Journal of Peptide Science
J. Peptide Sci. **7**: 641–649 (2001)
DOI: 10.1002/psc.355

Solid Phase Synthesis of Hydrophobic Difficult Sequence Peptides on BDDMA-PS Support

P. K. AJIKUMAR and K. S. DEVAKY*

School of Chemical Sciences, Mahatma Gandhi University, Priyadarsini Hills, Kottayam 686 560, Kerala, India

Received 22 November 2000
Accepted 16 August 2001

Abstract: This article illustrates the successful and efficient solid phase assembly of hydrophobic difficult sequence peptides following both *t*-Boc and Fmoc chemistry. The peptides were synthesized on an optimized 1,4-butanediol dimethacrylate-crosslinked polystyrene support (BDDMA-PS). Four difficult sequence test peptides, VAVAG, VIVIG, QVGQVELG and VQAAIDYING, were synthesized in relatively good yield and purity without any aggregation problems. The peptides were assembled on chloromethylated and 4-hydroxymethylphenoxymethyl (HMP) BDDMA-PS resins. The peptides were fabricated using Boc amino acid 1-hydroxybenzotriazolyl and Fmoc amino acid pentafluorophenyl active esters in coupling reactions. The peptides after synthesis were cleaved from the polymeric support by exposing the peptidyl resin to 90% trifluroacetic acid/5% thioanisole/5% EDT mixture. The HPLC and MALDI TOF MS studies of the peptides revealed the high homogeneity of the synthesized peptides. Chloromethylated resin having a functional group loading of 1.14 mmol Cl/g was used for the synthesis. The yield and homogeneity of these peptides synthesized using the new support were high when compared with the conventional DVB-PS resin.

Keywords: hydrophobic difficult sequences; BDDMA-PS; solid phase; peptide synthesis

INTRODUCTION

Solid phase synthesis is a rapid, efficient and reliable methodology for the chemical synthesis of peptides and proteins [1]. The assembly of some peptide sequences classified as 'difficult sequences' posed crucial synthetic problems in SPPS [2]. The solubilization of the resino peptide and reactivity of the terminal amino groups were reported to be significantly reduced during synthesis of these peptides. This sequence dependent phenomenon is believed to be due to the consequence of interchain association involving hydrogen bonding of the secondary amide groups of the peptide chain leading to β sheet formation [3]. But this problem can be reduced with a fully solvated peptidyl polymer matrix. Several amphiphilic and flexible supports such as, tetraethyleneglycol diacrylate (TTEGDA) and hexanediol diacrylate (HDODA) crosslinked polystyrenes have already been reported [4–5]. The systematic investigations on swelling and reactivity studies revealed that a 2% BDDMA-crosslinked polystyrene is an ideal polymeric support for solid phase peptide synthesis [6]. The synthetic efficiency of this support was compared with the conventional DVB-PS resin [7]. Here we report the synthesis of four difficult sequence peptides having chronic aggregating tendency [8-9] using a 2% 1–4, butanediol dimethacrylate crosslinked polystyrene

Abbreviations: The nomenclature and symbols used according to the IUPAC-IUB Commission of Biochemical nomenclature (*Eur. J. Biochem.* 1984; **138**: 9–37; *J. Biol. Chem.* 1989; **264**: 663–674). BDDMA, 1,4-butanediol dimethacrylate; Boc, *t*-Butoxycarbonyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, dicyclohexylurea; DIEA, N,N-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulphoxide; Fmoc, 9-fluorenylmethoxycarbonyl; HDODA, 1,6-hexanediol diacrylate; HOBt; 1-hydroxybenzotriazole; HMP, 4-hydroxymethylphenoxymethyl; NMP, N-methylpyrrolidone; PS, polystyrene; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TTEGDA, tetraethyleneglycol diacrylate.

* Correspondence to: Dr K. S. Devaky, School of Chemical Sciences, Mahatma Gandhi University, Priyadarsini Hills, Kottayam 686 560, Kerala, India; e-mail: mgu@vsnl.com
Contract/grant sponsor: DST, New Delhi.
Contract/grant sponsor: CSIR, New Delhi.

support. A 10-residue peptide VQAAIDYING, a fragment of the acyl carrier protein 65–74 was synthesized by both Boc and Fmoc strategies for a comparative study. A detailed description of the synthesis and characterization of the peptides is given in the paper.

MATERIALS AND METHODS

Fmoc amino acid pentafluorophenyl ester and DMAP were purchased from Nova Biochem (UK). Boc amino acids were prepared by Schnabel's method [10]. Polyvinyl alcohol (PVA MW 72 000–100 000), 1,4-butanediol dimethacrylate (BDDMA), trifluroacetic acid (TFA), 4-hydroxymethylphenol and thioanisole were supplied by Aldrich Chemical Co., USA and styrene by Fluka AG, Switzerland. Chloromethyl-methyl ether (CMME) was prepared according to the literature [11]. AR grade solvents were used after distillation and purification according to the literature procedures. The solvent DMF was purified as per the literature procedure for peptide synthetic conditions. IR spectra were recorded on a Shimadzu IR 470 spectrophotometer using KBr pellets and the ^{13}C CP-MAS NMR measurements were carried out on a Bruker 300MSL CP-MAS instrument. HPLC was performed using semipreparative HP 1100 series HPLC using Vydac reverse phase C_{18} semipreparative column. The mass spectral analysis was carried out on a Kratos analytical MALDI time of flight mass spectrometer.

