Journal of Peptide Science
J. Peptide Sci. **8**: 183–191 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.379

An Efficient Gel-Phase Synthesis of Peptides on a High Capacity Flexible Crosslinked Polystyrene Support: Comparison with Merrifield Resin

I. M. KRISHNA KUMAR, V. N. RAJASEKHARAN PILLAI and BEENA MATHEW*

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

Received 24 December 2001
Accepted 6 January 2002

Abstract: A highly solvating copolymer was prepared in high yield by introducing a flexible crosslinker, 1,4-butanedioldimethacrylate, into the polystyrene matrix by a free radical aqueous suspension polymerization. A 2 mol% crosslinked resin showed rigidity and mechanical characteristics comparable to those of divinylbenzene-crosslinked polystyrene (Merrifield resin, DVB-PS) support. Swelling and solvation characteristics of the new resin, BDDMA-PS, were much higher than DVB-PS support in all solvents used for solid phase peptide synthesis. The diacrylate crosslinks in the resin network were found to be highly stable even after 48 h treatment with neat TFA, 6 N HCl and 6 N KOH at 110 °C. To demonstrate the usefulness of the new resin in high capacity peptide synthesis, a typical difficult peptide, acyl carrier protein (ACP) fragment (65–74), was synthesized on commercially available 1 mol% crosslinked DVB-PS and 2 mol% crosslinked BDDMA-PS resins under identical conditions. A protocol using NMP/DMSO mediated coupling was employed for chain assembly. The yield and purity of the product from BDDMA-PS resin was higher than when the DVB-PS resin was used. The mechanistic reason behind the synthetic efficiency of the new resin was found to be its ability to induce random coil conformation to the growing peptide chains.

Keywords: solid-phase peptide synthesis; divinylbenzene-crosslinked polystyrene; flexible supports; difficult sequences; coupling difficulty; β-sheet structure

INTRODUCTION

Chemical synthesis is the method of choice for generating biologically active peptides and their analogues for the rapid screening of biological functions. The improvements and developments made in the Merrifield's solid-phase methodology and the commercial availability of the resins and automated peptide synthesizers have helped in replacing conventional solution phase methodology in many laboratories. The reagents and protocols for solid-phase technique have been well optimized to produce even the crude products with considerable purity. Despite these achievements in SPPS, a class of relatively short peptides have emerged as difficult sequences, which are resistant to easy synthesis owing to the sudden decrease of

Abbreviations: Abbreviations used for amino acids follow the IUPAC-IUB Commission of Biochemical Nomenclature, (*Eur. J. Biochem.* 1984; **138:** 937). ACP, acyl carrier protein; BDDMA, 1,4-butanediol dimethacrylate; BDDMA-PS, 2 mol% butanediol dimethacrylate-crosslinked polystyrene; Boc, tert-butyloxycarbonyl; CMME, chloromethyl methyl ether; CP, cross-polarized; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMSO, dimethylsulphoxide; DVB, divinylbenzene; DVB-PS, 1 mol% divinylbenzene-crosslinked polystyrene; ESI-MS, electrospray ionization mass spectrometry; FTIR, fourier transform infrared spectroscopy; HOBt, 1-hydroxybenzotriazole; MAS, magic angle spin; NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography.

* Correspondence to: Dr B. Mathew School of Chemical Sciences, Mahatma Gandhi University, Kottayam, Kerala, India-686 560; e-mail: mgu@md2.vsnl.net.in
Contract/grant sponsor: University Grants Commission, New Delhi.

reactivity of the resin bound peptides at various stages of synthesis [1–5]. The mechanistic reason behind the synthetic difficulty was recognized as the steric occlusion of the growing peptide chains within the polymeric network [3]. The steric effect is caused either by the physicochemical incompatibility of the growing peptide chains with the polymeric support [6] and/or by the formation of rigid secondary structures, especially β-sheet structure, by the pendant peptide chains [1]. The latter is the most serious difficulty which is highly sequence specific and irrespective of the resin type or strategy [2,3]. Although DVB-PS support has been widely used in SPPS with considerable success, its influence on the mass transport of reagents, solvation and swelling characteristics of the polymer as well as on the peptide are mostly negative due to the strong hydrophobic macromolecular environment of the resin [6]. Thus a support developed for peptide synthesis must be capable of suppressing the β-sheet forming tendency of the growing peptides and be compatible with the peptides towards all solvents used for synthesis.

