminal amino acid sequencing and to raise antibodies.

INTRODUCTION

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful technique for the separation of complex protein mixtures not only on an analytical scale but also on a preparative scale. Several methods for the extraction of proteins from gels have been reported: diffusion of protein from crushed gel slices [1], electroelution [2], electrophoretic transfer onto surface-modified glass fibre (SGF) [3–5] and extraction with solvents [6,7].

During our studies on a Cl^--translocating ATPase isolated from *Acetabularia acetabulum* [8,9], protein chemical characterization was carried out to obtain information on the primary structure. The N-terminal sequences of the subunits, a (54 kilodalton) and b (50 kilodalton), were obtained by the direct sequencing of the electroblotted proteins onto SGF sheets [3]. Both subunits separated by SDS-PAGE were subjected to chemical cleavage using CNBr, NCS and NH_2OH on gel slices, and fragments were also separated by SDS-PAGE and subjected to electroblotting and sequencing from the N-terminus. Several sequences were successfully obtained, but CNBr-cleaved fragments gave mixed sequences in most instances [10].

Preparative separation of the respective subunits was thus required. For this purpose, we applied chromatofocusing on Mono P and reversed-phase high-performance liquid chromatography (RP-HPLC) on C_4, C_8 and C_{18} columns, which were,

0021-9673/91/$03.50

however, unsuccessful. SDS-PAGE was the only means of separating the respective subunits. Sakakibara *et al.* [11] reported the electrophoretic separation of proteins on SDS-agarose gel and also described the extraction of proteins from agarose gels. We have applied this method to our proteins, but the recoveries were very low (< 5%). As reported by Tsugita *et al.* [7], extraction of proteins with 70% formic acid was also tried using bovine serum albumin (*ca.* 100 μg) and the recovery was less than 5%. Several types of apparatus for electroelution are commercially available, but a larger gel piece, *i.e.* a relatively large amount of protein, cannot be processed at one time. The aim of this study was to separate the a and b subunits on a preparative scale (> 100 μg), to digest the respective subunits by proteases, to purify the fragments by RP-HPLC and to subject them to automatic N-terminal sequencing. In this paper, a simple and preparative method for the electroelution of proteins from gel strips is described, which is applicable to the purification of proteins required for amino acid sequence analysis and to raise antibodies.

EXPERIMENTAL

Chemicals and instruments

Bovine serum albumin (BSA), ovalbumin (OVA), cytochrome *c*, *Staphylococcus aureus* V_8 protease (type XVIII) and trypsin (type III) were purchased from Sigma (St. Louis, MO, USA). Aldolase from rabbit muscle was obtained from US Biochemical (Cleveland, OH, USA) and Extracti-Gel D from Pierce (Rockford, IL, USA). Dialysis tubes were obtained from Sanko Junyaku (Tokyo, Japan) and closers of dialysis tubes from Spectrum (Los Angeles, CA, USA). Centricon YM-10 and YM-30 and YM-30 membranes from Amicon (Grace, Danvers, MA, USA) were used for the concentration of proteins, and a Konica Immunostain HRP kit (Konica, Tokyo, Japan) for detection of reactions with antibodies. Acetonitrile of HPLC grade was purchased from Kanto Chemical (Tokyo, Japan). Other reagents of analytical-reagent grade were purchased from Wako (Osaka, Japan).

A fast protein liquid chromatographic (FPLC) system and a Mono Q HR5 column from Pharmacia (Uppsala, Sweden) were used for the purification of proteins, and a Waters Assoc. Model 600E HPLC system for the separation of protease-digested fragments. A Wakosil 5C18 reversed-phase HPLC column (150 mm × 4 mm I.D.) was obtained from Wako.

Purification of proteins

Ca^{2+}-ATPase was isolated from rat skeletal muscle according to the method described by MacLennan [12]. However, Ca^{2+} – ATPase was not completely purified and the final preparation showed a major band around 100 kilodalton and several minor bands with molecular masses < 100 kilodalton on SDS-gel. The 100-kilodalton polypeptide was therefore purified by electroelution as described here.

