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Manual solid-phase syntheses of peptides on resins with high loading capacity requiring small volumes of solvents

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Abstract. The synthesis of peptides by manual solid-phase methods requiring very small volume of solvents at different steps is described. The syntheses have been carried out on polystyrene-based resins using fluorenylmethoxy carboxyl chemistry. All reactions were carried out in vials, of capacity 1.2 or 6 ml, with gentle stirring. The volumes of solvents used were 1–2 ml for each operation. The crude peptides after cleavage from the resin were reasonably pure. The total volume of solvents required for the entire synthesis is substantially less as compared to volumes required in semi-automated and fully automated peptide synthesizers which renders the described method cost-effective.

Keywords. Peptide synthesis; solid-phase; manual methods; polystyrene resins.

1. Introduction

Peptides composed of as few as three to about 50 amino acids, which are not primary translation products, play crucial roles in a large number of diverse biological processes. Although the initial characterization of biologically active peptides has been done on samples isolated from natural sources, chemical synthesis has been the method of choice to generate biologically active peptides as well as their analogues (Erickson and Merrifield 1976; Finn and Hofmann 1976; Barany and Merrifield 1980; Merrifield 1986). Although solution phase methods have been employed extensively for the syntheses of peptides, the current method of choice to obtain peptides is by solid-phase methods (Barany and Merrifield 1980; Merrifield 1986; Kent 1988). To-date, syntheses of peptides are conveniently accomplished by solid-phase methods on semi-automated and fully automated peptide synthesisers. In fact, syntheses of even small proteins have been achieved in automated peptide synthesisers (Wlodawer *et al* 1989; Millon *et al* 1992; Choma *et al* 1994; Liu *et al* 1996). Solid-phase protocols have also been adopted for simultaneous synthesis of peptides and peptide libraries (Fodor *et al* 1991; Blondelle *et al* 1995; Eichler and Houghten 1995). Protocols for synthesis as well as cleavage from solid supports have been optimized so that even crude peptides are of considerable purity. However, the use of protected amino acids preweighed and packaged in vials for use in automated peptide synthesis, large volumes of solvents required for washings and deprotection, and prepacked coupling and deprotection reagents, result in peptide synthesis being expensive, especially when synthesised on automated synthesisers. In this report, we describe a convenient, cost-effective method of manual, solid-phase

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Table 1. Primary structures of peptides.

PKLLKKFLKKWIG	1
PKLLKFLKWIG	2
PKLKTFLSKWIG	3
PKLKFLSKWIG	4
PKLLKTFLKWIG	5
PKLLKFLSKWIG	6
PKLLTKF(*p*-fluoro)LKSWIG	7
PKLLTKFLKSF(*p*-fluoro)IG	8
PKLLTKF(*p*-fluoro)LKSF(*p*-fluoro)IG	9

peptide synthesis using fluorenylmethoxycarbonyl (Fmoc) chemistry. The volumes of solvents used in this method are substantially less as compared to the volumes used in automated peptide synthesisers. The peptides chosen in the present investigations (table 1) are amphipathic peptides, which are analogues of 13-residue peptides (Sitaram *et al* 1992, 1995), possessing potent antimicrobial activity.

2. Materials and methods

2.1 *Materials*

Fmoc amino acids and dimethylamino pyridine (DMAP) were from Nova Biochem (UK). Resins, 4-hydroxymethylphenoxymethyl-isopolystyrene-1% divinylbenzene (HMP) and polyethylene grafted polystyrene with the handle hydroxymethylphenoxy acetic acid linker (PAC-PEG-PS) were from Applied Biosystems and PerSeptive Biosystems respectively. 1,1,3,3-tetramethyluronum hexafluorophosphate (HBTU) was from Applied Biosystems. Dimethylformamide was from Spectrochem (India) and diisopropyl ethylamine (DIEA) was from Sigma Chemicals Co., USA. 1-Hydroxybenzotriazole (HOBt) was prepared from *o*-chloro nitrobenzene.