Among the various supports developed to address these problems in SPPS, styrene- based supports showed high mechanical and chemical stability towards the various reagents and solvents used for peptide synthesis. The recognition that supports with hydrophobic–hydrophilic balance swell well in both polar and non-polar solvents and hence enhance the coupling and deprotection rate by effectively solvating the reactive species, led to the selection of flexible and polar crosslinking agents instead of rigid and hydrophobic DVB [7,8]. With this in mind, a 2 mol% crosslinked BDDMA-PS copolymer was prepared by suspension polymerization. In order to establish its applicability in high capacity synthesis, a test peptide, acyl carrier protein (65–74) fragment, Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly, was synthesized in high yield and excellent purity. This is a typical difficult sequence and its actual synthesis was reported to be very difficult due to the internal aggregation of the peptide, especially after the deprotection of the 9th amino acid residue [5]. So the attachment of the 10th amino acid, valine, has become very difficult and hence the target peptide is often associated with deletion sequences, which are very difficult to separate and characterize [4]. NMR studies of Merrifield resin carrying this peptide revealed that the segmental mobility of the polystyrene backbone is markedly decreased due to the β-sheet formation [3]. So many groups of peptide chemists have often used this sequence as a test peptide to check the validity of their support, strategy or reagent in peptide synthesis.

The present paper describes the synthetic results of ACP (65–74) fragment on both BDDMA-PS and DVB-PS resins under same experimental conditions. Coupling difficulty, yield and purity of the peptide products obtained from both supports were compared to establish the efficiency of the BDDMA-PS support. Solid state FTIR spectroscopy was used to probe the possible causes of the high synthetic efficiency of BDDMA-PS supports in terms of the conformational behaviour of the resin bound peptides.

MATERIALS AND METHODS

General

Styrene, BDDMA, 1% DVB-PS (200–400 mesh), TFA, thioanisol, ethanedithiol and *m*-cresol were obtained from Aldrich Chemical Company, USA. All side chain protected amino acids, HOBt and DIEA were purchased from Sigma Chemical Company, USA. All solvents were of commercial grade and purified before use. IR spectra were recorded on a Bruker IFS-55 spectrophotometer in the solid state, using KBr pellets. Pellets were prepared by grinding the polymer with KBr in the ratio 1 : 100 for 5 min under constant pressure. A solid-state ^{13}C-CP-MAS-NMR spectrum was recorded on a Bruker 300 MSL instrument operating at 75.47 MHz. HPLC analysis was conducted on a Shimadzu Model 6A instrument fitted with a UV/Vis spectrophotometric detector. A reverse phase C_{18} column (Bondapack, 4.6×250 mm, 5 μm) was used for the analysis using the binary solvent system (0.1% TFA containing acetonitrile and water). Amino acid analyses were performed on an LKB 4151 Alpha Plus amino acid analyser using *o*-phthalaldehyde detection. A micromass Quattro II triple quadrupole mass spectrometer was used for recording the electrospray ionization mass spectrum.

Preparation of BDDMA-PS Support

Styrene and BDDMA were washed with 1% NaOH solution (2×50 ml) followed by distilled water (3×30 ml) and dried over anhydrous Na_2SO_4 to remove the inhibitors. 11.22 ml of styrene, 0.44 ml of BDDMA and 500 mg dibenzoyl peroxide were dissolved in 20 ml toluene. The mixture was added to

175 ml of 1% polyvinyl alcohol$_{75000}$ solution taken in the reaction vessel equipped with a stirrer, water condenser and nitrogen inlet and kept at 85 °C on a water-bath. The mixture was stirred at 1500 rpm for 15 h and the precipitated copolymer was filtered, washed with hot water to remove the stabilizer, acetone (3 × 50 ml), toluene (3 × 50 ml) and methanol (3 × 50 ml). Finally the resin was soxhlet extracted using toluene, acetone, dichloromethane and methanol. The polymer beads were dried under vacuum at 45 °C. The yield was 9.2 g. The beads were sieved and 100–200 mesh sizes were used for peptide synthesis. IR(KBr) : 1720, 1490 cm^{-1} (ester); 700, 3010 cm^{-1} (aromatic); 2910, 2850 cm^{-1} (CH_2 str.)