CF_1-ATPase was purified from spinach leaves as described by Binder *et al.* [13].

Ribulose-1,5-bisphosphate carboxylase (RubisCo) was isolated from crude chloroplast-rich fraction of *A. acetabulum* prepared as follows. Axenic cells of *A. Acetabulum* (3–5 cm long, 58 g wet weight) were cut into small pieces with scissors, suspended in a homogenization buffer consisting of 50 m*M* piperazine-N,N′-bis-2-ethanesulphonic acid (PIPES)-Tris buffer (pH 7.6), 0.55 *M* sorbitol, 0.5 *M* sorbitol, 0.5 m*M* $MgSO_4$ and 2 m*M* dithiothreitol (DTT) (100 ml) and stirred for 2–3 min. The cell extracts were passed through four layers of gauze. Cell debris was treated twice in the same manner. All the cell extracts (ca. 300 ml) were centrifuged at 5000 *g* for 2 min. The precipitates were resuspended in the homogenization buffer (100 ml) and centrifuged as described above. The washed pellets were suspended in 10 m*M* NaCl (30 ml) and centrifuged at 12 000 *g* for 10 min. The pellets were washed twice with 10 m*M* NaCl. All the supernatants (*ca.* 30 ml) were combined and concentrated by ultrafiltration in an Amicon cell with a YM-30 membrane filter. The concentrated fraction was applied to a Mono Q HR 5 column, which had previously been equilibrated with a buffer containing 25 m*M* PIPES–Tris buffer (pH 7.0), 0.25 *M* sorbitol, 6 m*M* $MgSO_4$ 1 m*M* ethylene glycol l-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, 2 m*M* DTT and 0.125 m*M* phenylmethanesulphonyl fluoride (PMSF). Proteins were eluted by a linear increase in Na_2SO_4 from 0 to 0.2 *M* [8]. RubisCo was eluted at about 150 m*M* Na_2SO_4, concentrated and desalted by ultrafiltration and stored at −70°C.

SDS-PAGE

In most experiments 10% acrylamide gels were prepared according to the method of Laemmli [14] either on mini gels or preparative gels. For separation of cytochrome *c* 12.5% gels were used.

Electrophoretic elution of proteins from gel strips

After SDS-PAGE, proteins were revealed as transparent bands with 4 *M* sodium acetate solution [15] and excised using a razor blade. Proteins in gel strips were fixed in 50% (v/v) methanol solution for 15 min. They were then equilibrated twice in 0.125 *M* Tris–HCl buffer (pH 6.8) and 2% 2-mercaptoethanol for 15 min each. Equilibration of gel strips in the above buffer with 1% (w/v) SDS were performed as described above. The equilibrated gel strips were inserted in a dialysis tube with a minimum amount of the buffer with SDS (25 m*M* Tris, 190 m*M* glycine and 0.1% SDS, see below). The dialysis tubes were treated and electroelution was carried out in similar manners to those described by Findlay [16]. In this work, a horizontal flat-bed mini-gel electrophoresis apparatus (Renner, Darmstadt, Germany) was used for electroelution at 200 V for 3 h at 4°C. The buffer consisted of 25 m*M* Tris, 190 m*M* glycine and 0.1% SDS (pH 8.3). At the end of electrophoresis, the polarity of electrodes was changed for 30 s in order to avoid the adsorption of proteins on the dialysis tubes. The buffer inside the dialysis tubes was collected and the tubes were washed three times with a minimum volume of the buffer and concentrated by using Centricon YM-30 or YM-10.

Removal of SDS from the sample solution

The concentrated sample solution (100–200 μl, 1–2 mg protein/ml) was applied to an Extracti-gel D detergent-removing gel, which had previously been equilibrated with 50 m*M* Tris–HCl buffer (pH 8.0). The effluent was collected and concentrated by ultrafiltration as described above.

Digestion with proteases

The sample solution was added to 0.1% (w/v) SDS and proteases in a weight ratio of 50:1 (protein to protease) and digested at 37°C overnight. After digestion, the sample solution was stored at −20°C.