Preparation of BDDMA – Crosslinked Styrene Copolymer

A mixture of styrene (22.6 ml, 98 mol%), BDDMA (0.88 ml, 2 mol%), toluene (20 ml) and benzoyl peroxide (500 mg) was suspended in a 1% aqueous solution of polyvinyl alcohol (350 ml) and kept mechanically stirred at 600 rpm under a nitrogen atmosphere at a temperature of 85 °C. After 6 h the beaded resin was filtered and washed with hot water to remove PVA. It was then subjected to soxhlet extractions with acetone followed by methanol to remove all linear polymers and low molecular weight products. The beads were meshed to 200–400 range.

Chloromethylation of the BDDMA Crosslinked Styrene Copolymer

The dry resin (1 g) was swollen in DCM (10 ml), and to the swollen resin chloromethyl methyl ether (6 ml) and freshly prepared $ZnCl_2$ in THF (0.1 M, 0.3 ml) were added. The mixture was refluxed at 50 °C for 2.5 h with intermittent shaking. After reaction the resin was filtered, washed with THF, THF : H_2O (1 : 1), THF/2N HCl (1 : 1), hot water and finally with methanol. Further purification of the resin was done by soxhlet extraction with THF. The chlorine capacity was estimated by Volhard's titrimetric procedure and was found to be 1.14 mmol Cl/g.

Attachment of Boc-Gly-OH to the Chloromethylated Resin

Boc-Gly-OH (100 mg, 0.57 mmol) was dissolved in minimum amount of ethanol and the pH of the system was adjusted to between 7 and 8 by adding an aqueous solution of cesium carbonate. The solution was then co-evaporated with benzene and dried as the Boc-Gly-O-Cs salt powder and kept under vacuum over P_2O_5. The Cs-salt of Boc-Gly was then dissolved in minimum quantity of NMP (3 ml) and chloromethylated resin (200 mg, 0.228 mmol Cl) was added to this solution. The mixture was kept at 50°–60 °C for 32 h with occasional shaking. The resin was then filtered, washed with NMP, NMP : H_2O (1 : 1), methanol and DCM. The Boc amino acid appended resin was dried under vacuum. The amino group capacity was estimated by Gisin's picric acid method after deprotection of the Boc group and was found to be 1.08 mmol NH_2/g.

Preparation of 4-hydroxymethylphenoxymethyl BDDMA-PS Resin (HMP-BDDMA-PS)

The chloromethylated BDDMA-PS resin (1 g, 1.14 mmol Cl/g) was treated with DMA (15 ml), freshly recrystallized 4-hydroxymethylphenol (0.372 g, 3 mmol) and sodium methoxide (0.159 g, 3 mmol) at 50 °C for 20 h. HMP resin thus obtained was filtered and washed with dioxane (4 × 25 ml), dioxane/water (1 : 1 v/v, 4 × 25 ml), dioxane (4 × 25 ml), methanol (4 × 25 ml) and dried in vacuum. The extent of the reaction was measured by the determination of chlorine loss from the resin. The resin was subjected to pyridine fusion test and no residual chlorine was found. A quantitative result was obtained after 10 h. IR (KBr): 3380 (–OH stretching), 1280 (ether linkage).

Esterification of Fmoc-Gly to HMP-BDDMA-PS

Fmoc-Gly (345 mg, 1.16 mmol) was dissolved in dry DCM (3 ml) and a solution of DCC in dry DCM (120 mg, 1 ml, 0.58 mmol) was added. The mixture

was stirred for 30 min at 0 °C in dry conditions. The precipitated DCU was filtered off and the filtrate was rotaevaporated to dryness under reduced pressure. The residue was dissolved in minimum amount of DMF and the solution was added to preswelled 4-hydroxymethylphenoxymethyl BDDMA-PS resin (200 mg) in DMF. To the mixture DMAP (0.5 ml) was added and kept for 12 h under dry conditions with occasional stirring. The resin was thoroughly washed with DMF (10 × 5 ml). To protect any free –OH group, the resin was treated with acetic anhydride (1 ml) in DMF for 30 min. The resin was filtered and washed with DMF (5 × 5 ml), DCM (5 × 5 ml), methanol (5 × 5 ml) and dried under vacuum. The degree of incorporation of Gly was determined to be 0.96 mmol NH_2/g by spectrophotometric UV determination.

Synthesis of the Peptides VAVAG and VIVIG (1a and 2a)

For the synthesis of the peptide VAVAG (**1a**), Boc-Gly bound resin (100 mg, 0.108 mmol NH_2) was placed in a silanized glass reaction vessel clamped to a manually operated mechanical shaker. The Boc group was removed by treating with 30% TFA in DCM (4 ml, 30 min) followed by neutralization with 5% DIEA in DCM (4 ml, 2 × 5 min). The resin was then filtered and washed with DCM. The Kaiser test was performed to indicate free amino group and was found to be positive. The second amino acid, Boc-Ala-OH was coupled to the deprotected aminoacyl resin by the HOBt active ester method using NMP as the solvent. The subsequent amino acids were incorporated by repeating the same procedure deprotection, neutralization and coupling steps. For each coupling a three fold molar excess of the amino acid was taken. The weight of the peptidyl resin after synthesis was 130 mg. The same procedure was employed for the synthesis of peptide **2a** and the final weight of the peptidyl resin was 140 mg.