Chloromethylation of BDDMA-PS and DVB-PS Resins

The dry resin (1 g) was swelled in DCM (15 ml) for 1 h and then 6 ml CMME, 0.2 ml, 1 M anhydrous zinc chloride in THF were added and kept at 50 °C. DVB-PS resin was kept for 12 h and BDDM-PS resin for 3 h. The resin was filtered and washed with THF (10 ml × 3, 3 min), THF/4 N HCl (10 ml × 3 × 3 min), THF/water (1 : 1 v/v, 10 ml × 3 × 3 min), water (20 ml × 5 × 3 min) and finally with methanol (3 × 30 ml). The resin was then soxhlet extracted with THF and dried under vacuum. Chlorine capacity was estimated by the Volhard method [9]. Chlorine capacity: 2.36 mmol/g for BDDMA-PS and 2.28 mmol/g for DVB-PS. IR(KBr) : 1254, 690 cm^{-1}(CH_2-Cl).

Attachment of *C*-terminal Glycine on the Support – General Procedure

Boc glycine (10 mmol excess of chlorine capacity) was dissolved in a minimum quantity of ethanol and the pH was adjusted to 7.0 with a saturated solution of Cs_2CO_3 and kept for 1 h with stirring. Then the solvents were rotary evaporated by azeotropic distillation with dry benzene. A white powder of Cs-salt of Boc glycine was thus obtained and dissolved in NMP and chloromethylated resin was added. The reaction mixture was kept at 50 °C for 24 h in the case of BDDMA-PS and 40 h for DVB-PS resins. The resins were washed with DMF (3 × 30 ml), DMF/H_2O (1 : 1 v/v, 3 × 30 ml), DMF (5 × 30 ml), DCM (3 × 30 ml) and finally with methanol and dried under vacuum. The amino acid substitution level was then determined by the picric acid method [9]. The amino capacity was 2.20 mmol glycine/g for BDDMA-PS and 2.09 mmol glycine/g for DVB-PS.

Peptide Synthesis – General Procedure

Synthesis was performed manually on a silanized reaction vessel, using Boc/Bzl tactics. 100 mg of Boc glycine attached BDDMA-PS resin was taken and swelled in DCM for 30 min. 5 ml of 30% TFA/DCM (5 ml × 30 min) was used for the removal of the Boc group. For neutralization, 5 ml of 10% DIEA in DCM (5 min) was used. The resin was washed well with DCM (4 × 10 ml), NMP (3 × 10 ml) and then 2.5 mmol excess of the HOBt active ester of the next amino acid (asparagine) in NMP containing 10% v/v of DMSO was added and shaken for 60 min. The active ester was prepared by mixing Boc amino acids, DCC, and HOBt in 1 : 1 : 1 molar ratio in DMF under stirring for 20 min at 0 °C. The precipitated DCU was filtered to obtain the active ester. 2.5% v/v of DIEA was added and again shaken for 5 min. The resin was then washed with 30% MeOH/DCM (3 × 5 ml) and then with DCM (5 × 5 ml). The progress of the coupling step was monitored by picking up a single bead from the reaction mixture and performing the Kaiser test [10]. If positive, the next coupling was repeated in the same manner until the test was negative. This cycle of operation was repeated for the stepwise incorporation of the remaining amino acids. Finally the peptidyl resins were washed with NMP (6 × 10 ml), DCM (6 × 10 ml), methanol (6 × 10 ml) and ether (6 × 10 ml). The same protocol was adopted to assemble the peptide chain on the DVB-PS support. The weight of the peptidyl resin was DVB-PS (195 mg); BDDMA-PS (280 mg).

The completed peptide was cleaved from the support by the TFA/TFMSA method. 100 mg of peptidyl resin in TFA (5 ml), TFMSA (10 ml) was added dropwise with stirring. 150 µl of thioanisol, 150 µl of ethanedithiol and 100 µl of *m*-cresol were added and kept at 30 °C for 1.5 h and filtered. Filtrate was concentrated and cold diethyl ether was added to precipitate the peptide. It was washed repeatedly with ether and dried *in vacuo*. The peptide obtained was then dissolved in 5% acetic acid–water mixture, loaded onto a sephadex G_{25} column and eluted with 5% aqueous acetic acid. The fractions containing the peptide were collected and lyophilized. The yield on the basis of first amino acid substitution level: BDDMA-PS (94%), DVB-PS

