Journal of Chromatography, 308 (1984) 111—119
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2102

THE SEPARATION OF COLLAGEN α-CHAINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

COMPARISON OF COLUMN ALKYL STATIONARY PHASES AND TEMPERATURE EFFECTS

S.J.M. SKINNER*

Postgraduate School of Obstetrics and Gynaecology, National Women's Hospital, Claude Road, Epsom, Auckland 3 (New Zealand)

B. GREGO and M.T.W. HEARN

St. Vincent's School of Medical Research, Fitzroy, Melbourne 3065 (Australia)

and

G.C. LIGGINS

Postgraduate School of Obstetrics and Gynaecology, National Women's Hospital, Claude Road, Epsom, Auckland 3 (New Zealand)

(First received November 29th, 1983; revised manuscript received February 2nd, 1984)

SUMMARY

Procedures for the separation of α_1(I), α_2(I), α_1(II) and α_1(III) chains of human collagen by reversed-phase high-performance liquid chromatography are described. The influence of several different chromatographic parameters (stationary phase, mobile phase and temperature) has been examined and procedures to optimise resolution presented. These reversed-phase high-performance liquid chromatographic conditions also permit the separation of collagen α_1(I), α_2(I), α_1(II) and α_1(III) monomers from their corresponding dimeric β- and γ-components.

INTRODUCTION

The separation and quantitation of collagen α-chains by polyacrylamide gel electrophoresis (PAGE) has remained the most convenient and practical

0378-4347/84/$03.00

method for many years [1—3]. Recent reports have suggested that high-performance liquid chromatography (HPLC) may provide a suitable alternative [4—6] with the added attraction of easy recovery prior to characterisation of the purified materials. The speed of separation and the preparative options available with reversed-phase HPLC for polypeptide fractionation are also attractive when compared with classical chromatographic methods [7, 8]. We report here a comparison of the performance of several different reversed-phase supports with water—acetonitrile eluents at both room temperature and at 62°C, for the separation of collagen α-chains. The identity and purity of the separated collagen chains were confirmed by PAGE and by amino acid analyses.

MATERIALS AND METHODS

Reagents

Acetonitrile was obtained from Waters Assoc. (Milford, MA, U.S.A.). Trifluoroacetic acid was obtained form Pierce (Rockford, IL, U.S.A.). Human collagen standards (Types I, II and III) were donated by Professor A.L. Bailey (Bristol, U.K.). Samples of ^{3}H-labelled Type I collagen were donated by Dr. J. Bateman (Melbourne, Australia). Other collagen samples were prepared in this laboratory by established techniques

Chromatographic equipment

Two gradient elution HPLC systems were used. The HPLC system from Waters Assoc. consisted of two Model M6000A pumps, a gradient programmer Model M660, a Model U6K injector, and a Model M450 variable-wavelength detector. The second HPLC system was a DuPont Model 850 liquid chromatograph (Wilmington, DE, U.S.A.) equipped with a heated column compartment. Detection was performed with a Micromeritics Model 786 variable-wavelength detector (Norcross, GA, U.S.A.). Chromatograms from both HPLC systems were displayed on either an Omniscribe dual-pen recorder (Houston Instruments, Austin, TX, U.S.A.) or a Hewlett-Packard Model 3390A integrator (Waldbronn, F.R.G.). Samples were injected with SGE syringes (SGE, Melbourne, Australia). The *n*-alkyl silica reversed-phase packing material consisted of LiChrospher silica, 10-μm particle diameter with a nominal 50-nm pore diameter, bonded with *n*-butyldimethylchlorosilane or *n*-octyldimethylchorosilane to a ligand density of approximately 2.8 μmol/m^2 followed by maximal end-capping (with hexamethyldisilazane) and slurry packed into stainless-steel columns (15 × 0.40 cm) using procedures described elsewhere [9, 10]. Bakerbond wide-pore diphenyl columns (25 × 0.46 cm) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.).

The solvents used to chromatograph the collagen samples were water and water—acetonitrile (1:1), each containing 0.1% trifluoroacetic acid. New columns were equilibrated overnight in the aqueous solvent and prepared for chromatography by running 60-min gradients from 0 to 50% acetonitrile at 1 ml/min until a stable baseline was achieved. After each gradient run with different collagen samples the columns were equilibrated at initial conditions for 10 min.

