Journal of Chromatography, 240 (1982) 155–163
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,748

RAPID METHOD FOR PURIFICATION OF PLASMID DNA AND DNA FRAGMENTS FROM DNA LINKERS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON TSK-PW GEL

MICHAEL E. HIMMEL*
Biotechnology Branch, Solar Energy Research Institute, Golden, CO 80401 (U.S.A.)
PETER J. PERNA
Department of Biochemistry, Colorado State University, Fort Collins, CO 80423 (U.S.A.)
and
MICHAEL W. McDONELL
Molecular Genetics Group, Synergen, Boulder, CO 80301 (U.S.A.)
(First received November 19th, 1981; revised manuscript received January 19th, 1982)

SUMMARY

High-performance size exclusion chromatography (HPSEC) using TSK-G5000 PW in Tris buffer has been found to be a reliable method for the rapid fractionation of DNA ligation products. Plasmid and fragmented phage DNAs were found to elute in less than 2 min with recoveries greater than 98%. *Escherichia coli* transfection studies, using plasmid DNA that had been subjected to HPSEC column fractionation, showed high transformation efficiencies. $MgCl_2$, a component of the DNA ligation reaction, was found to produce DNA–column support interactions, which resulted in low DNA recoveries. Such interactions were eliminated by chelation with ethylenediaminetetraacetate prior to chromatography.

INTRODUCTION

The evolution of recombinant DNA technology has generated a requirement for a separation technique that will resolve from a DNA ligation reaction, insert DNAs and DNA linkers[1]. It is essential to remove the linker molecules from such a reaction in order to limit the probability of subsequently cloning DNA linkers without insert DNA. There are two approaches to removing linkers from the insert DNA. The first method is to treat the ligation mixture with a restriction endonuclease complementary to the DNA linker site in order to generate a cohesive end that can be joined to vector DNA and to eliminate linker oligomers. This step requires an extremely large and concentrated amount of the appropriate restriction endonuclease, since linkers not incorporated into insert DNA also compete for the endonuclease. After restriction endonuclease treatment the mixture is fractionated by methods with inherently low sample recoveries such as liquid–liquid partition[2,3], partition chroma-

0021-9673/82/0000–0000/$02.75

tography[4,5], gel electrophoresis[6], sedimentation velocity centrifugation[7], or molecular sizing chromatography[8–11]. The second approach is to fractionate first the ligation mixture by one of the methods mentioned above and then to generate cohesive ends by treating the fractionated DNA with the appropriate restriction endonuclease. The fractionation procedures necessary for the two general approaches given above are limited in that they do not meet all of the following requirements: (1) small sample dilution; (2) rapid separation of the ligation products; (3) efficient separation of the DNA–linker complex (1.0 to $5.0 \cdot 10^6$ daltons) from the greatly smaller DNA ligase enzyme, DNA linkers (*ca.* 5000 daltons) and adenosine triphosphate (ATP); and, (4) high recovery of the DNA in a buffer system appropriate for ethanol precipitation of the DNA–linker complex (especially critical when rare eukaryotic genes are being cloned). Recent work with high-performance size exclusion chromatography (HPSEC) has shown that TSK-SW[12] and TSK-PW[13] gels offer significant improvement in speed, resolution and buffer versatility over classical molecular sizing chromatography. However, the effects of high linear flow-rates, as generated in high-performance liquid chromatography (HPLC), on the biological activity of DNAs is largely unknown. These conditions led us to investigate the use of TSK-G5000 PW gel packing for the separation of DNA ligation products.