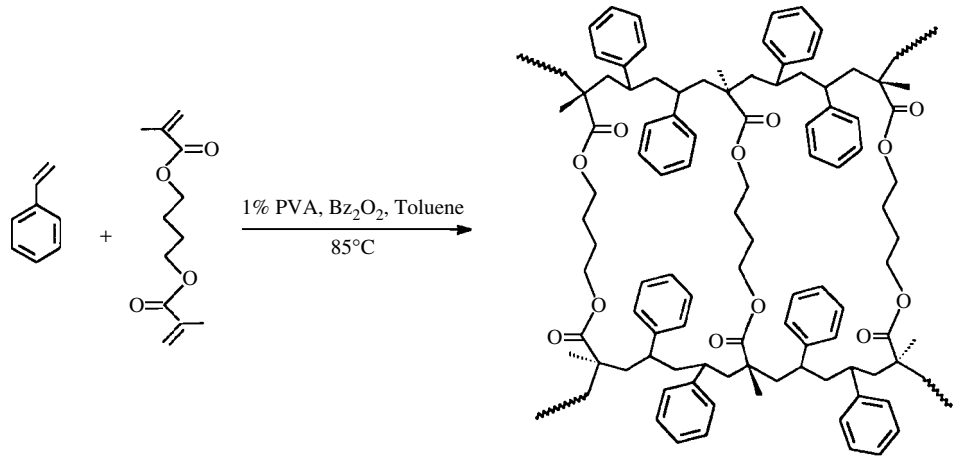


Figure 1 Preparation of BDDMA-PS support.

(62%). The amino acid analysis of the pure peptide was Val 0.98 (1.0); Ala 2.13 (2.0); Glu 0.96 (1.0); Ile 1.90 (2.0); Asp 2.08 (2.0); Tyr 0.74 (1.0); Gly 1.0 (1.0); the low value of Tyr is due to its partial degradation. Gln and Asn were hydrolysed to Glu and Asp.

RESULTS AND DISCUSSION

Preparation and Functionalization of BDDMA-PS Resins

The physicochemical characteristics and morphology of a polymer were determined by the chemical nature of the monomers, the mol percentage of the crosslinker, the monomer to diluent ratio, the amount of stabilizer, the nature of the diluent, the geometry of the vessel, stirring rate and temperature. Microporous beads of the copolymer, BDDMA-PS with physicochemical properties comparable to those of DVB-PS resins were obtained in 100–200 mesh size when the suspension polymerization was carried out in a cylindrical reaction vessel at 85 °C and 1500 rpm using toluene as the diluent (Figure 1). At 2 mol% crosslinking the resin exhibited sufficient mechanical integrity, polarity and swelling capacity necessary for the successful synthesis of polypeptides. The incorporation of the BDDMA crosslinker was confirmed from IR (KBr) at 1720 and 1490 cm^{-1} peaks characteristics of the ester carbonyl group. Peaks due to aromatic C–H str. (3020 and 700 cm^{-1}) and CH_2 str. (2910 and 2860 cm^{-1}) were also observed. Solid-state ^{13}C–CP–MAS–NMR (Figure 2a) showed peaks

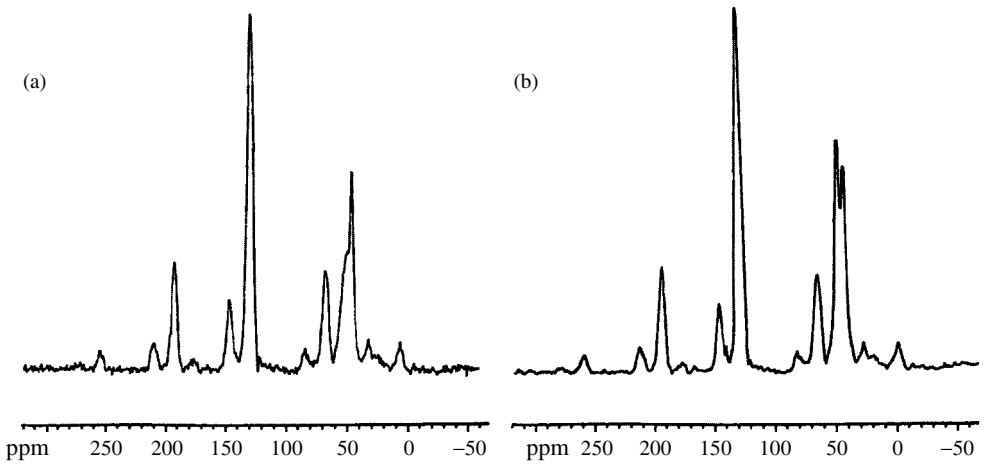


Figure 2 Gel-phase ^{13}C–CP–MAS–NMR spectrum of (a) BDDMA-PS and (b) Chloromethylated BDDMA-PS.

