polymer reports

Solid phase peptide synthesis on an acrylate copolymer attached to porous silica beads

R. Epton, G. Marr and R. G. Ridley
Department of Physical Sciences, The Polytechnic, Wolverhampton WV1 1LY, UK
(*Received* 20 *September* 1981)

Porous silica beads have been internally coated with a new copolymer prepared from *N*-[2-(4-acetoxyphenyl)ethyl]acrylamide and *N*-[3-(triethoxysilyl)propyl]acrylamide. The derivatized silica beads have been used as a solid phase support for the synthesis of the opoid heptapeptide H–Try–Gly–Gly–Gly–Lys–Met–Gly–OH.

Keywords Peptide; solid-phase; acrylate copolymer; silica beads

INTRODUCTION

Derivatized porous silica beads have been used as supports for the solid phase synthesis of peptides[1-3]. Silica beads are ideal for this purpose in that they are non-compressible and they can be used easily in reactors and columns. To date, silica beads have been derivatized with low molecular weight alcohols[1] and organo-silanes[2,3]. Titanium oxide coated silica was derivatized by successive treatment with 2-phenylethyltrichlorosilane, water and chloromethyl methyl ether and this support was used for the synthesis of a dipeptide[4].

Glass beads coated with polystyrene have been used successfully as a support for the synthesis of peptides. The reaction times were short and the washing procedures were simplified[5-7]. Porous silica beads internally coated with polymers have been used for bonded phase chromatography[8] and the immobilization of enzymes[9-11]. It has been suggested that the grafting of organic polymers onto inorganic materials would lead to supports that possessed the superior mechanical properties of the inorganic support and all the advantages associated with a gel type matrix[12].

We reported recently the coupling of triethoxysilane-substituted acrylate copolymers to porous silica beads and the use of these reagents for enzyme immobilization[13]. In this report we present the preparation of porous silica beads internally coated with a new triethoxysilane-substituted acrylate copolymer containing phenolic groups and the successful use of this support for solid phase peptide synthesis.

EXPERIMENTAL

Synthesis of a copolymer of N-[*2-(4-acetoxyphenyl)ethyl*]*acrylamide and* N-[*3-(triethoxysilyl)propyl*]*acrylamide (molar ratio 10:1)*

N-[2-(4-Acetoxyphenyl)ethyl]acrylamide[14] (18.64 g, 0.08 mol), *N*-[3-(triethoxysilyl)propyl]acrylamide[15] (2.20 g, 0.008 mol) and 2,2′-azobis(2-methylpropionitrile) (0.27 g) were dissolved in 1,1,2,2-tetrachloroethane (180 cm^3). The solution was deoxygenated with nitrogen for 1 h and then heated for 4 h at 65°C. Dry diethyl ether was added to the viscous reaction mixture and the resultant precipitate was collected by filtration to yield Copolymer A (14.2 g, 69%). Analysis by ^1H n.m.r. indicated a ratio of N-[2-(4-acetoxyphenyl)ethyl]acrylamide to N-[3-(triethoxysilyl)propyl]acrylamide of 10:1. (Found: Si, 1.12%. 10:1 mole ratio requires: Si, 1.08); M_n(membrane osmometry) = 49,600; [η](chloroform, 25°C) = 0.10.

Pre-treatment of porous silica beads

Spherosil porous silica beads (Rhone-Poulenc Industries) Type XOB 015 (internal surface area, S_A, = 25 $m^2\ g^{-1}$, average pore diameter, $\overline{dp}$, = 125 nm, pore volume, 1 $cm^3\ g^{-1}$) were treated with 0.2M nitrous acid at 80°C with continuous sonication in a Dowe Sonicleaner Type 6442 A, for 4 h. The beads were washed to pH 7 with distilled water and heated to 640°C in an electric furnace for 18 h.