MATERIALS AND METHODS

Apparatus

For the collection of careful analytical data in this study, a sophisticated solvent delivery system, consisting of a Hewlett-Packard Model 1084B liquid chromatograph interfaced with a Waters Associates Model 450 variable-wavelength monitor, was used. For routine laboratory use, however, a less elaborate system was used. This consisted of an LKB Model 2138 Uvicord UV chromatography column monitor equipped with the HPLC flow cell option and a Model 39-500 low-pressure HPLC pump from Rainin Instruments. The samples were loaded on-column directly with a Rainin model 5020 PTFE rotary injection valve fitted with 0.030 in. I.D. PTFE tubing. A stainless-steel column (200 × 3.2 mm I.D.) was packed by the high-pressure gel filtration slurry method described by Kirkland[14] with 18-μm TSK G5000 PW gel packing (Toyo Soda, Tokyo, Japan). Samples were loaded by suction filling the injection valve loop. After initial calibration of flow-rates with the UV monitor, the system may be monitored using a stopwatch for DNA collection.

Enzymes

The restriction endonuclease *Eco*RI was the gift of Marj Thomas. All other restriction endonucleases were purchased from commercial sources. Bacteriophage T4 DNA ligase was generously provided by S. Scherer and J. Widom. Bacterial alkaline phosphatase (*E. coli*) was from Bethesda Research Labs and bacteriophage T4 polynucleotide kinase was from New England Biolab.

Enzymatic reactions

Restriction endonucleases were used as recommended by the manufacturer. Plasmid and phage DNAs were cleaved at concentrations of 100–200 μg/ml. DNA

ligations were performed in 50 m*M* Tris–HCl (pH 7.4), 10 m*M* $MgCl_2$, 10 m*M* dithiotreitol (DTT), 1 m*M* adenosine triphosphate (ATP) and 100 n*M* T4 DNA ligase. The *Eco*RI cut pBR325 DNA [5.45 kilobase pairs (kb)[15]] was dephosphorylated enzymatically with bacterial alkaline phosphatase and the 5′ end was labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase[16]. *Eco*RI synthetic linker DNA (Collaborative Research) was similarly labeled.

Preparation of bacteriophage T7 DNA

Wild-type T7[17] was grown on *E. coli* B at 37°C in a shaking incubator. Phage particles were purified from the lysate by polyethylene glycol precipitation and CsCl banding[18]. The purified phage were dialyzed against 10 m*M* Tris–HCl, 1 m*M* ethylenediaminetetraacetate (EDTA) (pH 7.4) and extracted with phenol. Residual phenol was removed by ether extraction.

Isolation of plasmid pBR325 DNA

Plasmid pBR325 DNA[15] was prepared by a modification of the procedure of Wensink *et al.*[19]. An overnight culture of *E. coli* containing plasmid pBR325 was used to innoculate 1 l of L-broth. The culture was grown to saturation at 37°C with vigorous aeration. The cells were harvested by centrifugation at 3330 *g* for 10 min in a GSA rotor (Sorvall) at 5°C. The cell pellet was resuspended in 250 ml TE buffer (10 m*M* Tris–HCl, pH 7.4; 1 m*M* EDTA) and collected by centrifugation. The cell pellet was resuspended in 30 ml of 15 (w/v) sucrose in 50 m*M* Tris–HCl (pH 7.4), 50 m*M* Na_2EDTA and 1 mg/ml freshly prepared lysozyme. This mixture was incubated at room temperature for 30 min. Next, 32 ml of Triton solution [0.1 % Triton X-100; 50 m*M* Tris–HCl (pH 8); 50 m*M* Na_2EDTA] was added and lysis was allowed to proceed for 10 min. The mixture was poured into polypropylene tubes and centrifuged at 36,900 *g* for 1 h in an SS34 (Sorvall) rotor at 5°C. The supernatant was poured into a graduated cylinder, the volume was adjusted to 60 ml, and 6 ml of 10 mg/ml ethidium bromide and 57 g of CsCl were added (density = 1.59 g/ml). This solution was centrifuged for 72 h at 112,000 *g* and 20°C in the 50.2 Ti rotor (Beckman). After equilibrium banding the DNA was visualized by fluorescence under long-wavelength UV light, and the lower band (containing the covalently closed plasmid DNA) was removed by side puncture of the polyallomer tube. The DNA-containing fractions were pooled; the density was adjusted to 1.59 g/ml with the CsCl–ethidium bromide solution in a final volume of 39.5 ml, and then re-banded in the 50.2 Ti rotor at 112,000 *g* for 72 h at 20°C. The re-banded DNA was collected and the ethidium bromide was removed by repeated isopropanol extraction. The CsCl was removed by diluting the sample with four volumes of TE buffer followed by ethanol precipitation. The DNA pellet was washed with ethanol, dried in a vacuum, and resuspended in TE buffer.