Purification of the digested fragments by RP-HPLC

The digested solution was added to formic acid at a final concentration of *ca.* 10% (v/v) and centrifuged at 15 000 *g* for 5 min, then the supernatant was applied to an RP-HPLC column. The solvents used for HPLC were 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A), and 0.1% TFA and 60% (v/v) acetonitrile (solvent B), with a linear gradient from 0 to 100% solvent B in 60 min. The flow-rate was 1 ml/min. Chromatography was performed at 40°C. The peaks were collected and rechromatographed by RP-HPLC with a minute change in the linear gradient of acetonitrile. The collected sample solutions were evaporated under reduced pressure by using a Speed-Vac concentrator (Savant). The residues were dissolved in doubly distilled, deionized water and subjected to N-terminal sequencing.

N-Terminal sequencing based on Edman degradation

Amino acid sequence analysis based on Edman degradation was performed on a Model 470A gas-phase protein/peptide sequencer and a Model 120A-01 on-line PTH analyser (Applied Biosystems, Foster City, CA, USA).

Protein determination

Proteins were determined by the method described by Heil and Zillig [17]. BSA was used as a standard protein. When the recoveries of standard proteins (BSA, OVA, aldolase and cytochrome *c*) were determined, the respective protein solution was used as standard.

Antibodies

The CF_1 complex (α, β, γ, δ and ε subunits) was prepared as described above, and the respective subunits were separated by electroelution as described here. Ca^{2+}-ATPase from rat skeletal muscle was also purified by electroelution.

The respective protein solution (200–500 μg of protein in 0.5 ml) was added to 0.5 ml of Freund's Complete Adjuvant (Difco) and an emulsion was prepared. Male albino rabbits (*ca.* 2.5 kg body weight) were immunized intramuscularly and subcutaneously. After 2 weeks, rabbits were again immuned with 125–250 μg protein in the same manner. After 1 week, whole blood was collected and the serum was separated and stored frozen at

−20°C. The reactions of the proteins with the antibodies were tested by Western blotting as described by Ikeda *et al.* [8], except that a Konica Immunostain HRP kit was used for detection of the reactions.

RESULTS

Recoveries of standard proteins from gel slices by electroelution

Recoveries of standard proteins from gel slices by the present method are summarized in Table I, and ranged from 70 to 75.2% for 14–66-kilodalton proteins. The 100-kilodalton protein Ca^2-ATPase was eluted at 200 V for 6 h, after SDS-PAGE on 10% acrylamide gel, and the recovery was above 60%. Recoveries of proteins from the Extracti-gel D column were about 90%, when removal of SDS is required.

Application to raise antibodies

The results are shown in Fig. 1. The respective antiserum against the α, β, γ, δ and ε subunits of the the CF_1 complex from spinach and the antiserum against Ca^{2+}-ATPase showed high specificity as in Fig. 1. The antisera against the respective CF_1 component did not show any cross-reactivity with Ca^{2+}-ATPase and *vice versa* (data not shown).