Sample and chromatographic fraction treatment

The samples of Types I, II and III collagens were dissolved in acetic acid (0.5 *M*, 2 mg/ml) and diluted with an equal volume of 0.1% trifluoroacetic acid. The sample was heated to 50°C for 15 min and allowed to cool to room temperature. The sample was injected onto the column within 20 min of heating at 50°C.

The collagen fractions separated by HPLC were collected in glass tubes and evaporated under nitrogen to remove most of the acetonitrile. The aqueous material was then frozen and lyophilized.

Sodium dodecyl sulphate—polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)—PAGE separation of collagens was carried out according to the method of Sykes et al. [1] as modified by Sodek and Limeback [3] with a 4% stacking gel and a 7.5% separating gel. The gels were stained according to the method of Fairbanks et al. [11] with Coomassie Blue R 250.

Amino acid analysis

Aliquots of fractions identified as α_1(I) and α_2(I) by SDS—PAGE were hydrolysed with 6 *M* hydrochloric acid for 24 h at 110°C. The hydrolysates were dried, dissolved in the starting buffer and analysed with a Durrum amino acid analyser, Model D502 (Palo Alto, CA, U.S.A.).

Quantitation of tritium

Fractions (1 ml) containing ^{3}H-labelled collagens were mixed with 10 ml Soluene (Packard Instruments, Downers Grove, IL, U.S.A.) and counted for a minimum of 4 min using a β-counter (Packard Tricarb).

RESULTS

Type I collagen

In preliminary experiments designed to optimise resolution and recovery, samples of human Type I collagen (20—500 μg) were chromatographed on the LiChrospher *n*-butyl and Bakerbond diphenyl columns at both 20°C and 62°C. In these experiments a 90-min linear gradient from 0 to 50% acetonitrile in 0.1% trifluoroacetic acid with a flow-rate of 1 ml/min at 20°C was used. Using these conditions the human Type I collagen samples eluted from the LiChrospher *n*-butyl column as two peaks (with retention times 46.4 ± 0.3 min and 51.5 ± 0.1 min over the sample loading range 20—500 μg) which contained the collagen chains α_1(I) and α_2(I) respectively (Fig. 1a). There was, however, contamination as assessed by SDS—PAGE of these main peaks by dimeric β- and trimeric γ-components. With the Bakerbond diphenyl column, using the same elution conditions, similar selectivity was observed (Fig. 1b). The presence of collagen chain dimers and trimers in many collagen preparations has been recognised previously [12]. The fact that these aggregates exhibit similar hydrophobicities as isolated collagen chains under the above reversed-phase conditions is thus not unexpected. However, by employing similar criteria for the optimisation of gradient elution conditions which have been