2.2 *Methods*

Syntheses using Fmoc chemistry (Atherton and Sheppard 1989) were carried out in a Nalgene cryovial with dimensions 1.2 cm × 4 cm and of volume 1.2 ml with HMP resin, and in a glass tube of dimensions 6 cm × 1.2 cm and volume 6 ml with PAC-PEG-PS resin. Gentle stirring was done with a magnetic stirrer. The loading capacities of HMP resin was 0·8 mmol/gm while that of PAC-PEG-PS was 0·2 mmol/gm. The scale of synthesis was ~ 50 mg (0·04 mmol) when HMP resin was used and ~ 200 mg (0·04 mmol) with PAC-PEG-PS. The first amino acid was attached to the resin by the symmetric anhydride procedure (Atherton and Sheppard 1989). The protocols for the addition of subsequent amino acids are described in table 2. Coupling of Fmoc amino acids was mediated by HBTU/HOBt and DIEA. Subsequent to deprotection by piperidine (20% in DMF), it was ensured that no piperidine was present during the coupling step. The number of washes indicated in table 2 was sufficient for the complete removal of piperidine. At the end of the synthesis, the resin was washed thoroughly with DMF, isoamyl alcohol and ether and air dried. Deprotection from the resin was effected by TFA: phenol : H_2O thioansole: ethane dithiol (82·5:5:5:5:2·5 v/v) at room temperature for 5 h. The yields of crude peptides were ~ 90% by weight. The peptides

were analysed by FPLC on a pep RPC 5/5, reversed phase column (Pharmacia). The composition of peptides were confirmed by amino acid analysis on an LKB Alpha Plus amino acid analyser and liquid secondary ion mass spectrometry on a VG autospec–M mass spectrometer.

3. Results and discussion

The FPLC traces of 12–15 residue peptides synthesised on HMP resin by the protocol described in table 2 is shown in figure 1. While peptides 5 and 6 show heterogenity, the other peptides are reasonably pure. The major peak in all the cases showed an amino acid analysis characteristic of the peptide. The minor peaks in the FPLC traces of peptides 5 and 6 have one L-residue less than the parent peptides. The FPLC traces of peptides prepared on PAC-PEG-PS is shown in figure 2. It is evident that the crude peptides are of considerable purity. The total volumes of solvents consumed for each cycle in the present method were ~ 15 ml when HMP resin was used and 30 ml when PAC-PEG-PS resin was used. In LKB/NovaSyn semi-automated peptide synthesisers the volume of solvent used for each cycle is ~ 130 ml. In the Applied Biosystems Model 431 A peptide synthesiser the total volumes of solvents required for one cycle are 273 and 95 ml for synthesis on 0·25 and 0·1 mmol scales respectively. Thus, the volumes used in the present manual method are substantially less as compared to volumes used in semi-automated peptide synthesisers.

Although in synthesis on automated machines, constant attention is not required, unlike in the present method, the cost of Fmoc amino acids, which are pre-weighed and packed, are substantial as compared to bulk packing. The volumes of solvents required for the various steps are also considerable. In spite of significant efforts at optimisation of protocols that ensure relatively pure products even in the crude state, synthesis on such automated machines could conceivably yield impure peptides needing purification. In the present method, Fmoc amino acids used are from bulk packing from the manufacturers, and hence considerably less expensive than those that

Table 2. Protocols employed for the synthesis of peptides on HMP and PAC-PEG-PS resins.