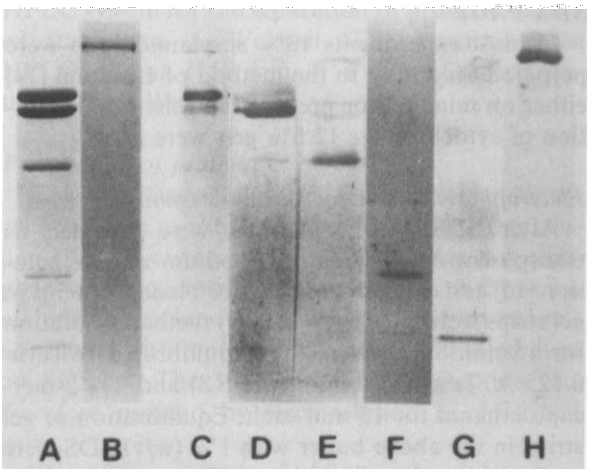

Fig. 1. Immunoblot of spinach CF_1 components (α, β, γ, δ and ε subunits) and rat skeletal muscle Ca^{2+}-ATPase. Purified CF_1 complex (*ca.* 4 μg) and Ca^{2+}-ATPase (*ca.* 2 μg) were subjected to SDS-PAGE and to immunoblotting. The reactions with the respective antiserum (diluted 1:250 to 1:500) were performed at room temperature for 1 h, and were revealed by using a Konica Immunostain HRP kit. Lanes: A and B = Coomassie Blue stain of the isolated CF_1 complex and Ca^{2+}-ATPase, respectively; C = reaction of the CF_1 complex with the antiserum against the α subunit (diluted 1:500); D = reaction of the CF_1 complex with the anti-β serum (1:500); E = reaction of the CF_1 complex with the anti-γ serum (1:500); F = reaction of the CF_1 complex with the anti-δ serum (1:250); G = reaction of the CF_1 complex with the anti-ε serum (1:500); H = reaction of Ca^{2+}-ATPase with the antiserum against Ca^{2+}-ATPase (1:500).

Application to N-terminal sequencing

RubisCo was isolated from *A. Acetabulum* and the large subunit (53 kilodalton) was separated by electroelution as described. The recovery was 77% when RubisCo (272 μg of protein) was separated by preparative SDS-PAGE (3 cm wide × 11 cm long × 1.5 mm thick) (10%) and electroeluted.

The elution patterns of RubisCo large subunit from *A. Acetabulum* are shown in Fig. 2a and b

TABLE I

RECOVERIES OF STANDARD PROTEINS FROM GEL STRIPS

BSA, OVA and aldolase were subjected to SDS-PAGE using 10% gel and cytochrome *c* using 12.5% gel.

Standard protein	Amount of protein applied (μg)	Amount of protein recovered (μg)	Recovery (%)
BSA (66 kilodalton)	110	81.1 ± 4.4 (n = 4)	73.7
OVA (45 kilodalton)	110	82.5 ± 2.8 (n = 4)	75.0
Aldolase (40 kilodalton)	75	52.7 ± 7.1 (n = 3)	70.3
Cytochrome *c* (14.3 kilodalton)	150	113 ± 6.3 (n = 3)	75.2