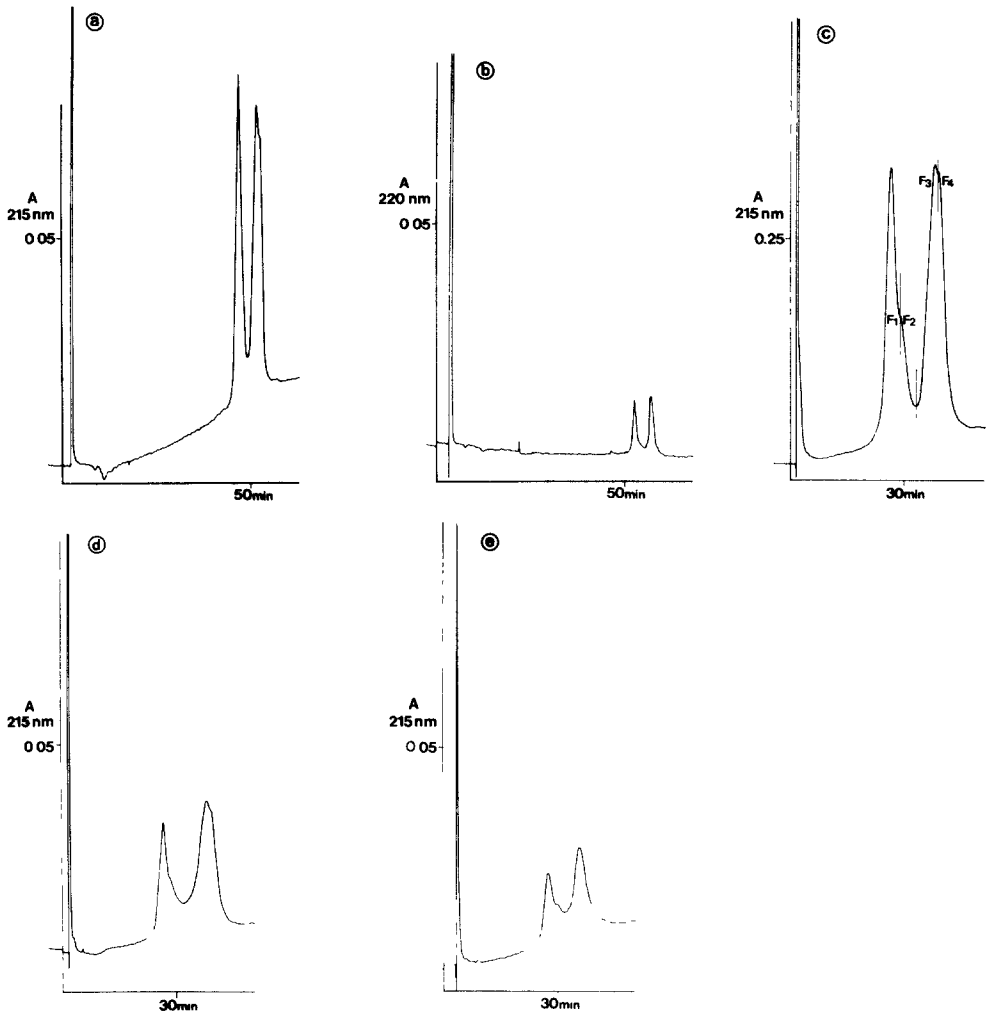

Fig. 1. The separation of type I collagen by HPLC using the *n*-butyl LiChrospher and the Bakerbond diphenyl columns at 20°C and at 62°C. The samples of Type I collagen were heated in 0.5 *M* acetic acid—0.1% trifluoroacetic acid (1:1) at 50°C for 15 min, cooled to 20°C and injected onto the column within 20 min of cooling. Solvent A was 0.1% trifluoroacetic acid in water. Solvent B was 0.1% trifluoroacetic acid in water—acetonitrile (1:1). The traces represent the chromatograms of: (a) 60 μg Type I collagen, linear gradient from 0 to 100% B in 90 min at 1 ml/min, butyl column, 20°C; (b) 60 μg Type I collagen, linear gradient from 0 to 100% B in 90 min at 1 ml/min, diphenyl column, 20°C; (c) 500 μg Type I collagen, linear gradient from 35 to 70% B in 70 min at 1.2 ml/min, butyl column, 62°C; (d) 60 μg Type I collagen, linear gradient from 35 to 70% B in 70 min at 1.2 ml/min, butyl column, 20°C; (e) 40 μg Type I collagen, linear gradient from 40 to 70% B in 50 min at 1 ml/min, diphenyl column, 20°C.

applied [13] to the separation of other high-molecular-weight proteins, improved resolution of Type I collagen chains could be obtained using the LiChrospher *n*-butyl column at 62°C. The elution conditions which were finally selected to take advantage of the large *s*-values [10] of collagen chains involved a shallower gradient, namely a 70-min linear gradient from 17.5 to 35% aceto-