Plasmid transfection

Plasmid DNAs were used to transform *E. coli* LE392 essentially as described by Mandel and Higa[20]. The efficiency of transformation is about $4 \cdot 10^5$ transformants/μg of Form I DNA. Saturation occurs between 50 and 100 ng of Form I DNA per 10 cm plate ($1 \cdot 10^9$ cells).

Agarose gel electrophoresis

Column fractions of T7 DNA cleaved by restriction endonuclease were electrophoresed from a 6 mm × 15 mm sample well in a 6-mm-thick 1.8% horizontal agarose gel at 4 V/cm in 40 m*M* Tris–acetate (pH 8.1), 2 m*M* EDTA buffer as described by McDonell *et al.*[21]. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed using long-wavelength UV light and Kodak Plus-X film.

Chromatography and scintillation counting of labeled DNA and linkers

The pBR325 DNA (5.45 kb) was purified from *E. coli* and cut with *Eco*RI restriction endonuclease. The DNA and *Eco*RI linkers were labeled independently as described above. The unincorporated [γ-^{32}P]ATP was removed from the labelled DNA and labelled linkers by Sephadex gel filtration prior to HPLC. A mixture of labeled plasmid DNA (0.5 μg) and labeled linkers (0.15 μg) was loaded in a volume of 50 μl. Fractions were collected as drops and were spotted on Whatman DE-52 discs (about 30 μl each). Discs were then washed five times with phosphate buffers, followed by one wash of distilled water, ethanol and diethyl ether. The dried discs were counted in a 2,5-diphenyloxazole–toluene liquid scintillation fluid.

Elution standards and column calibration

Tobacco mosaic virus was a gift of Dr. A. T. Tu. The bovine serum albumin was obtained from Armour and the dinitrophenyl (DNP)-alanine was purchased from Sigma. Column parameters are defined as described by Pharmacia[22]. Here, V_0 (the void volume) refers to the interstitial volume between the beads; V_i and V_s refer to the volume within the beads that is accessible and inaccessible to solvent, respectively. The total column volume, V_t, is the sum of V_0 and V_i. V_e is the elution volume of the sample under study.

Chromatography parameters

Chromatography was performed at 25°C with a mobile phase flow-rate of 0.5 ml/min, a chart speed of 1.0 cm/min and detection at 260 nm. Sample injection volume was 50 μl for both protein and DNA samples. Under these conditions, the chromatography column pressure did not exceed 18 kg/cm^2 (17 bar).

RESULTS

The elution profiles of several standard molecular weight markers are shown in Fig. 1. Tobacco mosaic virus ($40 \cdot 10^6$ daltons)[23], bovine serum albumin ($6.63 \cdot 10^4$ daltons)[24] and DNP-alanine (256 daltons) were found to elute at 1.0 ml, 1.52 ml and 1.75 ml, respectively. These standards were run in 100 m*M* KCl, 10 m*M* $NaPO_4$ (pH 7.0) buffer at a flow-rate of 0.5 ml/min. Although extensive calibration of this column was not made (a procedure we have undertaken on commercially packed TSK columns[12,13]), the approximate values $V_0 = 1.0$ ml and $V_t = 1.75$ ml were obtained. The total geometrical column volume, V_g, from column dimensions, was 2.01 ml.