The free peptides **1a** and **2a** were obtained from the resin by suspending the peptidyl resin (100 mg) in a mixture of 95% TFA/5% thioanisole mixture (10 ml) for 8 h at room temperature. The mixture was filtered and rotaevaporated to reduce the volume of filtrate. The peptide was precipitated by adding cold ether. This precipitated peptide was centrifuged on an ultracentrifuge with repeated washing by ice cold ether until free of all contaminating agents. Finally the peptide was lyophilized with 10% acetic acid solution and

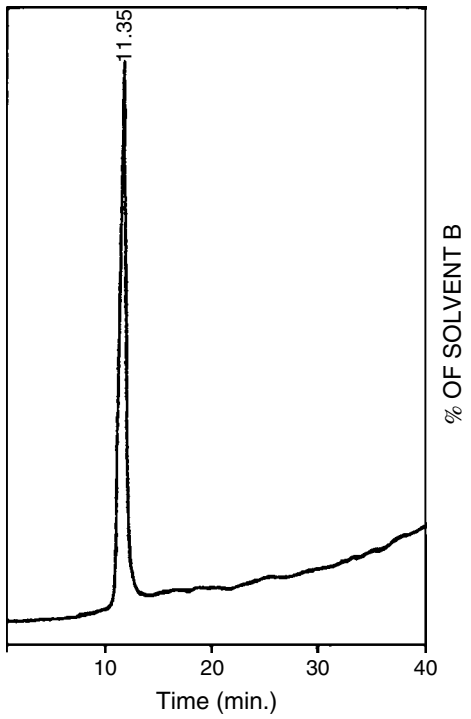

Figure 1 HPLC of the peptide **1a**. Conditions: Solvent A: water containing 0.1% TFA, Solvent B: 80% Acetonitrile containing 0.1% TFA. The gradient used was 25–50% B in 25 min, 95% B at 40 min. Flow rate 1.5 ml/min; detection 226 nm.

obtained as white powder. The purity of the crude peptide was checked by HPLC (Figures 1 and 2) and the parent peptides were confirmed by MALDI TOF mass spectral analysis. The MALDI TOF mass spectral analysis of the HPLC fraction collected of peptide **1a** showed m/z values at 415.5 [$(M+H)^+$], 436.9[$(M+Na)^+$], 452.8 [$(M+K)^+$] and peptide **1b** m/z 500.4 [$(M+H)^+$], 522.4[$(M+Na)^+$], 538.6 [$(M+K)^+$].

Synthesis of the Peptide VQAAIDYING (3a)

Boc synthesis. Boc-Gly appended resin (100 mg) was washed with DCM (5 × 2.5 ml) and the Boc group was removed by 30% TFA in DCM (4 ml, 30 min) followed by a neutralization with 5% DIEA in DCM (4 ml, 2 × 5 min). The subsequent amino acids were coupled to the deprotected resin by the HBTU/HOBt active ester method. The active esters of the amino acids were prepared by stirring the mixture of Boc amino acid, HBTU, HOBt (1 : 1 : 1, 3 equiv.) and DIEA (6 equiv.) in NMP for 10 min. The solution was added to the N^{α} deprotected peptidyl resin and agitated gently for 60 min, the solution was filtered and washed the resin with NMP (5 × 2.5 ml) and

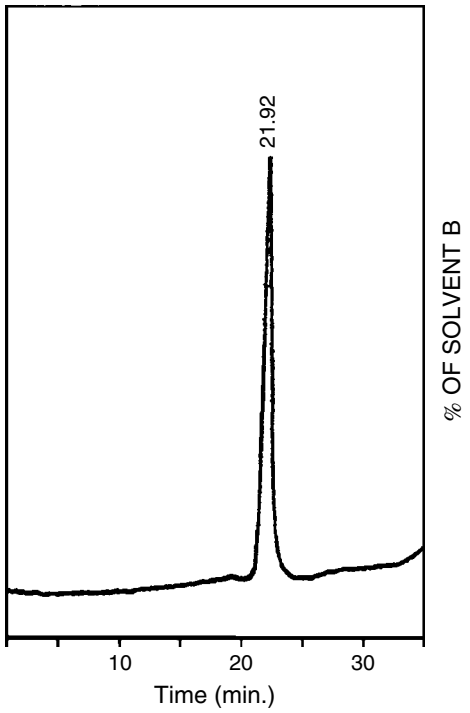

Figure 2 HPLC of peptide **2a**. Conditions: as for Figure 1.