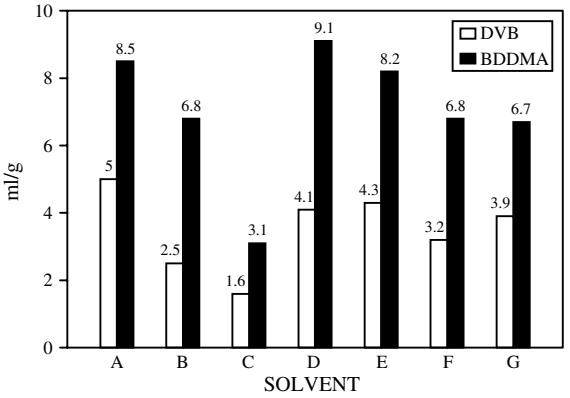
at 130.48 ppm (aromatic carbons), 148.26 ppm (para carbon of styrene), 66.87 ppm (methylene carbon of BDDMA) and 42.73 ppm (polymer backbone methylenes).

Both DVB-PS and BDDMA-PS resins were functionalized by Fridel-Craft's reaction using chloromethyl methyl ether (CMME, carcinogen) and 1 M anhydrous zinc chloride catalyst [11]. The BDDMA-PS resin gave a chlorine capacity of 2.36 mmol/g in 3 h and DVB-PS resin required 12 h to obtain a capacity of 2.28 mmol/g. That is, functionalization of the BDDMA-PS resin was relatively easy, probably due to its flexible nature and high swelling capacity. Unlike the other Lewis acid catalysts, $ZnCl_2$ catalysed reactions can be easily controlled to effect low capacity functionalizations under mild conditions. While the other catalysts vigorously react to give brownish yellow resins, this reaction gave white resins upon washing with 4 N HCl/dioxane. The resin was characterized by IR(KBr) : 1420, 668 cm^{-1} (C–Cl str.) and solid-state ^{13}C–NMR (Figure 2b): 48.30 ppm (methylene carbon of chloromethyl group); 135.62 ppm (C–6 carbon of polystyrene ring).

Microscopic examination of the resins was done by placing a small amount of the resin on a glass slide with a few drops of ethanol. The beads were examined at 180 X magnification for the extent of size variation and the tendency of the beads to clump together. Beads of uniform size with no significant tendency to aggregate or clump were selected since the uniform mixing, washing and especially complete draining of solvents may be problematic with resins of clumping nature. The chemical stability of BDDMA-PS resins were also checked by IR spectroscopy. The resin showed no change in the IR (KBr) spectrum, after 48 h treatment with 6 N HCl, TFA and 6 N KOH at 110 °C.

Swelling Studies

BDDMA-PS resins exhibited better swelling than Merrified resin in solvents used for peptide synthesis (Figure 3). Flexible and polar BDDMA-crosslinks rendered the polymer network more open and hydrophilic than the conventional DVB-PS resins. When suspended in polar aprotic solvents like NMP, DMF etc., the reagents can well penetrate into the matrix for quantitative availability of the reactive functional sites. This can also increase the solvation of the polymer and the peptide chains reducing the incompatibility to a minimum. The functionalization



A-Tetrahydrofuran, B-N,N-Dimethyl formamide, C-Methanol, D-Dichloromethane, E-N-Methyl pyrolidone, F-Dioxane, G-Benzene

Figure 3 Swelling capacity of 2 mol% DVB-PS and BDDMA-PS resins.

of BDDMA-PS resins does not show any decrease in solvent uptake so that the crosslinking network remains distinct resulting in the higher porosity and easy mass transport of reagents. Thus the new resin may facilitate the reactions performed on it even at a high substitution level. Swelling studies were carried out in a graduated cylinder by measuring the dry volume and the solvent imbibed volume of the polymer beads.