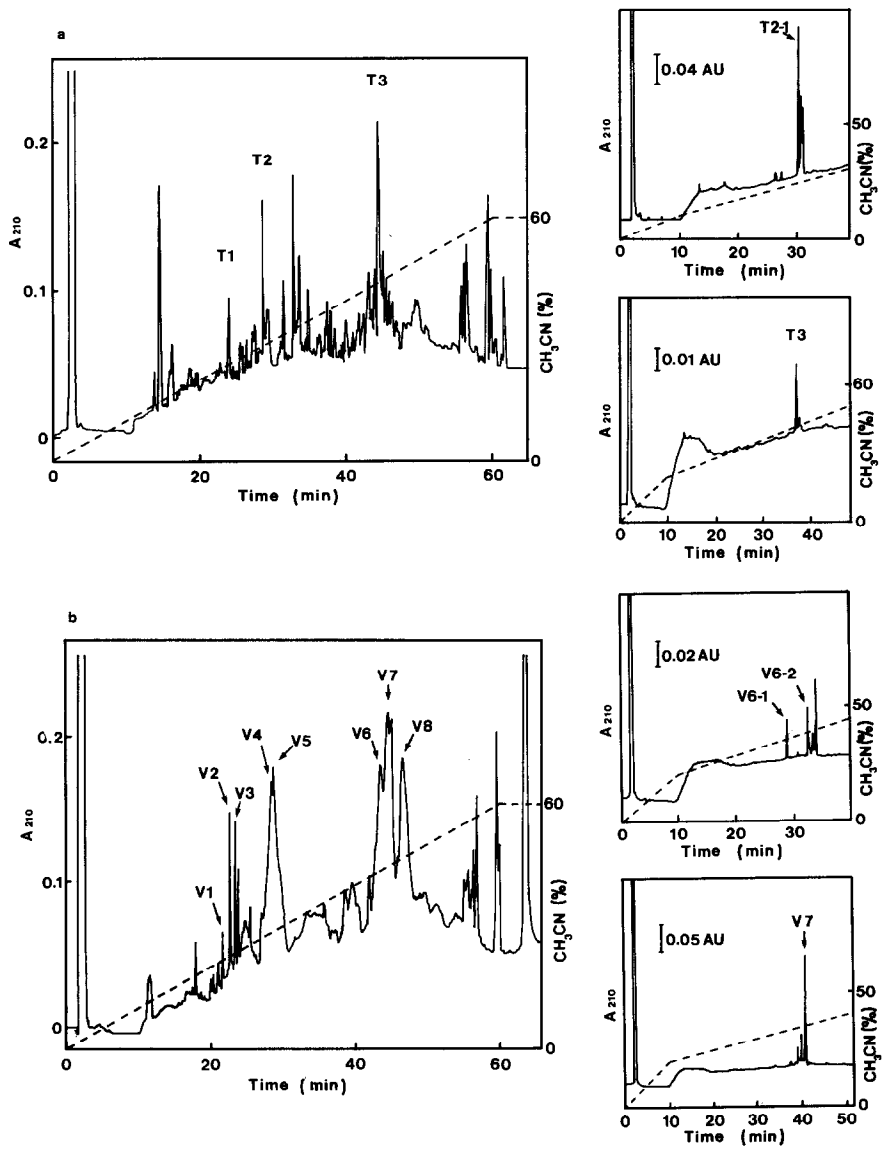

Fig. 2. High-performance liquid chromatograms of RubisCo large subunit after (a) Trypsin and (b) V_8 protease digestions. (a) Rubisco large subunit (*ca.* 180 μg) was digested with trypsin at 37°C overnight and chromatographed as described under Experimental. In the two chromatograms on the right, examples of rechromatography of peaks T_2 and T_3 are shown. (b) Rubisco large subunit (*ca.* 300 μg) was digested with V_8 protease at 37°C overnight. In the two chromatograms on the right examples of rechromatography of peaks V_6 and V_7 are shown.

after trypsin and V_8 protease digestion, respectively. The amino acid sequences obtained from the purified fragments are summarized in Table II. The data support the close similarity (56–100%) of RubisCo from *A. acetabulum* to those of alfalfa RubisCo large subunit.

DISCUSSION

The electroelution method presented here is a combination of SDS-PAGE, detection with sodium acetate (but not with Coomassie Blue dyes), which facilitate evaluation of the protein concentrations

TABLE II

AMINO ACID SEQUENCES OBTAINED FROM *A. ACETABULUM* RUBISCO LARGE SUBUNIT

Single-letter coding for amino acids used.

Digestion		Amino acid sequence[a]	Alignment[b]
Trypsin	T1	*AGAGF*	ATVGF
	T2-1	*LNATAPT*(S)*ETMLQR*	LNATAGTCEEMMKR
	T3	*LTYYTPDYQ*VLG*TXVL*	LTYYTPDYETKDTDIL
V_8 protease	V1-1	*AXVQARNE*	ACVQARNE
	V1-2	*AIYK*	AIYK
	V2	YA*AAVAAE*	AGAAVAAE
	V3-1	*LAAAXE*	LAAACE
	V4-2	*G*/N*GDIIR*	GGDHIH
	V6-2	*IF*(T/*G*)*DDAXLQFGGGTLG*	IFGDDSVLQFGGGTLG
	V7	*SSTGT* (*W*)*TTV*(*W*)*T*(*D*)*GXT*	SSTGTWTTVWTDGLT
	V8-1	*TKAGAGFXAGVXXYRL*	TKATVGFKAGVKDYRL
	V8-2	(*D*/N)*LRIPQ*(S)*FVTTFLGVV*	DLRIPAAYVKTFQGPP

[a] Identical amino acids are italicized.
[b] Alfalfa chloroplast RubisCo large subunit.

and electropheretic elution of proteins from gel strips with high recoveries, which is similar to electroelution of DNA from agarose gel strips. The most important point was the fixation of proteins in gel strips by 50% methanol, as without fixation diffusion of proteins occured, which led to lower recoveries of proteins.