When the first attempts were made at chromatographing the DNA ligation mixture, which contains 10 m*M* $MgCl_2$, 10 m*M* DTT and 1 m*M* ATP, the DNA was greatly retarded on the column and was found to elute at the void volume only after adding EDTA to the mobile phase buffer. It was found that complete recoveries could

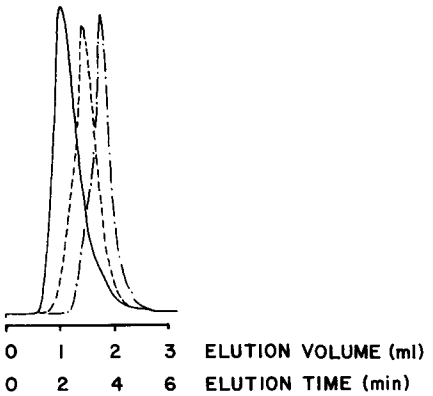

Fig. 1. Chromatography of calibration standards on TSK-G5000 PW. Tobacco mosaic virus (———), bovine serum albumin (------) and DNP-alanine (– · – ·) were used to mark the void volume, intermediate included volume and total column volume, respectively.

be obtained when the ligation buffer was "quenched" with EDTA (final EDTA concentration in the sample $\geqslant$ 15 m*M* before chromatography). When DNA samples had no $MgCl_2$ added, the subsequent chromatography showed no signs of retention and the DNA eluted normally at V_0. These results are summarized in Table I.

A mixture of *Hae*III endonuclease treated T7 DNA[25], and *Eco*RI DNA linkers was chromatographed and collected as drop fractions from G5000 PW. Agarose gel electrophoretic analysis (Fig. 2) showed that DNA fragments from 2.7 to approximately 0.10 kb eluted in the void volume (after 2 min of chromatography). DNA

TABLE I

BUFFER EFFECTS ON DNA ELUTION FROM G5000 PW

Buffer A = 10 m*M* phosphate (pH 7.0) and buffer B = 50 m*M* Tris (pH 7.4).

DNA elution behavior	*Column buffer*	*Injection buffer*
40% recovery —elutes past V_t*	Buffer A + 100 m*M* KCl	Buffer B + 100 m*M* $MgCl_2$ + 10 m*M* DTT**
40% recovery —elutes past V_t	Buffer A	Buffer B + 100 m*M* $MgCl_2$ + 10 m*M* DTT**
20% recovery —elutes past V_t	Buffer B + 10 m*M* $MgCl_2$	Buffer B + 100 m*M* $MgCl_2$ + 10 m*M* DTT**
40% recovery —elutes past V_t	Buffer B	Buffer B + 10 m*M* $MgCl_2$ + 10 m*M* DTT**
98% recovery —elutes at V_0	Buffer B + 15 m*M* EDTA	Buffer B + 10 m*M* $MgCl_2$ + 10 m*M* DTT**
98% recovery —elutes at V_0	Buffer B	Buffer B
98% recovery —elutes at V_0***	Buffer B	Buffer B + 10 m*M* $MgCl_2$ + 10 m*M* DTT + 15 m*M* EDTA

* Recovery estimated by area under elution profile at 260 nm.