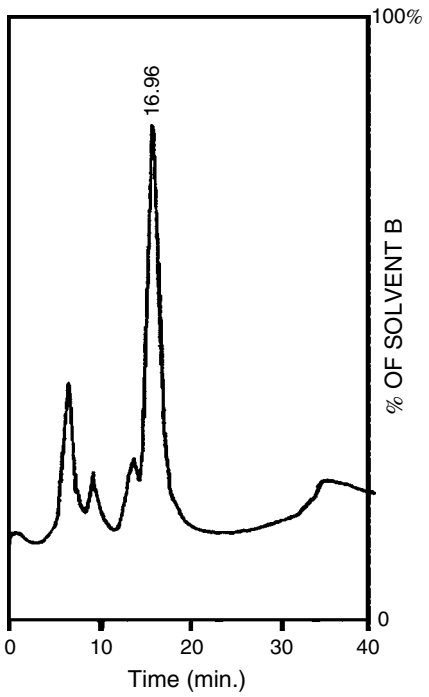

Figure 3 HPLC of peptide **3a** from Boc synthesis. Conditions: as for Figure 1.

DCM (5 × 2.5 ml). After the elongation of the peptide chain the resin was thoroughly washed with DCM (5 × 2.5 ml), methanol (5 × 2.5 ml) and dried under vacuum.

The peptide was cleaved from the resin by suspending the resin in 90% TFA/5% thioanizole/5% EDT mixture for 10 h. The resin was filtered off using a sintered funnel and the volume of the filtrate was reduced by rotaevaporation. To the residue ice cold ether (10 ml) was added to precipitate the peptide. The precipitated peptide was centrifuged on an ultracentrifuge with repeated washing using ice-cold ether. Finally the peptide was lyophilized with 10% acetic acid solution and obtained as white powder. The yield of the peptide obtained from 100 mg of peptidyl resin was found to be 45 mg. The purity of the peptide was checked by HPLC analysis (Figure 3) and the parent peptides authenticity was confirmed by ESI MS analysis; m/z value: 1063.5 $(M+H)^+$, 1064.5 $(M+2H)^+$.

Fmoc synthesis. The peptide synthesis was carried out in a silanized glass reaction vessel clamped to a manually operated mechanical shaker. The Fmoc-Gly appended resin (100 mg, 0.096 mmol NH_2) was allowed to swell in DMF for 30 min before starting peptide synthesis. Fmoc protection was removed by 20% piperidine in DMF (10 ml, 20 min). The second residue Asn in peptide **3a** was incorporated by agitating the deprotected resin with Fmoc-Asn-OPfp (165 mg, 0.318 mmol) and HOBt (43 mg, 0.318 mmol) for 60 min with DMF as the solvent. Three equivalents of Fmoc amino acid pentafluorophenyl ester were used for coupling reactions. All the subsequent amino acids were coupled by the same procedure. Fmoc cleavage and the extent of coupling reactions were monitored by the Kaiser test. After incorporating all amino acids, *N*-terminal Fmoc protection of the peptidyl resin was removed by agitating with 20% piperidine (10 ml) in DMF for 20 min. The peptidyl resin was washed with DMF (5 × 5 ml), DCM (5 × 5 ml), methanol (5 × 5 ml) and dried in vacuum. During the assembly of the peptide up to residue Glu^{66}, the Kaiser test was negative after 60 min. The final coupling reaction Val^{65}-Gln^{66} was done with an increased reaction time of 2 h and only after this was a negative Kaiser test observed. The final weight of the resin after assembly of the peptide was 170 mg.

Synthesis of the Peptide QVGQVELG (4a)

Peptide **4a** was assembled on HMP-BDDMA-PS resin using the Fmoc amino acid OPfp method. Fmoc-Gly attached resin (100 mg, 0.96 mmol NH_2) was used for the elongation of the peptide chain. All

the experimental procedures were the same as in the synthesis of peptide **3a**. All the couplings were undertaken without any difficulty and the Kaiser test was negative after 30 min at each acylation step. The final weight of the resin after assembly of the peptide was 150 mg.

Cleavage of the Peptides from the HMP-BDDMA-PS Support

The peptidyl resin (100 mg) was suspended in a mixture of 90% TFA/5% thioanisole/5% EDT mixture (10 ml) for 5 h at room temperature. The mixture was filtered and the volume of the filtrate was reduced by rotaevaporation. The peptide was precipitated by adding ice-cold ether. This precipitated peptide was centrifuged on an ultracentrifuge with repeated washing using ice-cold ether. Finally the peptide was lyophilized with 10% acetic acid solution and obtained as white powder. The yield of the crude peptides **3a** and **4a** were 51 mg and 46 mg, respectively. The purity of the crude peptides were checked by HPLC (Figures 4 and 5) and the parent peptides were confirmed by MALDI TOF mass spectral analysis (Figures 6 and 7).

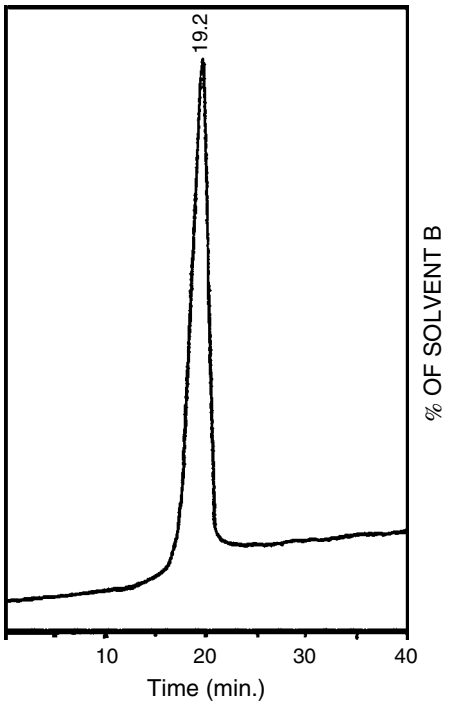

Figure 5 HPLC of peptide **4a**. Conditions: as for Figure 1.