The technique presented here has following advantages: it does not require special expensive apparatus and reagents, preparative-scale amounts of proteins can be processed, it is not time consuming and takes about 8 h to obtain pure proteins or subunits, proteins are eluted under mild conditions and therefore little degradation of proteins occurs, it is applicable not only to soluble proteins but also to membrane-bound proteins and it is applicable to raise antibodies and to the N-terminal sequencing.

Of course, as mentioned in the Introduction, numerous methods for the isolation of proteins or subunits have been reported. We have also tried several of those methods for our purposes, but were unsuccessful. For example, an electroendosmotic preparative electrophoresis unit (Genefit) purchased from Funakoshi Pharmaceutical (Tokyo, Japan) did not allow the separation of OVA (45 kilodalton) and BSA (66 kilodalton), the procedure is troublesome and the apparatus is expensive. The extraction of proteins from gel slices with 70% formic acid was not suitable on a preparative scale (>100 μg of protein). Feick and Shiozawa [6] reported extraction of proteins with formic acid–acetonitrile–isopropanol–water, and this method gave high recoveries for several hydrophobic proteins. This method, however, has the disadvantage that acid-labile proteins cannot be processed by the method. SDS-agarose gel electrophoresis as described by Sakakibara *et al.* [11] gave poor resolution of proteins such as α (56 kilodalton and β (50 kilodalton) subunits of CF_1-ATPase from spinach. In addition, 3–4% agarose gels are difficult to prepare and the use of, for example, NuSieve or low-melting agarose is too expensive for the separation of proteins on a preparative scales. A similar method to that reported in this paper has already been presented by Findlay [16]. However, no details of recoveries and no application data were reported.

With the present method, one could readily obtain milligram amounts of proteins or polypeptides, which is applicable to raise antibodies and N-terminal sequencing after protease digestion.

REFERENCES

1 D. A. Hager and R. R. Burgess, *Anal. Biochem.*, 109 (1980) 76.
2 R. M. Hewick and M. W. Hundkapiller, in A. Darbre and M.

D. Waterfield (Editors), *Practical Protein Chemistry —A Handbook*, Wiley, Chichester, 1986, pp. 473–490.
3 R. H. Aebersold, D. B. Teplow, L. E. Hood and S. B. H. Kent, *J. Biol. Chem.*, 261 (1986) 4229.
4 C. Vandekerckhove, G. Bauw, M. Puype, J. Van Damme and M. Van Montagu, *Eur. J. Biochem.*, 125 (1985) 9.
5 C. Eckerskorn, W. Mewes, H. Goretzki and F. Lottspeich, *Eur. J. Biochem.*, 176 (1988) 509.
6 R. G. Feick, and J. A. Shiozawa, *Anal. Biochem.*, 187 (1990) 205.
7 A. Tsugita, T. Ataka and T. Uchida, *J. Protein Chem.*, 6 (1987) 121.
8 M. Ikeda, R. Schmid and D. Oesterhelt, *Biochemistry*, 29 (1990) 2057.
9 M. Ikeda and D. Oesterhelt, *Biochemistry*, 29 (1990) 2065.
10 T. Ohhashi, C. Moritani, F. Lottspeich, D. Oesterhelt and M. Ikeda, unpublished results.
11 R. Sakakibara, N. Tominaga, A. Sakai and M. Ishiguro, *Anal. Biochem.*, 162 (1987) 150.
12 D. H. MacLennan, *Methods Enzymol.*, 32 (1974) 291.
13 A. Binder, A. Jagendorf and E. Ngo, *J.Biol. Chem.*, 253 (1978) 3094.
14 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
15 R. C. Higgins and M. E. Dahmus, *Anal. Biochem.*, 93 (1979) 257.
16 J. B. C. Findlay, in B. D. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins —A Practical Approach*, Academic Press, New York, 2nd ed., 1990, pp. 83–89.
17 A. Heil and W. Zillig, *FEBS Lett.*, 11 (1970) 165.