Synthesis of ACP (65–74) Fragment

The present paper describes the synthesis of the ACP (65–74) fragment on high capacity DVB-PS and BDDMA-PS supports under identical conditions. The first amino acid substitution level was kept constant to keep the overall distribution of the growing peptide chains the same on both supports. Thus, it was assumed that the probability of aggregation of the peptide chains due to the local density of functional sites on both the supports is the same. The *C*-terminal amino acid, glycine, was attached to both supports as its caesium salt and the extent of esterification was determined by the picric acid method [9]. The amino capacity of the BDDMA-PS resin was 2.20 mmol/g and that of DVB-PS was 2.09 mmol/g. A reaction time of 24 h was given to the BDDMA-PS resin and 40 h to DVB-PS resin, to produce almost the same level of amino acid substitution (>90%). The relatively easy esterification on the BDDMA-PS support indicates the easy availability of all the reaction sites. In the DVB-PS resin, the reaction may be suppressed by the poor swelling and steric effect developed towards

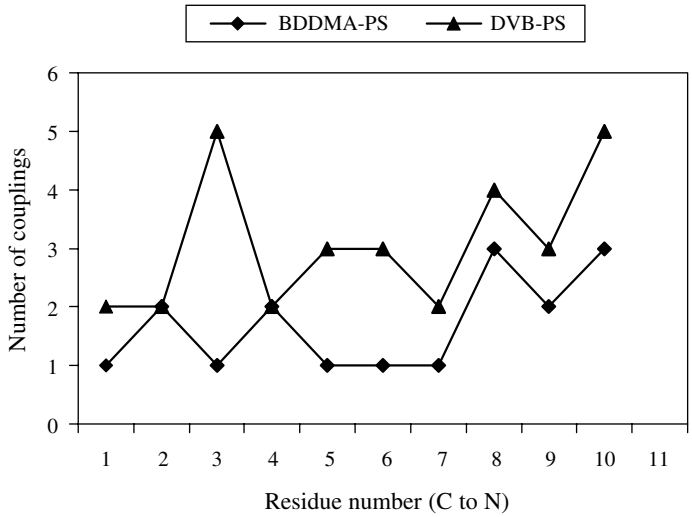


Figure 4 The number of couplings given on both DVB-PS and BDDMA-PS supports for the assembly of different amino acids.

the end of the reaction, especially at higher loading levels.

The subsequent amino acids were attached using a 2.5 mmol excess of HOBt active esters of N^{α}-Boc-protected amino acids in NMP containing 10% v/v of DMSO. NMP and DMSO were reported to be effective for the destabilization of the resin bound β-sheet structure [4]. In BDDMA-PS resin, all the couplings except Tyr to Ile and Val to Gln were completed in the first time itself. But, in the second coupling these amino acids was quantitatively attached. In each step, even a very slight blue colour in the Kaiser test, in either the solution or the resin was taken to be positive. However, more couplings had to be given for each step on the DVB-PS support. The number of couplings given for the complete incorporation of different amino acids is shown in Figure 4. It was observed that a total acylation time of 30 h was required to assemble the peptide on DVB-PS resin while 14 h was enough to build on the BDDMA-PS resin. The efficiency of the chain assembly on both supports was also compared by subjecting the peptidyl resin to the amino acid analysis. The amino acid composition of the peptide obtained from BDDMA-PS resin agrees well with the theoretical value. But the product from DVB-PS was contaminated with impure sequences, as evident from the low value of Tyr, Gln, Val and the high value of Ala (Table 1).

The purity of the crude peptide was ascertained by TLC and HPLC techniques. On TLC analysis, using (a) pyridine : water : acetic acid (85 : 10 : 5) and (b) *n*-butanol : acetic acid : water (66 : 12 : 26), the peptide

Table 1 Amino Acid Analysis of DVB-PS and BDDMA-PS Resins Carrying ACP (65–74) Fragment

Amino acid	Theoretical	DVB-PS	BDDMA-PS
Ala	2	3.37	2.07
Asp	2	1.47	2.04
Glu	1	0.48	0.89
Gly	1	1.0	1.0
Ile	2	1.61	1.97
Tyr	1	0.85	0.98
Val	1	0.41	0.88

obtained from the BDDMA-PS resin gave a single spot, $R_{f(a)}$: 0.52 and $R_{f(b)}$: 0.14. The product from the DVB-PS also gave a major spot at the same R_f value. The homogeneity was further ascertained by HPLC analysis. The crude peptide from the BDDMA-PS resin was more homogeneous than the product from the DVB-PS resin (Figure 5). Moreover, the amino acid analysis of the peptidyl resin indicated that the additional peaks obtained in the HPLC profile of the crude product obtained from DVB-PS were the unwanted peptides produced during the synthesis of the target sequence.