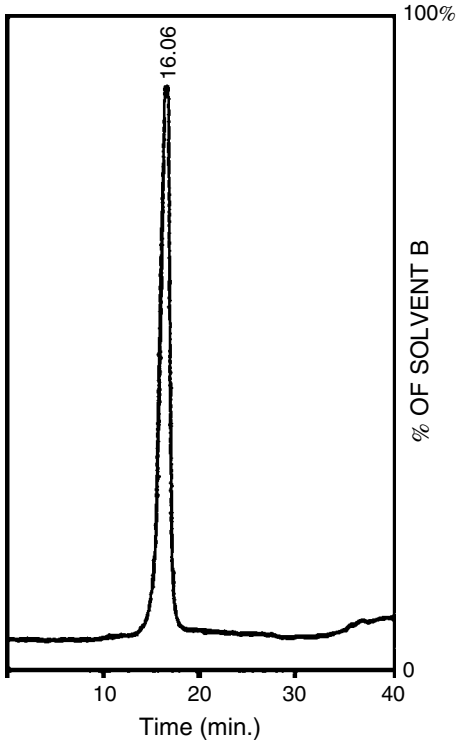

Figure 4 HPLC of peptide **3a** from Fmoc synthesis. Conditions: as for Figure 1.

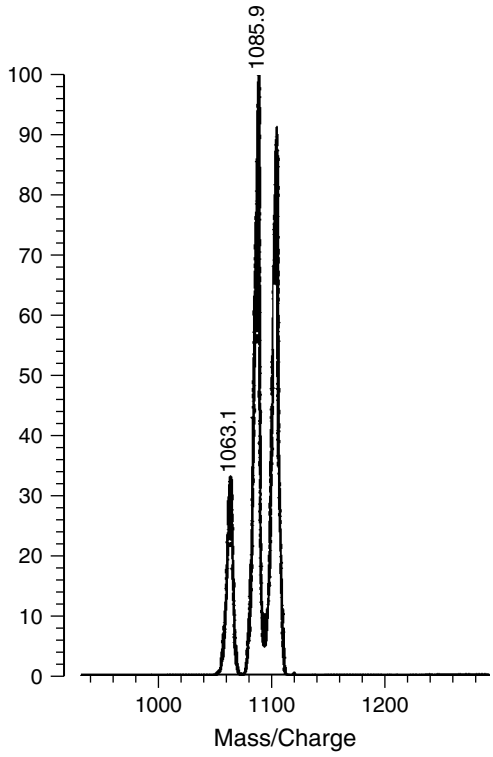

Figure 6 MALDI TOF mass spectrum of the HPLC fraction of peptide **3a**.

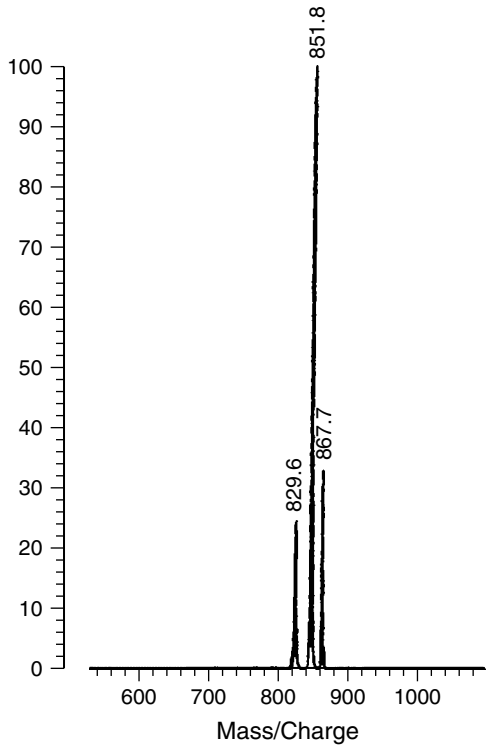

Figure 7 MALDI TOF mass spectrum of the HPLC fraction of peptide **4a**.

RESULTS AND DISCUSSION

Preparation and Functionalization of BDDMA-PS Resin

The BDDMA-crosslinked polystyrene supports were prepared by the radical initiated suspension polymerization of the monomers in toluene, at 85 °C [6]. Systematic studies on synthesis, characterization and optimization of the polymer revealed that a 2% crosslinked resin is suitable for peptide synthesis. The swelling and reactivity studies revealed that the polar flexible crosslinking makes the polymeric backbone more compatible with the solvation properties of the growing peptide chain [7]. The polymer was functionalized via Friedel Crafts chloromethylation using chloromethyl methyl ether in the presence of dry $ZnCl_2$ in THF as catalyst at 50 °C. The capacity of the resin was determined using Volhard's titrimetric method [12] and was found to be 1.14 mmol Cl/g resin. To improve the purity of the final peptide and the rate of release from the resin we introduced the 4-hydroxymethylphenoxymethyl (HMP) linker to the BDDMA-PS resin. The HMP linker was attached to the resin by treating chloromethylated resin with 4-hydroxymethylphenol using $NaOCH_3$ in DMA at 50 °C [13]. The HMP linker attached BDDMA-PS resin was characterized by IR spectroscopy (–OH stretching vibrations at 3380 cm^{-1} and the ether linkage at 1280 cm^{-1}). The hydroxyl group capacity was determined by the esterification of Fmoc-Gly using preformed symmetric anhydride in the presence of DMAP and the extent of Gly incorporation was calculated by the standard spectrophotometric measurements. The observed result was quantitative with an amino group capacity of 0.96 mmol NH_2/g.