Derivatization of pretreated porous silica beads with Copolymer A

A 0.1% w/v solution of 4-toluenesulphonic acid in chloroform was prepared and dried (calcium chloride). Copolymer A (10 g) was dissolved in this solution (100 cm^3) and added to the pre-treated spherosil porous silica beads. The mixture was placed under vacuum several times, to ensure entry of the polymer solution into the pores of the support. The beads were heated under reflux, with mechanical stirring, for 18 h. The coated beads were washed exhaustively with hot chloroform and dried under vacuum. The support was shaken in a mixture of chloroform, methanol and water (5:4:1) for 24 h. The solvent mixture was removed and the coated beads were heated to 100°C under high vacuum for 24 h. (Found, C,

0032-3861/82/020306-04$03 00

2.30; H, 0.20; N, 0.28%). This corresponded to a polymer content of 35 mg g^{-1}; phenol content was 0.13 mmol g^{-1}.

De-O-acetylation of derivatized porous silica beads

The derivatized porous silica beads were shaken in morpholine for 2 h at room temperature. The removal of the acetate protecting group was monitored by disappearance of the 1760 cm^{-1} absorption peak in the infra-red spectrum. The beads were washed three times with ethanol and then three times with diethylether.

Determination of phenol content[16]

The de-*O*-acetylated derivatized porous silica beads (0.5 g) were added to a mixture of methanol (100 cm^3), concentrated hydrochloric acid (5 cm^3) and bromate–bromide reagent (25 cm^3). [The bromate–bromide reagent was prepared by dissolving potassium bromate (0.56 g, 0.003 mol) and potassium bromide (2.0 g, 0.017 mol) in water (1 dm^3)]. After 3 minutes, potassium iodide (0.5 g) was added and the iodine was titrated with standard 0.025M sodium thiosulphate. A blank was determined by titrating an identical solution, containing uncoated beads, with 0.025M sodium thiosulphate. Phenol content of the derivatized beads determined titrimetrically was 0.12 mmol g^{-1} (value obtained by elemental analysis was 0.13 mmol g^{-1}).

Preparation of H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH

Synthesis of Boc-Gly-Gly-Gly-OH. Di-tert-butyl dicarbonate (7.7 g, 0.036 mol) was added dropwise, over 1 h, to a solution of *N*-(*N'*-glycylglycyl)-glycine (5.7 g, 0.03 mol) and sodium hydroxide (1.2 g, 0.03 mol) in water (9 cm^3) and 2-methyl propan-2-ol (6 cm^3) when a white, turgid suspension formed. After 1 h 2-methylpropan-2-ol (6 cm^3) was added and the mixture was stirred for 16 h when the suspension dissolved. Water (15 cm^3) was added and the solution was washed with petroleum ether (b.p. 40–60°C) (3 × 20 cm^3). The aqueous layer was acidified to pH 2–3 by the addition of potassium bisulphate (approx. 4.2 g), to produce a turgid suspension, and this was extracted with ethyl acetate (4 × 25 cm^3). The extracts were dried over magnesium sulphate and the solvent evaporated under reduced pressure to yield a white solid. Recrystallisation from acetone gave *N*[*N'*-(*N''*-*tert*-butoxycarbonylglycyl)glycyl]-glycine (6.55 g, 75%), m.p. 160°–161°C (lit. 157°–159°C)[17] ν_{max} (KBr disc) 3300 (multiplet, N–H str.) 1740, 1710, 1680, 1640 (Boc and glycyl C=O str.) and 1550 cm^{-1} (N–H bend); δ 60 MHz, DMSO deut.) 8.4 (1H, broad, –NH–), 8.0 (1H, broad, –NH–), 6.9 (1H, broad, –NH–), 3.8 [6H, m, (–NHCH$_2$CO–)$_3$] and 1.4 ppm [9H, s, $(CH_3)_3C$–]. Found: M^+, 216 [$C_{11}H_{19}N_3O_6$–$(CH_3)_3CO$– requires M, 216].