The homogeneity of the product from BDDMA-PS resin was further confirmed by recording the electrospray ionization mass spectrum (ESI-MS). [M + 1 : 1046.2 (100%); M + 2 : 523.1 (46%), $C_{47}H_{74}N_{12}O_{16}$ requires M + 1045.9]. The formation of deletion sequences on DVB-PS resin even after checking

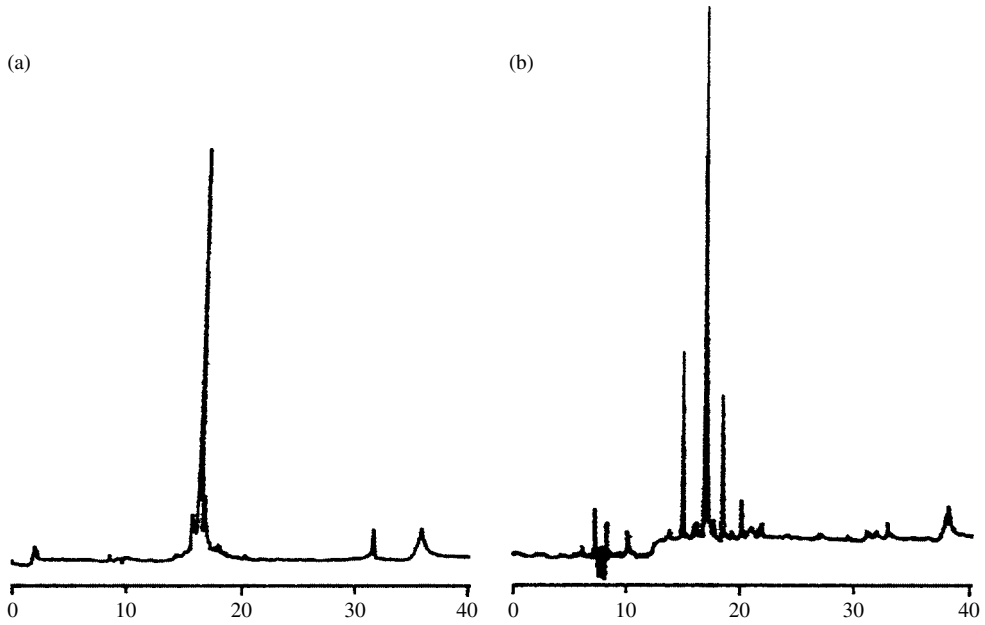


Figure 5 Analytical HPLC profile of ACP (65–74) fragment synthesized on (a) BDDMA-PS and (b) DVB-PS supports.

each coupling for completion may be due to the lack of sensitivity in the Kaiser test. The DVB-PS resin possesses poor swelling in methanol in which the ninhydrin solution was prepared for the Kaiser test. So some of the amino groups buried inside the polymer matrix might not have been available for the ninhydrin solution to react. Thus it can be seen that the high capacity and 2 mol% crosslinking affected the efficiency of synthesis on DVB-PS to a considerable extent. This is clear from the yield, purity and coupling difficulty observed during the synthesis of ACP (65–74) fragment on this support. But, when the support was changed to BDDMA-PS, the synthesis became more easy.

FTIR Investigation

To understand the possible causes of synthetic efficiency of the BDDMA-PS support, conformational developments of the growing peptide chains on both the supports were followed using solid-state FTIR spectroscopy. Narita *et al.* [12] have used this technique extensively for conformational analysis of peptides bound to linear and crosslinked supports. Though there can be nine bands of characteristic stretching frequencies for an isolated planar amide bond, the most significant amide I (1600–1700 cm^{-1}) region was selected for conformational assignments. Boc-protected peptide fragments collected after the attachment of 8, 9 and 10 amino acid residues were subjected to FTIR analyses as described by Narita [12]. DVB-PS samples collected after the attachment of the 8th residue gave a sharp peak at 1639 cm^{-1} and a small peak at 1658 cm^{-1}. Similarly peaks were obtained at 1646 cm^{-1} and 1638 cm^{-1} in the amide I region for samples collected after the attachment of the 9th and 10th residues, respectively. These peaks are assigned to the β-sheet structure, in accordance with the early reports [13]. The same sequences when built on the BDDMA-PS resin had peaks observed at 1660, 1662 and 1665 cm^{-1}, respectively. These are characteristic of the random coil conformation [13]. The shoulder found at 1648 cm^{-1} for the sample collected after the attachment of the 9th residue shows the presence of a fraction of β-sheet aggregation in the BDDMA-PS bound sample also (Figure 6a–c). This indicates that, as the support was changed from DVB-PS to BDDMA-PS, a major conformational transition from β-sheet to random coil conformation was observed. A similar result was reported in the case of 1,6-hexanediol diacrylate-crosslinked polystyrene support [14]. Since the random structure is more prone to solvation than β-sheet structure especially in solvents like NMP, DMSO etc. amino acylations on BDDMA-PS were fairly easy. In the case of DVB-PS resin the suppression of β-sheet formation is very difficult due to the poor swellability of the resin in NMP and DMSO. The rigid and hydrophobic polymer