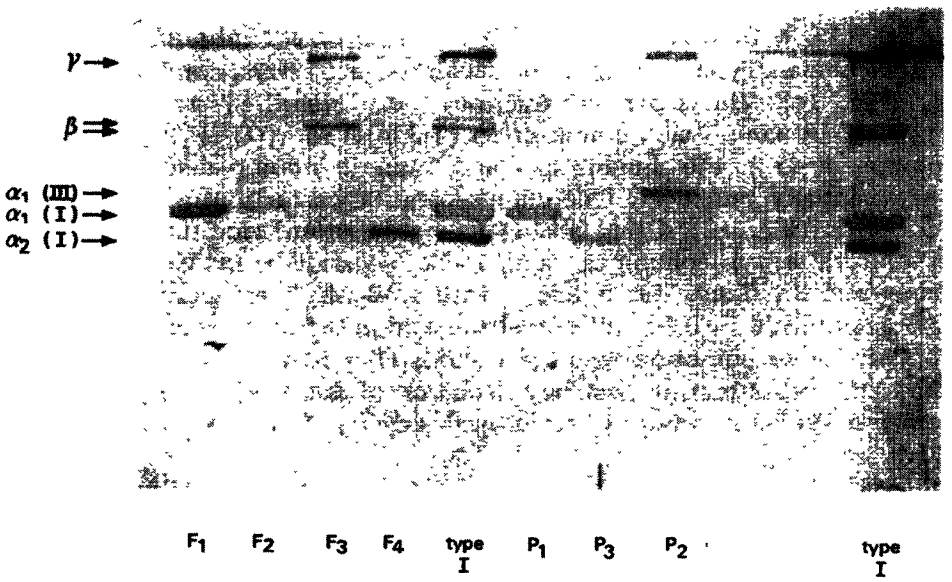

Fig. 2. SDS—PAGE of HPLC fractions of Type I and Type III collagens. SDS—PAGE was performed as described in Materials and methods. The fractions F_1—F_4 were collected from the separation of Type I collagen shown in Fig. 1c. The fractions P_1, P_2 and P_3 (not in sequence) were collected from the separation of Types I and III collagen shown in Fig. 3a.

TABLE I

AMINO ACID ANALYSIS OF α_1(I) AND α_2(I) ISOLATED BY HPLC (RESIDUES/1000 TOTAL RESIDUES)

	α_1(I)		α_2(I)	
	Found	Reported*	Found	Reported*
Aspartic acid	46.2	42	49.4	44
Threonine	17.2	16	19.5	19
Serine	37.2	34	39.3	30
Glutamic acid	73.4	73	73.7	68
Proline	114.9	124	104.1	113
Hydroxyproline	110.3	109	93.6	94
Glycine	332.6	333	321.0	338
Alanine	114.3	115	97.1	102
Half-cystine	—	0	—	0
Valine	21.1	21	34.6	35
Methionine	3.0	7	7.0	5
Isoleucine	10.3	6	20.5	14
Leucine	20.1	19	31.9	30
Tyrosine	—	1	—	4
Phenylalanine	11.6	12	9.6	12
Histidine	4.2	3	12.8	12
Lysine	26.0	26	20.2	18
Arginine	52.5	50	52.3	50
Hydroxylysines	5.1	9	13.6	12

*As reported by Miller and Gay [12].

nitrile in 0.1% trifluoroacetic acid at a flow-rate of 1.2 ml/min. With these conditions human Type I collagen was separated into several component peaks F_1—F_4 (Fig. 1c). The separated fractions were identified by SDS—PAGE (Fig. 2) and by amino acid composition (Table I) as α_1(I) (F_1), the dimeric β_{11}-component (F_2), the dimeric β_{12}- and trimeric γ-components (F_3) while F_4 was essentially pure α_2(I). The same separation carried out at 20°C gave a similar result (Fig. 1d). Using analogous optimisation procedures with the Bakerbond diphenyl column a comparable separation between the α_1(I) monomer and β_{11} dimer was obtained with a 50-min linear gradient from 20 to 35% acetonitrile in 0.1% trifluoroacetic acid at a flow-rate of 1 ml/min at 20°C. With these conditions the α_1(I) and α_2(I) peaks eluted at 28.0 ± 0.1 min and 36.6 ± 0.2 min, respectively. Isolation of the β_{11} dimer from the α_1(I) monomer could be achieved by careful peak shaving of the second eluting peak (Fig. 1e).

The recovery of the Type I collagen from the column was determined by running a sample of [^{3}H]Type I collagen. The majority of the radioactivity (74%) was eluted with the α_1(I) and α_2(I) peaks in a ratio of 2.3:1 which corresponds well to the expected ratio α_1(I):α_2(I) of 2:1.

Type III collagen

Type III collagen is composed of a trimer of α_1(III) chains which, unlike Type I and II collagens, is not dissociated into its monomeric components unless a sulfhydryl-reducing agent, such as β-mercaptoethanol, is present. The unreduced Type III trimer eluted between the α_1(I) and α_2(I) peaks of the type I collagen (Fig. 3a) on the LiChrospher *n*-butyl and *n*-octyl columns as well as on the Bakerbond diphenyl columns at both 20°C and 62°C. After reduction of Type III collagen with β-mercaptoethanol the α_1(III) monomer