Synthesis of Peptides 1a and 2a

For the synthesis of peptides **1a** and **2a**, the *C*-terminal amino acid Boc-Gly-OH was appended as benzyl ester to the chloromethylated 2% BDDMA-PS resin (1.14 mmol Cl/g) by the cesium salt method. The Boc deprotection was performed by treatment with 30% TFA in DCM followed by neutralization with 5% DIEA in DCM. The peptide chain was built by sequentially coupling the successive amino acids towards the amino terminus by the manual solid phase method using Boc amino acid strategy [14]. The synthesis was carried out by the HOBt active ester coupling method. In each step, HOBt active ester of the Boc amino acid was employed and NMP was used as the solvent. The extent of Boc deprotection and amino acylation reactions were monitored by Kaiser's test [15]. A three fold molar excess of Boc amino acid was used for the coupling reaction. At the end of the synthesis, the resin was washed thoroughly with methanol DCM mixture (33 : 67), DCM and methanol and dried under vacuum. The peptides were cleaved from the resin by 95% TFA/ 5% thioanisole. The yield of the crude peptides obtained from 100 mg of the peptidyl resins were 93% and 94%, respectively.

The purity of each peptide was checked by HPLC and in each case only one major peak was obtained (Figures 1 and 2). The parent peptide was confirmed by MALDI TOF MS. The MALDI TOF mass spectral analysis of the HPLC fraction collected of peptide **1a** gave m/z values, 415.5 [$(M+H)^+$], 436.9 [$(M+Na)^+$], 452.8 [$(M+K)^+$] and peptide **2a** m/z 500.4 [$(M+H)^+$], 522.4 [$(M+Na)^+$], 538.6 [$(M+K)^+$].

Synthesis of the Peptides 3a and 4a

The 10 residue peptide, VQAAIDYING (**3a**), a fragment of the acyl carrier protein is a classic difficult sequence peptide that has been used to assess the performance of new coupling procedures,

additives, resin supports, backbone protection etc. The major difficulty during the assembly of this peptide was the strong interchain association after the addition of the penultimate glutamate residue leading to incomplete N^{α} deprotection and coupling with the final ^{65}Val to the sequence. Here we attempted the solid phase synthesis of this peptide by Boc and Fmoc N^{α}-protecting strategies using 2% BDDMA-PS support having a loading capacity of 1.14 mmol Cl/g.

First we tried the synthesis of this peptide on Boc-Gly scaffolded BDDMA-PS resin (1.08 mmol NH_2/g) using the HBTU/HOBt coupling method. The Boc α-amino protection was removed by agitating the resin with 30% TFA in DCM for 30 min followed by neutralization using 5% DIEA in DCM. After washing with DCM, the NMP solution of the next amino acid Boc-Asn-OH/HBTU/HOBt (1 : 1 : 1) was added and agitated for 60 min. A three fold molar excess of the reagents was used for the acylation reactions. After 30 min, the extent of the coupling reaction was monitored by the Kaiser test and found to be negative. The successive amino acids were incorporated by following the same procedure of deprotection and coupling steps. During the acylation of the residues Asp, Ile and Val the Kaiser test was positive after 30 min coupling but in the case of Asp and Ile, the reaction was complete after 1 h reaction time. The most difficult coupling between Val^{65} to Gln^{66} was not complete even after an extended reaction time of 2 h. The beads gave faint blue colour with the Kaiser reagents. Capping was not given at any stages of difficult couplings. After the assembly of the peptide, 100 mg of the peptidyl resin was treated with 90% TFA/5% thioanisole/5% EDT mixture for 10 h to obtain the free peptide. The purity of the crude peptide was checked by HPLC (Figure 3) and was confirmed by ESI MS analysis; m/z value: 1063.5 $(M+H)^+$, 1064.5 $(M+2H)^+$.

Peptide **3a** was fabricated by the milder Fmoc chemistry also using the Fmoc amino acid OPfp/HOBt coupling method. For an optimal condition for a deletion free homogeneous product peptide, the judicious selection of resin support is very important. The so-called Wang resin, 4-hydroxymethylphenoxymethyl linker attached polystyrene support with a combination of milder acid cleavage was proved to be very effective in SPPS. To improve the purity of the final peptide and the rate of release from the resin, the 4-hydroxymethylphenoxymethyl linker was introduced to the BDDMA-PS resin.

The *C*-terminal residue, Gly, was esterified to the HMP-BDDMA-PS resin using preformed symmetric anhydride in the presence of DMAP and the extent of esterification reaction was calculated by the standard spectrophotometric methods [16]. A quantitative result was obtained after 12 h of reaction and the amino capacity was estimated to be 0.96 mmol NH_2/g. The unreacted hydroxyl groups if any were acetylated to avoid unwanted side reactions during synthesis.