Experimental procedures used in solid phase peptide synthesis

Peptide synthesis was performed manually in a vessel adapted from that used by Corley *et al.*[18], N_α-Boc-amino acids were obtained from Sigma, London. Thin layer chromatography (t.l.c.) was carried out on silica gel (Merck Kieselgel 60 F_{254}) in the following systems (ratios by volume); A: ethyl acetate, 1-butanol, acetic acid, water (1:1:1:1); B: pyridine, 1-butanol, acetic acid, water (4:1:1:2). Fluorescamine reagent was used to monitor the coupling of N_α-Boc-amino acids to the growing peptide chain[19]. Amino acid analyses were performed by the Birmingham University Macromolecular Analysis Service. Samples for amino acid analysis were prepared by heating the peptide at 130°C for 4 h in 1:1 (v/v) propionic acid/12M hydrochloric acid containing phenol (0.05% w/v), thioglycolic acid (0.1% w/v) and a standard quantity of D, L-norleucine in a sealed tube under vacuum.

Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-O-C_6H_4[porous silica support] Boc-Gly-O-C_6H_4[porous silica support]. De-*O*-acetylated, copolymer derivatized porous silica beads (5.0 g, 0.12 mmol g^{-1} phenol content) were equilibrated in DMF and a solution of Boc-glycine (1.4 g, 0.008 g mol) and DMAP (0.48 g, 0.004 mol) in DMF (20 cm^3) was added. After 1 minute of vigorous stirring a solution of DCC (2.06 g, 0.01 mol) in DMF (10 cm^3) was added and the reaction allowed to proceed for 16 h at 25°C. The excess reagents were removed and the beads were washed thoroughly according to Sequence A: CH_2Cl_2/EtOH(1/3)(3 ×), Et_2O(2 ×), DMF (3 ×), Et_2O(2 ×), AcOH(3 ×), Et_2O(2 ×). A small sample of beads gave a negative fluorescamine test and the infra-red spectrum confirmed the presence of phenyl ester (1760 cm^{-1}) and Boc (1710 cm^{-1}) groups. Amino acid analysis showed a glycine substitution of 0.06 mmol g^{-1}.

Acetylation. The beads were equilibrated in DMF and a solution of acetic anhydride (4.0 g, 0.04 mol) and triethylamine (6.0 cm^3, 0.44 mol) in DMF (30 cm^3) was added and left for 1.5 h at 25°C. After an intermediate wash of CH_2Cl_2(3 ×), DMF(3 ×) and Et_2O(3 ×) the acetylation was repeated and followed by a further wash cycle. A sample of the derivatized beads (0.2 g) was removed for amino acid analysis and showed a glycine substitution of 0.06 mmol g^{-1}.

Boc-Met-Gly-O-C_6H_4[porous silica support]. The Boc-Gly-O-C_6H_4[porous silica support] (0.06 mmol g^{-1} glycine) was equilibrated in benzyl alcohol and an excess of HCl in benzyl alcohol (3.0M) was added. The mixture was stirred manually for 5 minutes, the reagent was removed and a fresh solution of HCl in benzyl alcohol added, and stirred for 10 minutes. The reagent was removed and the beads were washed with Et_2O(3 ×). The deprotection and the ether wash were repeated, followed by a wash according to Sequence B: benzyl alcohol (2 ×), Et_2O(2 ×), DMF(3 ×), Et_2O(3 ×) [the beads gave a positive fluorescamine test; in the infra-red spectrum the band at 1710 cm^{-1} (Boc group) disappeared].

The beads were equilibrated in DMF and a solution of Boc-methionine (1.60 g, 0.006 mol) and 4-methylmorpholine (0.32 g, 0.003 mole) in DMF (15 cm^3) was added. After vigorous stirring for 1 minute a solution of DCC (1.6 g, 0.008 mol) and HOBr (0.11 g, 0.0008 mol) in DMF (10 cm^3) was added and the reaction was left for 2 h. The reaction was stopped by removal of the reagents and the beads were washed according to Sequence A. The beads gave a negative fluorescamine test after 0.5 h; and in the infra-red spectrum the band at 1710 cm^{-1} indicated the presence of a Boc group.

Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-O-C_6H_4[porous silica support]. The deprotection, coupling and wash cycles were repeated with N_α-tert-butoxycarbonyl-N_ε-benzyloxycarbonyl-lysine, Boc-Gly-Gly-Gly-OH and Boc-tyrosine to give Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-O-C_6H_4[porous silica support] (Amino acid analysis, found: Gly, 4.00; Met, 0.81; Lys, 0.91, Tyr, 1.20).

Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly—DMAE ester. Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-O-C_6H_4[porous silica support] (1 g) was shaken in DMAE/DMF (1/1) for two days, washed with DMF(4 ×), and the filtrate and washings were evaporated under reduced pressure to give an oily residue. Cleavage from the support was monitored using infra-red spectroscopy by disappearance of the phenyl ester peak (1760 cm^{-1}). The residue was re-dissolved in DMF (1 cm^3) and passed through a Sephadex LH20 column (80 × 2.5 cm) eluted with DMF at a flow rate of 13 cm^3 h^{-1}, collecting 5 cm^3 fractions. The eluent was monitored at 280 nm and the elution profile is given in *Figure 3*. Fractions 36–38 were combined to give Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly—DMAE ester as a colourless glass (27 mg, 46%; based on C-terminal glycine). T.l.c. System A gave one major spot $R_fA = 0.46$ (Amino acid analysis gave: Gly, 4.00; Met, 0.85; Lys, 1.00; Tyr, 1.05).

Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-OH. Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-DMAE ester (13.5 mg, 0.013 mmol) was dissolved in DMF/H_2O(1/1)(6 cm^3) and the pH maintained at 9.7 for 2 days by the addition of 0.1 M sodium hydroxide. The hydrolysis was monitored using t.l.c. by the disappearance of the ester, $R_fA = 0.46$, and the appearance of the free acid, $R_fA = 0.74$. After 2 days, water (3 cm^3) was added and the solution acidified to pH 4.0 by careful addition of saturated potassium bisulphate solution. The solution was evaporated under reduced pressure at room temperature. The residue was suspended in DMF, filtered and extracted several times with DMF. The extracts were combined and evaporated under reduced pressure to give a glass (17.6 mg, 39%). T.l.c., $R_fA = 0.77$ (Amino acid analysis gave: Gly, 4.00; Met, 0.93; Lys, 1.02; Tyr, 0.90).

H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH.2HBr. Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-OH (8.8 mg, 0.010 mmol) was treated with HBr in acetic acid (45% w/v, 1 cm^3). After 2 h, dry diethyl ether was used first to precipitate and then to wash the dihydrobromide salt, (6.7 mg, 32%). T.l.c. studies showed one major product in Systems A and B. $R_fA = 0.16$, $R_fB = 0.28$. (Amino acid analysis gave: Gly, 4.10; Met, 1.00; Lys, 0.96; Tyr, 1.00).

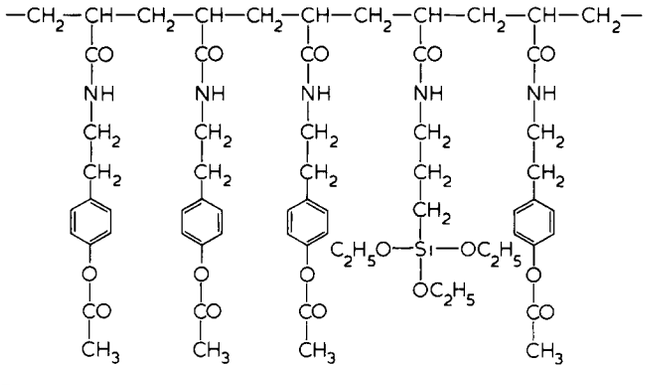

Figure 1 Schematic representation of (*N*-[2-(4-acetoxyphenyl)ethyl]-acrylamide and *N*-[3-(triethoxysilyl)propyl] —acrylamide) copolymer (Copolymer A)