** Buffer system used in ligation reaction.

*** Elution at V_0 indicates absence of DNA–column support interactions.

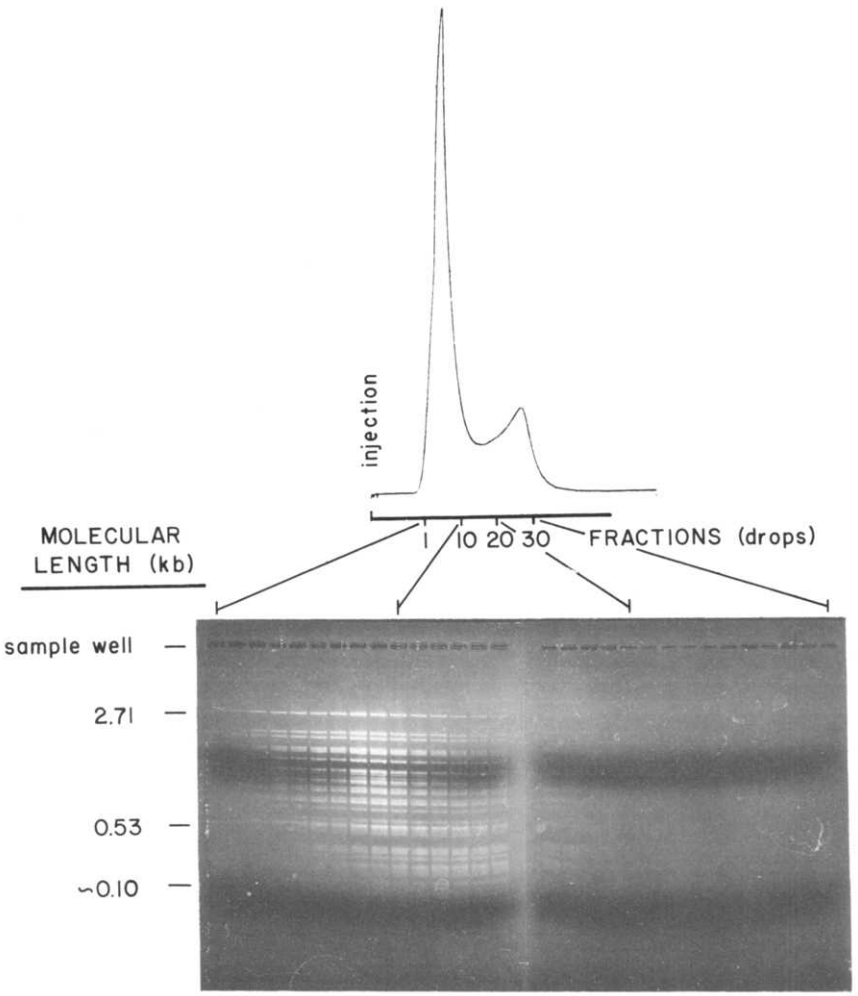

Fig. 2. Composite elution diagram of *Hae*III-treated T7 DNA and *Eco*RI linkers showing correlation between specific elution from G5000 PW and molecular length of DNA fragments. Note that DNA linkers are not visualized by the ethidium bromide stain.

linkers failed to stain with ethidium bromide because they were in single stranded form during electrophoresis and therefore did not bind the stain.

The DNA ligation system was also chromatographed with *Eco*RI cleaved ^{32}P end-labeled pBR325 DNA and ^{32}P end-labeled *Eco*RI DNA linkers. These elution profiles, plotted both as a function of counts per minute and $A_{260\ nm}$ per fraction, are shown in Fig. 3. Here 82,700 total cpm as plasmid DNA and 400,900 total cpm as *Eco*RI linkers were loaded on the column in one injection. Examination of the ^{32}P cpm from peak A on Fig. 3 yielded 81,000 total cpm for an apparent DNA recovery of 98%. The recovery from peak B on Fig. 3 was somewhat lower with 354,000 cpm giving a recovery value of 88% for the DNA linkers.

Studies with *Eco*RI-cleaved pBR325 DNA recovered from the void volume

peak showed that this DNA could be religated and was capable of transforming *E. coli* with an efficiency equivalent to that found for unaltered plasmid DNA.

DISCUSSION

HPSEC of the DNA ligation reaction mixture (containing pBR325 DNA, *Eco*RI DNA linkers, T4 DNA ligase and ATP) on TSK G5000 PW, was found to provide very low column residence times and excellent separation of DNA from oligonucleotide linkers and ATP.

Chromatography of the DNA ligation mixture in this study is effective because the plasmid DNA ($3.6 \cdot 10^6$ daltons) and DNA fragments are large when compared to the DNA linkers (5,300 daltons) and the T4 DNA ligase (65,000 daltons)[26]. Although the small-bore column (3.2 mm I.D.) used in this study provides the gross size fractionation required, it is evident that we are using this column in a "desalting" mode. TSK G5000 PW gel beads have an average pore diameter of about 260 Å[12]; therefore, the DNA is certainly excluded from the gel pores. Partial gel permeation of smaller DNAs may be possible using the noncommercially available gel beads (pore diameter 3500 Å) described by Kirkland[27]. However, we suspect that subjecting medium-sized DNAs to true gel permeation chromatography will generate extensive DNA shearing.