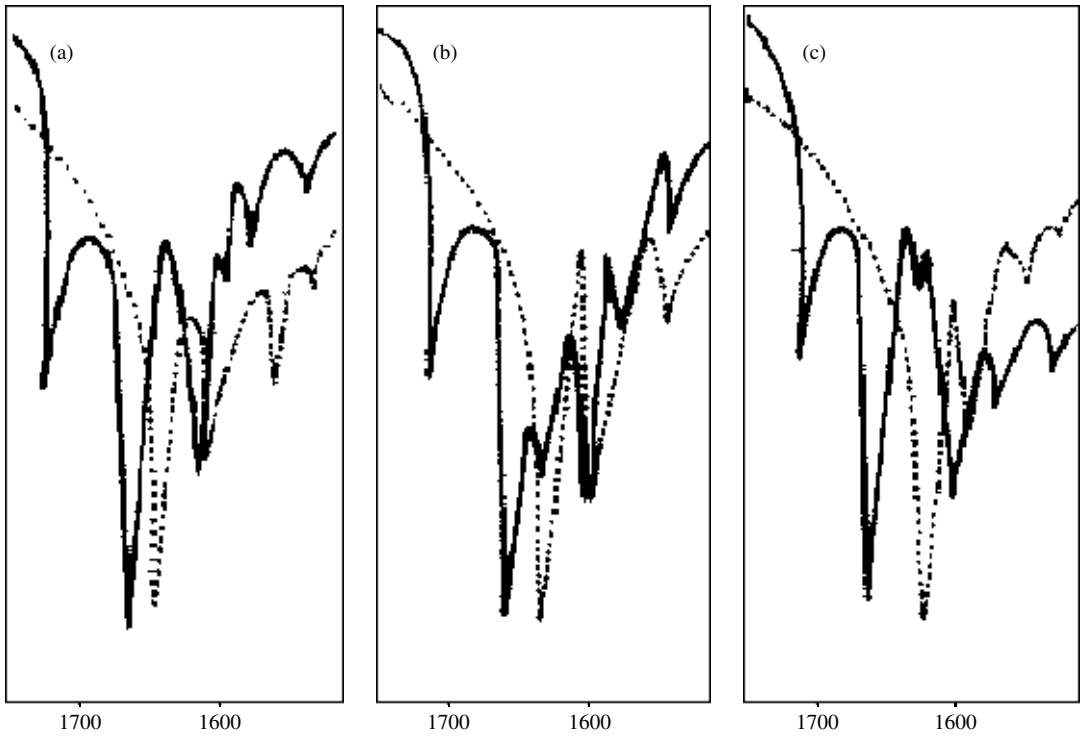


Figure 6 Solid state FTIR spectra of resin bound ACP fragments after the attachment of (a) 8th, (b) 9th (c) 10th amino acid residues. — BDDMA-PS and - - - - DVB-PS.

matrix and high substitution further increased the inter- and intra-molecular interaction of the peptide chains to make additional crosslinking.

CONCLUSIONS

In summary, a satisfactory synthesis of the difficult sequence peptide, ACP (65–74) fragment was achieved on the more polar and flexible BDDMA-PS support. The resin can be easily prepared and functionalized. It has all the physicochemical characteristics such as mechanical strength, chemical stability, high reactivity and high swelling capacity necessary for the successful synthesis of peptides, even at high functional capacity level. The suppression of β-sheet forming tendency of the pendent peptide chains on the polymer support when used in conjugation with NMP/DMSO renders the support promising for the synthesis of difficult sequences.

Acknowledgements

The authors thank the University Grants Commission (UGC), New Delhi for the financial assistance to one of them (IMK).

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