		HMP resin (50 mg, 0·04 mmol)		PAC-PEG-PS resin (200 mg, 0·04 mmol)	
Step	Reaction	Volume	Time	Volume	Time
1	DMF wash, twice	2 ml	2′	4 ml	2′
2	Deprotection 20% piperidine in DMF	1 ml	20′	2 ml	20′
3	DMF, wash 5 times	5 ml	1′	10 ml	5′
4	Coupling	1 ml	60′	1 ml	60′
	Aminoacid + HBTU + HOBt + DIPEA in DMF (3 equivalents excess)				
5	DMF wash	5 × 1 ml	5′	10 ml	5′
6	Repeat steps 2–5				

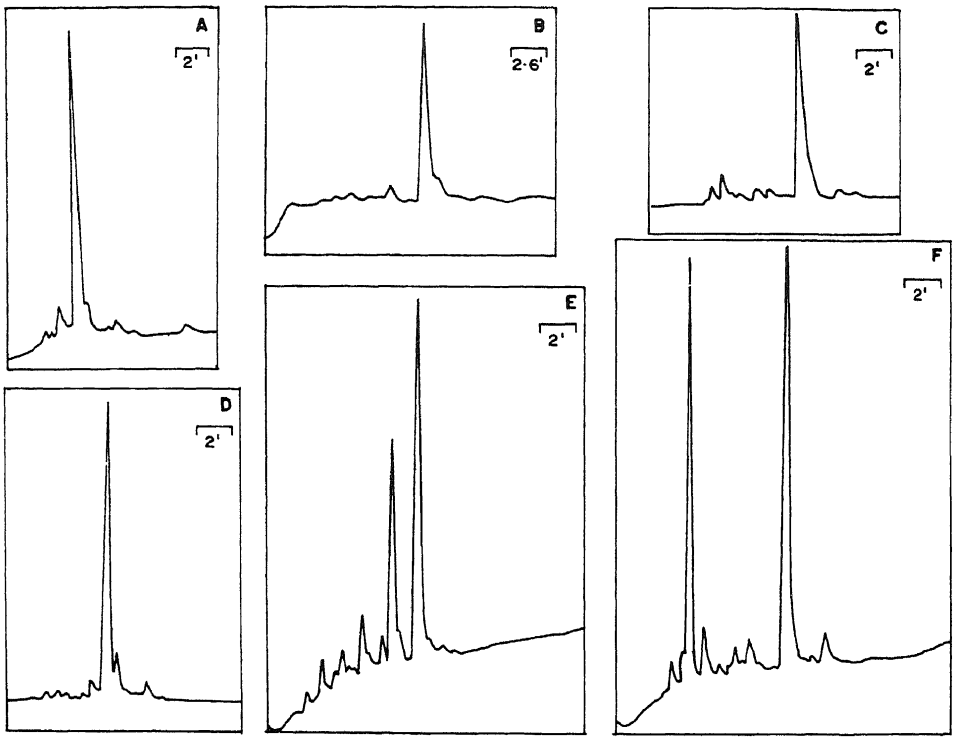

Figure 1. Analytical FPLC of peptides 1–6. Conditions: 30 min linear gradient 0–100% B at a flow rate of 0·5 ml/min through a Prep RPC 5/5 Pharmacia column. A – 0·1% TFA in water, B = 0·1% TFA in CH_3CN. Detection at 214 nm. (**A**) 1, (**B**) 2, (**C**) 3, (**D**) 4, (**E**) 5, and (**F**) 6.