Small-bore columns of the type used in this analysis show peak shape irregularities (as seen with the chromatography standards in Fig. 1) because the injection "bolus" does not approach infinite diameter during chromatography and wall effects occur[28]. However, the inherent high speed and low sample dilution attained in this study are more important to the separation problem discussed here, than peak shape aberrations. The sample dilution is small (relative to column volume) as a 50-μl

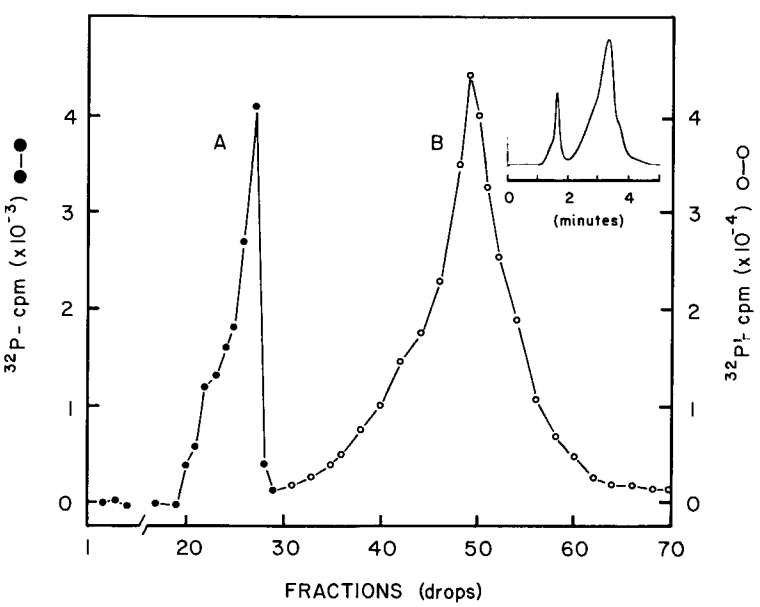

Fig. 3. Elution diagram of ^{32}P-labeled *Eco*RI-cut pBR325 DNA and labeled *Eco*RI DNA linkers on G5000 PW. Insert shows elution time as a function of $A_{260\ nm}$.

sample of DNA may be collected after chromatography in 250 μl; a volume from which it may be easily precipitated with ethanol.

The small-bore column used in this study generates a linear flow-rate of *ca.* 370 $ml \cdot cm^{-2} \cdot h^{-1}$ at 0.5 ml/min. This may be compared to other supports where the maximum linear flow-rates are: Sephadex G-75–G-200 (77–12 $ml \cdot cm^{-2} \cdot h^{-1}$), Sephacryl S-200 and S-300 (30 and 25 $ml \cdot cm^{-2} \cdot h^{-1}$)[22]. The effect of such a high flow-rate on DNA shearing as used in this system is negligible because full-length T7 DNA (40 kb) was unaltered by the chromatography (data not shown). Evidence for the survival of unaltered 5.45 kb linear DNA from possible HPLC shearing effects is shown in this study by the successful transfection of *E. coli* with HPLC treated material. Furthermore, chromatography of other extended macromolecules (7.1×10^6 MW polystyrene) in tetrahydrofuran, showed little evidence of shear sensitivity at linear flow-rates of less than 400 $ml \cdot cm^{-2} \cdot h^{-1}$ (ref. 27).

Electrophoresis of *Hae*III endonuclease treated T7 DNA collected from G5000 PW indicated that all DNA fragments greater than about 150 base pairs were excluded from the gel pores and elute at the void volume. Therefore, elution samples as large as ten linker multimers (*ca.* 100 base pairs) generated during ligation, are separated from DNA fragments as small as 150 base pairs. Similar effects were also observed with plasmid DNAs as large as 5.45 kb. However, in preliminary experiments with *Hind*III digested bacteriophage lambda DNA, a 23.8 kb DNA fragment was found to elute as a third peak at a slightly greater elution volume than the void volume (data not shown). The apparent retention of this larger DNA may be due to a type of physical entrapment within the chromatography packing. We are currently investigating this phenomenon.