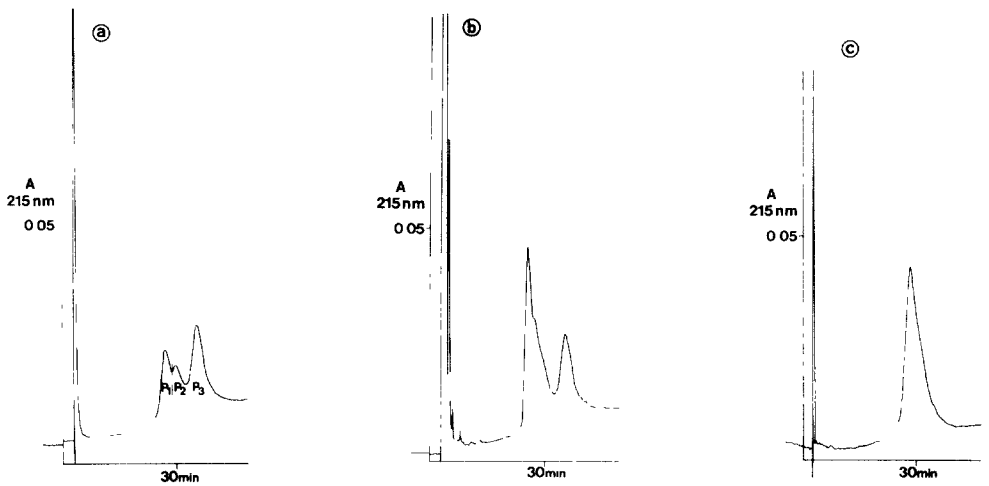

Fig. 3 The separation of types I, II and III (monomer and trimer) collagens. The samples were prepared as in Fig. 1 and were separated on the Bakerbond diphenyl column at 20°C with a linear gradient from 40 to 70% B in 50 min at 1 ml/min (as in Fig. 1e). Detection was at 215 nm. The chromatograms represent: (a) Types I and III collagens (40 μg of each), the fractions P_1, P_2 and P_3 were collected manually; (b) types I and III collagens (reduced with β-mercaptoethanol, 40 μg of each); (c) type II collagen (40 μg).

elutes as a sharply defined peak immediately before the α_1(I) peak (Fig. 3b). The identity of the fractions obtained from the run shown in Fig. 3a was confirmed by SDS—PAGE (Fig. 2).

Type II collagen

Type II collagen is composed of three α_1(II) chains which are dissociated into the monomeric form on heating. Using either the LiChrospher *n*-butyl packing material or the commercial Bakerbond diphenyl columns at both 20°C and 62°C the α_1(II) monomer was found to elute immediately after the α_1(I) peak (Fig. 3c).

DISCUSSION

Recent reports on the separation of collagen chains by reversed-phase HPLC have suggested a need for high column temperatures [4] or have advocated [5] the use of the UV opaque pyridine—formate elution conditions to maintain adequate chromatographic selectivity. However, these elution conditions require sophisticated post-column derivatization procedures for sample detection. Since these requirements are not easily accommodated in many laboratories, a simplified elution system for collagen chain separation by HPLC was sought and a comparison of widely differing column stationary phases made.

As has been noted in previous studies [10, 13] on the separation of proteins on large-pore alkyl silicas, the *n*-alkyl chain length did not have a significant influence on the resolution of collagen chains under gradient elution conditions with low pH water—acetonitrile combinations. In addition, there appeared to be very little effect of temperature on the separation of α_1(I), α_2(I), α_1(II) and α_1(III) chains provided the samples were initially heated to 50°C to dissociate the collagen chains before injection. One practical caveat in this approach, however, is that if pepsin (a likely preparation contaminant) is present in the collagen sample, heating to 50°C will lead to rapid proteolysis of the dissociated collagen triple helix. Under the chromatographic conditions employed in this study there appeared to be no discernible improvement in resolution if the column temperature is raised to 62°C.

In the comparison of the *n*-butyl and diphenyl stationary phases no major selectivity differences were evident. The slightly higher efficiency of the diphenyl silica support was in accord with the smaller particle size of the parent silica matrix than was used for the preparation of the *n*-butyl silica support. Despite the slightly sharper peaks obtained with the Bakerbond diphenyl silica column, the *n*-butyl LiChrospher column gave better overall resolution, i.e. higher selectivity for the β_{12}- and γ-components from α_2(I). Preliminary attempts to separate collagens on standard octadecyl silica columns which were neither macroporous nor fully capped were without success.