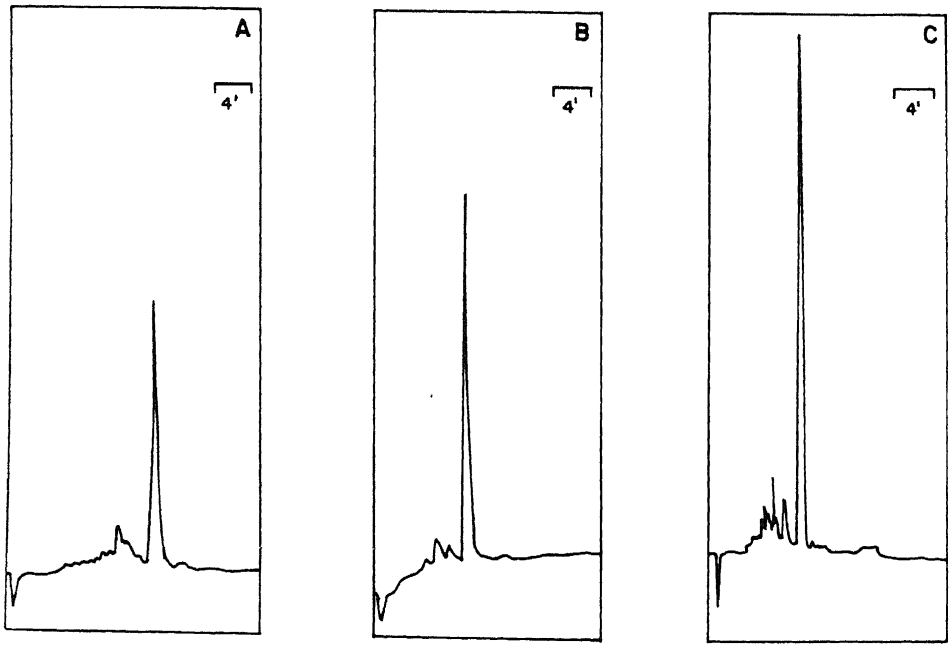

Figure 2. Analytical FPLC of peptide 7–9. Conditions as described in legend to figure 1. (**A**) 7, (**B**) 8, (**C**) 9.

are pre-weighed and packaged for automated synthesis. The solvent volumes are also less. Although the present synthesis has been restricted to only 13-residue peptides, it should be possible to synthesise longer peptides. The present method can also be adopted for multiple synthesis as well as combinatorial methods. The availability of high loading capacity resins permits the use of small amounts of resin ($\sim$ 50–200 mg) to obtain 0·05 mmols of peptide. The reagents (i.e. Fmoc amino acids and coupling reagents) to be used can be adjusted according to initial substitution. The method should be applicable to all polystyrene-based resins which are rigid and do not disintegrate on mild agitation.

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References

Atherton E and Sheppard R C 1989 In *Solid phase peptide synthesis: A practical approach* (Oxford: IRL)

Barany G and Merrifield R B 1980 In *The peptides: Analysis, synthesis biology* (eds) I Gross and J Meienhofer (New York: Academic Press) vol. 2 pp 3–285

Blondelle S E, Perez-Paya E, Dooley C T, Pinilla C and Houghten R A 1995 *Trends Anal. Chem.* **14** 83

Choma C T, Lear J P, Nelson M J, Dutton R L, Robertson D E and DeGrado W F 1994 *J. Am. Chem. Soc.* **116** 856

Eichler J and Houghten R A 1995 *Mol. Med. To-day* **1** 174

Erickson S W and Merrifield R B 1976 In *The proteins* (eds) H Neurath and R L Hills (New York: Academic Press) vol. 11, pp. 257–528

Finn F M and Hofmann K 1976 In *The proteins* (eds) H Neurath and R L Hills (New York: Academic Press) vol. 11, pp. 2–105

Fodor S P A, Leighton Read J, Pirrung M L, Stryer L, Tsai Lu A and Solas D 1991 *Science* **251** 767

Kent S B H 1988 *Ann. Rev. Biochem.* **57** 957

Liu C F, Rao C and Tam J P 1996 *J. Am. Chem. Soc.* **118** 307

Merrifield R B 1986 *Science* **232** 341

Millon de L R C, Milton S C F and Kent S B H 1992 *Science* **256** 1445

Sitaram N, Chandy M, Pillai V N R and Nagaraj R 1992 *Antimicrob. Agents Chemother.* **36** 2468

Sitaram N, Subbalakshmi C and Nagaraj R 1995 *Int. J. Pept. Protein Res.* **46** 166

Wlodawer A, Miller M, Jaskolski M, Sathyanarayana B K, Baldwin E, Weber I T, Selk L M, Clawson L, Schneider J and Kent S B H 1989 *Science* **245** 616