The Fmoc-Gly incorporated HMP-BDDMA-PS resin (100 mg, 0.096 mmol NH_2) was allowed to swell in DMF for 30 min before starting the peptide synthesis. The Fmoc group was removed by treating with 20% piperidine in DMF for 20 min. The successive peptide bond forming reactions were carried out with the Fmoc amino acid pentafluorophenyl ester/HOBt (1 : 1) coupling method. Three equivalents of Fmoc amino acid active esters were used in all chain extension acylations. The extents of deprotection and acylation reactions were monitored by the Kaiser test.

Here in the assembly of the peptide, up to the 9th residue Gln^{66}, all acylations were complete after a single coupling of 60 min. Coupling of the last residue Val^{65} with Gln^{66} was given an increased reaction period of 2 h and after this a negative Kaiser test was observed. After the cleavage of final Fmoc protecting group, the peptidyl resin was thoroughly washed with DMF, DCM, methanol and dried under vacuum.

The completed peptide was deprotected from the resin using 90% TFA/5% thioanisole/5% EDT mixture. A trial deprotection was performed with 10 mg peptidyl resin, in order to identify any undesired side reactions. No considerable side reactions were observed within a period of 5 h for the peptide synthesized by Fmoc chemistry using a more acid labile HMP linker attached resin. But for a quantitative deprotection of the peptide assembled by the Boc chemistry the TFA treatment was extended to 10 h. The yield of the crude peptide from Boc and Fmoc synthesis were 86% and 91%, respectively. The purity of the crude peptides obtained was checked by HPLC. From the HPLC profiles of the crude peptides from Fmoc (Figure 4) and Boc syntheses (Figure 3), it was found that the former contained only one major peak. But the purity of the peptide from the Boc synthesis was less compared with the Fmoc synthesis, which may be due to the accumulation of impurities by the incomplete acylation and deprotections during the assembly and also by the increased TFA exposure

during the final cleavage of the peptide. Peptide **4a** was also assembled by the Fmoc chemistry using the same procedure employed in the synthesis of peptide **3a**. Here all the acylations were straightforward without any coupling difficulty. The yield of the peptide obtained was 93%. The HPLC profile of the crude peptide **4a** showed only one major peak (Figure 5). The peak fraction was collected and the peptides were confirmed by MALDI TOF mass spectral analysis (Figures 6 and 7). Peptide **3a** gave m/z values 1063.1 [$(M+H)^+$], 1085.9 [$(M+Na)^+$], 1101.3 [$(M+K)^+$] and peptide **4a**, m/z values 829.6 [$(M+H)^+$], 851.8 [$(M+Na)^+$], 867.7 [$(M+K)^+$].

The exceptionally sluggish peptide bond formation of Val^{65}-Gln^{66} in peptide **3a** has already been reported and this constitutes formation of deletion peptides. Even though the Fmoc group plays the role of facilitated nucleation of β sheet formation, the excellent purity of the synthesized peptide showed the efficiency of peptide synthesis on a high capacity 2% BDDMA-PS resin. All the amino acylations were complete after a single coupling itself and the use of 3 molar excess reagents with a single coupling revealed a cost reduced Fmoc peptide synthesis on BDDMA-PS. The improved swelling of the resino peptide and the suppression of the aggregation of peptide chains can be attributed to the optimum physicochemical characteristics of the polymer support. The studies proved that the support can be used for an efficient assembly of chronic difficult sequence peptide.

For the past 30 years, the phenomenon of difficult sequence continues to be one of the most troublesome aspects of contemporary SPPS [2,17]. In the solid phase synthesis of difficult sequence peptides using both Boc and Fmoc chemistries, there is considerable evidence that aggregation of the resino peptide can lead to low coupling yields and an accumulation of single amino acid deletion impurities which are difficult to separate from the target peptide. Two general strategies were successfully used to overcome the coupling difficulties due to interchain interactions in the synthesis of β aggregating peptides. The first involves the use of physical methods including the use of elevated temperatures, solvent mixtures containing trifluoroethanol, dimethylsulphoxide, hexafluoroisopropanol and potent β disaggregating additives of chaotropic salts such as LiBr, KSCN and $NaClO_4$ [18]. The second strategy uses the chemical means involving the replacement of a secondary amide bond by a reversible tertiary bond, thus eliminating the amide hydrogen that forms the interchain hydrogen bonding network [17,19]. One more factor, which minimizes these limiting factors, was the use of low loading resins. The efficient synthesis of a chronic difficult sequence like ACP 65-74 using HDODA-PS resin has been recently reported [20]. Here it is believed that the interaction between the crosslinker and NH of the growing peptide suppressed the tendency of peptide to acquire β sheet structure, which can lead to improved synthetic efficiency without any additives or introducing tertiary amide bonds. The 2% BDDMA crosslinked polystyrene also showed such optimum characteristics. In the present investigations of the synthesis of the well known test sequence peptide ACP 65-74, the most difficult coupling between Val and Gln was completed after a total reaction time of 120 min on a BDDMA-PS resin with relatively high loading capacity. The HPLC and mass spectral studies showed the absence of desvaline peptide usually observed in this synthesis.