The apparent interaction of DNA with the TSK gel support in the presence of Mg^{2+} ions is most intriguing. It is unclear at this time whether the nature of the interaction is purely electrostatic or a complex feature of both DNA charge and shape.

ACKNOWLEDGEMENT

The authors wish to thank Karel Grohmann who was the source of many fruitful discussions.

REFERENCES

1 T. Maniatis, R. C. Hardison. E. Lacy, J. Lamer, C. O'Connell, D. Quon, G. K. Sim and A. Efstratiadis, *Cell*, 15 (1978) 687–701.
2 G. Bloomquist and P.-A. Albertsson, *Anal. Biochem.*, 30 (1969) 222.
3 L. Rudin and P.-A. Albertsson, *Biochim. Biophys. Acta*, 134 (1967) 36.
4 D. A. Usher, *Nucl. Acids Res.*, 6 (1979) 2289.
5 C. Kidson, *Biochemistry*, 8 (1979) 4376.
6 H. Smith, *Methods Enzymol.*, 65 (1980) 371–380.
7 K. McCarty, R. Vollmer and K. McCarty, *Anal. Biochem.*, 61 (1974) 165–183.
8 G. R. Shepperd and D. F. Petersen, *J. Chromatogr.*, 9 (1962) 445.
9 J. Attonen and E. Kulonen, *J. Chromatogr.*, 24 (1966) 197.
10 J. E. Loeb and J. Chauveau, *Biochim. Biophys. Acta*, 182 (1969) 225.
11 J. Satava, S. Zadrazil and Z. Sormova, *Collect. Czech. Chem. Commun.*, 38 (1973) 2167.
12 M. Himmel and P. G. Squire, *Int. J. Peptide Prot. Res.*, 17 (1981) 365–373.

13 M. Himmel and P. G. Squire, *J. Chromatogr.*, 210 (1981) 443–452.
14 J. J. Kirkland, *Chromatographia*, 8 (1975) 661.
15 F. Bolivar, *Gene*, 4 (1978) 121–136.
16 G. Chaeonas and J. H. Van de Sande, *Methods Enzymol.*, 65 (1980) 75–85.
17 F. W. Studier, *J. Mol. Biol.*, 79 (1973) 227–236.
18 F. W. Studier, *J. Mol. Biol.*, 79 (1973) 237–248.
19 P. C. Wensink, D. J. Finnegan, J. E. Donelson and D. S. Hogness, *Cell*, 3 (1974) 315–325.
20 M. Mandel and A. Higa, *J. Mol. Biol.*, 53 (1970) 159–162.
21 M. W. McDonell, M. N. Simon and F. W. Studier, *J. Mol. Biol.*, 110 (1977) 119–146.
22 *Sephadex Filtration in Theory and Practice*, Pharmacia Fine Chemicals, Uppsala, 1979, pp. 30–35.
23 C. Caspar, *Advan. Protein Chem.*, 18 (1963) 37.
24 M. O. Dayhoff, L. T. Hunt, P. J. McLaughlin and W. E. Barker, in M. O. Dayhoff (Editor), *Atlas of Protein Sequence and Structure: Data Section*, The National Biomedical Research Foundation, Silver Spring, MD, 1972, pp. D1–D163.
25 A. H. Rosenberg, M. N. Simon, F. W. Studier and R. J. Roberts, *J. Mol. Biol.*, 135 (1979) 907–915.
26 A. Panet, J. H. van de Sande, P. C. Loewen, H. G. Khorana, A. J. Raae, J. R. Lillehaug and K. Kleppe, *Biochemistry*, 13 (1973) 5045–5050.
27 J. J. Kirkland, *J. Chromatogr.*, 125 (1976) 231–250.
28 J. H. Knox, *J. Chromatogr. Sci.*, 15 (1977) 353.