RESULTS AND DISCUSSION

One of the most common reagents for the derivatization of silica is 3-aminopropyltriethoxysilane which couples to the silica *via* a siloxane linkage. This reagent was treated with acryloyl chloride to give *N*-[3-(triethoxysilyl)propyl]acrylamide (Monomer I)[15]. Copolymerization of Monomer I with N-[2-(4-acetoxyphenyl)ethyl]acrylamide (Monomer II)[14,20] (molar ratio 1:10) gave the Copolymer (A) (*Figure 1*) in good yield. Activated porous silica beads were heated with a 10% w/v chloroform solution of Copolymer A in the presence of *p*-toluenesulphonic acid as catalyst to effect the coupling of Copolymer A to the porous silica beads. Unreacted ethoxysilyl groups were hydrolyzed with a chloroform–methanol–water mixture which also served to remove any excess uncoupled Copolymer A from the pores of the beads[3]. The derivatized silica beads where then heated at 100°C under high vacuum to effect further condensation of the attached and hydrolyzed copolymer with the silica surface. The phenol content of the derivatized beads was found to be 0.13 mmol g^{-1} by elemental analysis. This figure was derived assuming complete copolymerization of the original monomers I and II. Titrimetric estimation of bromine uptake by the phenol group, after de-O-acetylation, gave a phenol content of 0.12 mmol g^{-1}. This value was in good agreement with the phenol content as estimated by elemental analysis and indicated that most of the phenol groups were available for further chemical reaction.

The usefulness of this support for pepetide synthesis was tested by the preparation of the heptapeptide H–Tyr–Gly-Gly-Gly-Lys-Met-Gly-OH. This is a model opoid peptide, first prepared by Goldstein *et al.*[21], with proven opiate properties. The synthesis of the heptapeptide is outlined in *Figure 2*. The first step in the preparation was the attachment of Boc-glycine to the de-O-acetylated porous silica support by a *N,N'*dicyclohexylcarbodiimide mediated coupling in the presence of 4-dimethylaminopyridine as the catalyst. Amino acid analysis showed that the loading of Boc-glycine on the support was 0.06 mmol g^{-1} which indicated that 50% of the available phenol groups had undergone reaction. The free phenol groups were acetylated by treatment of the support with acetic anhydride in the presence of triethylamine. After acetylation, treatment with hydrogen chloride in benzyl alcohol removed the *N*-terminal Boc groups from the glycine residues. This deprotection was monitored by infra-red at 1710 cm^{-1} (ester carbonyl absorption) which showed when the Boc group had been removed. Boc-Methionine, N_α-Boc-N_ε-Z-lysine, Boc–Gly–Gly–Gly–OH and Boc-tyrosine were attached successivly to the glycine substituted support *via* DCC mediated couplings in the presence of 4-methylmorpholine, to neutralize the hydrochloride salt, and 1-hydroxybenzotriazole, to suppress racemization (*Figure 2*). Each coupling reaction was deemed to be complete when a small sample of the support gave a negative fluorescamine test. The anchored heptapeptide, Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-O-C_6H_4[porous silica support], was cleaved from the support by treatment with 2-dimethylaminoethanol to give Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-$OCH_2CH_2NMe_2$ which was purified by gel permeation chromatography using dimethylformamide as the eluent (*Figure 3*)[22]. Fractions 36–38 were combined to give the product in 46% yield