The successful solid phase synthesis of these highly aggregating difficult sequence peptides on a moderate loading level (BDDMA-PS with 1.14 mmol Cl/g) by both Boc and Fmoc chemistries and extremely high homogeneity of the product peptide proves the synthetic efficiency of this novel polymeric support. The synthesis could be achieved in a cost-effective manner by using small amounts of resin with high functional group loading, thereby minimizing the quantity of solvents employed. Further, complete coupling of amino acids could be achieved in a single coupling step.

CONCLUSION

The solid phase syntheses of hydrophobic difficult sequence peptides were carried out. The peptides were synthesized with high purity and homogeneity and the yields were relatively high. The synthesis could be achieved in a cost-effective manner by using a small amount of resin with high functional group loading, thereby minimizing the quantity of solvents employed. Further, complete coupling of amino acids could be achieved in a single coupling step. The studies led to a convenient and cost effective method of manual solid phase synthesis of peptides using both Boc and Fmoc chemistry on a 2% BDDMA-PS.

Acknowledgements

The authors thank DST, New Delhi for financial support (KSD) and CSIR, New Delhi for the award of

a Senior Research Fellowship (PKA). Thanks are due to Professor P. Balaram, Molecular Biophysics Unit, Indian Institute of Sciences, Banglore for providing the laboratory facilities.

REFERENCES

1. Fields GB. Solid phase peptide synthesis. *Methods Enzymol.* 1997; **289**: 3–336.
2. Quibell M, Johnson T. *Difficult Peptides: Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Chan WC, White PD (eds). Oxford University Press: New York, 2000; 115–117.
3. Hyde C, Johnson T, Owen D, Quibell M, Sheppard RC. Some difficult sequences made easy: A study of interchain association in solid phase peptide synthesis. *Int. J. Peptide Protein Res.* 1993; **43**: 431–440.
4. Renil M, Pillai VNR. Synthesis, characterisation and application of tetraethyleneglycol dimethacrylate crosslinked polystyrene support for gel phase peptide synthesis. *J. Appl. Polym. Sci.* 1996; **61**: 1885–1894.
5. Arunan C, Pillai VNR. Solid phase synthesis of hydrophobic peptides on 1,6-hexanediol diacrylate-crosslinked polystyrene resin: Comparison with Merrifield resin. *Protein Peptide Lett.* 1999; **6**: 391–398.
6. Ajikumar PK, Devaky KS. Solid phase synthesis of diazepam binding inhibitor fragments 32–38 and 45–50 on a flexible butanediol dimethacrylate crosslinked polystyrene support. *Indian J. Chem. Section B* 1999; **38**: 783–788.
7. Ajikumar PK, Devaky KS. Optimization of butanediol dimethacrylate crosslinked polystyrene: a novel polymeric support for solid phase peptide synthesis. *Lett. Peptide Sci.* 2000; **7**: 207–215.
8. Narita M, Isokawa S, Honda S, Umeyama H, Kakei H, Obana S. Individuality of amino acid residues in protected peptides. *Bull. Chem. Soc. Jpn.* 1989; **62**: 773–779.
9. Bedford T, Hyde C, Johnson T, Jun W, Owen W, Quibell M, Sheppard RC. Amino acid structure and difficult sequences in solid phase peptide synthesis. *Int. J. Peptide Protein Res.* 1992; **40**: 300–307.
10. Schnabel E. Improved synthesis of t-butyl carbonyl amino acids by a constant pH reaction. *Justus Leibigs Ann. Chem.* 1967; **702**: 188–190.
11. Marvel CS, Porter PK. *Organic Synthesis Collective* Volume 1. John Wiley & Sons: New York, 1967; 377.
12. *Vogel's Text Book of Quantitative Inorganic Analysis*, 4th edn. Longman Group: Essex, 1978; 342.
13. Lu G, Mojosov S, Tam JP, Merrifield RB. Improved synthesis of p-alkoxybenzyl alcohol resin. *J. Org. Chem.* 1981; **46**: 3433–3436.
14. Barany G, Merrifield RB. Solid phase peptide synthesis. *Peptides*, Gross E, Meienhofer J (eds). Academic Press: New York, 1979; **2**: 1–284.
15. Kaiser E, Colescott RC, Bossinger CD, Cook PI. Colour test the detection of free terminal amino groups in the solid phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
16. Chan WC, White PD. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach.* Oxford University Press: New York, 2000; 41–76.
17. Miranda LP, Meutermans WDF, Smythe ML, Alewood PF. An activated O→N acyl transfer auxiliary: efficient amide-backbone substitution of hindered 'difficult' peptides. *J. Org. Chem.* 2000; **65**: 5460–5468.
18. Penninggton MW, Byrnes ME. Procedures to improve difficult couplings. *Methods Mol. Biol.* 1994; **35**: 1–16 and ref. cited theirin.
19. Johnson T, Quibell M, Owen D, Sheppard RC. A reversible protecting group for amide bond in peptides: Use in the synthesis of 'difficult sequences'. *J. Chem. Soc. Chem. Commun.* 1993; 369–372.
20. Arunan C, Pillai VNR. Synthesis of acyl carrier protein fragment 65–74 on a flexible crosslinked polystyrene support: comparison with Merrifield resin. *Tetrahedron* 2000; **56**: 3005–3011.