As part of the evaluation of elution conditions several solvent systems and gradients were tried in this investigation. Increasing the mobile phase pH to 6.0 at constant low ionic strength resulted in no eluted components and we assume the collagens precipitated or adhered to the column matrix. Similar results were obtained when a 60 m*M* phosphate buffer gradient from pH 2.5 to 7.0 was

run in conjunction with an acetonitrile gradient (0 to 50%). A primary mobile phase based on 0.1% phosphoric acid also appears to be a suitable eluent for the reversed-phase HPLC of collagens but in contrast to 0.1% trifluoroacetic acid this ionic modifier lacks volatility and cannot be removed by lyophilisation.

Although it appears from this and other investigations [4—6] that collagens may be chromatographed with a variety of reversed-phase columns and solvent systems, the procedures do not yet allow the complete resolution of all differing collagen types. The greatest separation is between α_1(I) and α_2(I) while α_1(II) and the α_1(III) trimer elute just after α_1(I) and the α_1(III) monomer elutes just before α_1(I). These differences are sufficient to allow micropreparative separations of individual chains which can be recovered in high purity. However, reliable quantitative analytical separations will only be obtained when the contamination from β- and γ-components is adequately controlled, possibly by preliminary salt precipitation [14] or by gel permeation chromatography [8, 15]. The relatively minor contribution which the molecular size differences between the collagen species (100,000—300,000) make on the separation with reversed-phase columns is noteworthy. As has been extensively documented [16] retention to *n*-alkyl silicas by macromolecules is dominated by the hydrophobic contact area with molecular weight playing a minor role on resolution, mainly through its influence on column efficiencies.

The advantages of HPLC of proteins are well recognized and may apply particularly in collagen biochemistry. Newly discovered collagen types and genetic variants have recently resulted in a three-fold increase in known collagenous proteins all of which may potentially be purified by rapid reversed-phase HPLC techniques. The high resolution and rapidity of HPLC should find ready acceptance in the clinical diagnosis of genetic defects in collagen synthesis and post-translational processing. There is also the evidence from this investigation that the relatively neglected β- and γ-components may also be separated by reversed-phase HPLC. Availability of these components should lead to new insights into α-chain cross-linking patterns and hence the structural complexity which results in the unique architecture of the collagenous tissue framework.

ACKNOWLEDGEMENTS

This investigation has been supported by grants from the Medical Research Council of New Zealand and The National Health and Medical Research Council of Australia. The excellent technical assistance of F. Lambrou with the amino acid analyses is acknowledged. We are grateful to Dr. G. Gibson, Department of Surgery, University of Auckland for his advice and discussion.

REFERENCES

1 B. Sykes, B. Puddle, M. Frances and R. Smith, Biochem. Biophys. Res. Commun., 72 (1976) 1472.
2 H. Sage and P. Bornstein, Methods Enzymol., 82 (1982) 96.
3 J Sodek and H.F. Limeback, J. Biol. Chem., 254 (1979) 10496.
4 M. van der Rest and P. Fietzek, Eur. J. Biochem., 125 (1982) 491.

5 A. Fallon, R.V. Lewis and K.D. Gibson, Anal. Biochem., 110 (1981) 318.
6 K.A. Smolenski, A. Fallon, N.D. Light and A.J. Bailey, Biosci. Rep. 3 (1983) 93.
7 T.F. Kresina and E.J. Miller, Biochemistry, 18 (1979) 3089.
8 K.A. Piez, E.A. Eigner and M.S. Lewis, Biochemistry, 2 (1963) 58.
9 B.L. Karger, K. Benedek, N. Miller, B. Feibush and S.A. Cohen, in I. Chaiken, M. Wilchek and I. Parikh (Editors), Affinity Chromatography and Biological Recognition, Academic Press, New York, 1983, p. 479.
10 M.T.W. Hearn and B. Grego, J. Chromatogr., 296 (1984) in press.
11 G. Fairbanks, T.L. Steck and D.S.H. Wallach, Biochemistry, 10 (1971) 2606.
12 E.J. Miller and S. Gay, Methods Enzymol., 82 (1982) 3.
13 M.T.W. Hearn and B. Grego, J. Chromatogr., 255 (1983) 125.
14 G. Chandrakasan, D.A. Torchia and K.A. Piez, J. Biol. Chem., 251 (1976) 6062.
15 Z. Deyl, K. Macek, M. Adam and M. Horáková, J. Chromatogr., 230 (1982) 409.
16 M.T.W. Hearn, Adv. Chromatogr., 20 (1982) 1.