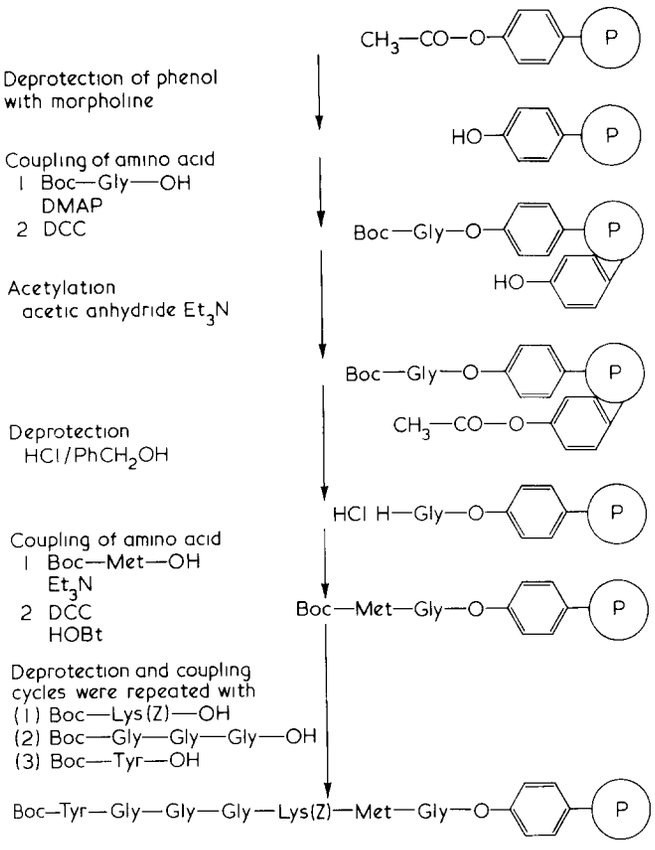

Figure 2 Synthesis of the heptapeptide on the porous silica support derivatized with the Copolymer A (P-porous silica support)

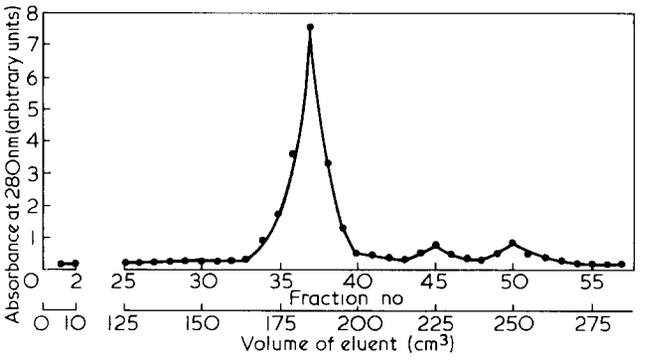

Figure 3 Elution profile showing Boc—Tyr—Gly—Gly—Gly—Lys(Z)—Met—Gly—DMAE ester purification by g.p.c. on Sephadex LH20, eluted with DMF. The eluent was monitored by ultra-violet adsorption at 280 nm

which was shown to be pure by thin layer chromatography. The 2-dimethylaminoethyl ester of the heptapeptide was hydrolysed by water in dimethylformamide at pH 9.7. The product, Boc-Tyr-Gly-Gly-Gly-Lys(Z)-Met-Gly-OH, was obtained in 39% yield and it was shown to be pure by thin layer chromatography. The *N*-tert-butyloxycarbonyl protected heptapeptide was treated with hydrogen bromide in acetic acid to remove the protecting groups. The resultant dihydrobromide salt was obtained in 32% yield and it was shown to be pure by thin layer chromatography.

We conclude that porous silica supports derivatized with triethoxysilane-substituted copolymers can be used successfully for solid phase peptide synthesis. The phenylester linkage is ideal for attaching the peptide to the copolymer in these systems and the peptide can be cleaved from the copolymer under mild conditions.

ABBREVIATIONS

Boc,	N-tert-butyloxycarbonyl
Z,	benzyloxycarbonyl
DMF,	*N*,*N*-dimethylformamide
DMAE,	2-dimethylaminoethanol
DMAP.	4-dimethylaminopyridine
DCC,	*N*,*N*′-dicyclohexylcarbodiimide
HOBt,	1-hydroxybenzotriazole
Gly,	glycine
Met,	methionine
Lys,	lysine
Tyr,	tyrosine

REFERENCES

1 Bayer, E., Jung, G., Halasz, I. and Sebastian, I *Tetrahedron Lett.* 1970, 4503
2 Parr, W and Grohmann, K. *Tetrahedron Lett.* 1971, 2633
3 Parr, W. and Grohmann, K. in 'Chemistry and Biology of Peptides' (Ed. J Meienhofer), Ann Arbor Sc., Ann Arbor, Michigan, 1972, 169
4 Postnov, V. N , Makarov, K. A., Aleskovskii, V. B. and Kolt'sov, S. I. *Dokl. Akad. Nauk. S.S.S R.* 1977, **235**, 599
5 Horvath, C. G., Preiss, B. A. and Lipsky, S. R., Anal. Chem., 1967, **39**, 1422; Bayer, E., Eckstein, H , Hägele, K , König, W. A., Brüning, W., Hagenmaier, H and Parr, W. *J Am Chem. Soc.* 1970, **92**, 1735
6 Bayer, E., Hagenmaier, H., Jung, G., Parr, W., Eckstein, H., Hunziker, P and Sievers, R. E. in 'Peptides 1969' (Ed. E. Scoffone), North-Holland, Amsterdam, 1971, 65
7 Scott, R. P. W , Chan, K. K., Kucera, P. and Zolty, S. *J. Chromatogr Sci* 1971, **9**, 577
8 Haken, J. K *J. Chromatogr.* 1977, **141**, 247
9 Royer, G P , Andrews, J. P and Uy, R. *Enzyme Technol. Digest* 1973, **1**, 99
10 Royer, G. P., Liberatoire, F. A. and Green, G. M. *Biochem. Biophys Res. Commun.* 1975, **64**, 478
11 Royer, G. P , Green, G. M. and Sinha, B. K. *J. Macromolec. Sci , Chem.* 1976, **A10(1–2)**, 289
12 Goldstein, L. and Manecke, G. in 'Applied Biochemistry and Bioengineering Vol. 1' (Eds. L B. Wingard Jr., E. Katzir-Katchalski and L Goldstein), Academic Press, New York, 1976, p. 23
13 Epton, R , Marr, G. and Ridley, R. G. *Polymer* 1979, **20**, 1447
14 Goddard, P , *Ph.D. Thesis*, The Polytechnic, Wolverhampton, 1980
15 Van den Heuvel, W. A., Willems, J F., Philippaerts, H. A., Beels, R F. and Hegeln, R. F.; Ger. Offen. 1957, 625 (1970)
16 Scott, W. in 'Standard Methods of Chemical Analysis' 1939, Vol. 2, p 2253
17 Channabasavaiah, K. and Sivanandaiah, K M. *Int. J Peptide Protein Res* , 1976, **8**, 323
18 Corley, L., Sachs, D. H. and Anfinsen, C. B. *Biochem. Biophys. Res. Commun* 1972, **47**, 1353
19 Felix, A. M , Jiminez, M H., Vergona, R. and Cohen, M. R., *Int. J Peptide Protein Res.*, 1973, **5**, 201; Felix, A M. and Jiminez, M. H , *Analyt. Biochemistry* 1973, **52**, 377
20 Epton, R., Goddard, P., Marr, G., McLaren, J V and Morgan, G. J *Polymer* 1979, **19**, 1444
21 Goldstein, A , Goldstein, J S. and Cox, B M *Life Sci.* 1975, **17**, 1643
22 Hudson, D and Kenner, G W *Int J Biol. Macromol.*, 1980, **2**, 63