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Solid-phase synthesis of partial sequences of rubber elongation factor protein on hexanediol diacrylate crosslinked polystyrene support

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ABSTRACT

Peptide synthesis has presently emerged as a powerful tool for studying protein/peptide structures and their functions, initiating biomedical researches in immunology, pharmacology, enzymology and molecular biology. Peptides are present in very small amount in their biological sources and it is very difficult to isolate and purify them. The invention of SPPS is the real land mark in synthetic peptide chemistry. Though many modifications have been done on Merrifield synthesis, many a peptide chains, particularly hydrophobic peptide chains have not been prepared successfully using this method. The difficulties observed in the synthesis can be attributed to change in solvation of the peptide-polymer at different stages in the synthesis giving rise to both truncated and deletion sequences. Also, the strong association of hydrophobic peptides via β-sheet formation within the peptide-polymer matrix is a major problem. Herein we report the synthesis and usage of 2% hexanediol diacrylate-crosslinked polystyrene support for the production of highly hydrophobic peptides. The synthetic utility of the support was demonstrated by the quantitative synthesis of the hydrophobic fragments of rubber elongation factor protein on the chloromethylated support. The first successful attempt to synthesize partial sequences of REF protein by solid phase peptide synthesis is reported. The free peptide was obtained in high yield and purity as checked by reverse phase HPLC. The synthesized peptides were characterized by amino acid analysis and electronspray ionization mass spectrometry.

KEYWORDS

Solid-phase peptide synthesis;
1,6-hexanediol diacrylate;
Hydrophobic peptides;
β-sheet conformation;
Rubber elongation factor protein.

INTRODUCTION

Synthesis of a peptide with a well defined sequence of amino acid residues was a fairly complex process till the introduction of solid phase peptide synthesis (SPPS) by Merrifield[1] in 1962. Methods for SPPS involve the use of an insoluble support onto which the protected peptide chain is assembled. The accelerating discovery of animal and plant peptide constituents of potential biological[2,3] and pharmacological[4] importance has led to immense demands for synthetic peptides. Although the Merrifield solid-phase approach has been improved and generalized for the synthesis of a variety of complicated

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molecules, the highly hydrophobic macromolecular environment of the polymer matrix can induce the adoption of unfavorable conformations by growing peptide chains. During hydrophobic peptide chain synthesis, secondary structures like β-sheet conformation are formed due to hydrogen bonding among the pendant chain which brings additional crosslinking resulting in significant decrease in the solvation of hydrophobic peptide chains[5,6]. Thus synthesis of hydrophobic peptide chain is still a long-standing problem for peptide chemists.

Rubber Elongation Factor (REF) protein, which is responsible for the elongation of rubber particles, contains highly hydrophobic fragments which are difficult to synthesize. REF is 137 amino acids long and has a molecular mass of 14.6 kd[7]. The protein is tightly bound to serum-free rubber particles and is necessary for the addition of multiple cis-isoprene units to rubber molecules[8]. The sequence determination of REF was done by digesting it with trypsin, acetic acid, hydroxylamine, staphylococcus protease and chymotrypsin by Mark S. Dennis *et al*[7]. But all the fragments were not recovered due to their hydrophobic nature.

Herein, we describe the use of an efficient support for the synthesis of highly hydrophobic peptide chains. The elucidation of relationship between preferred conformation of growing peptide chain and its physicochemical properties are of considerable practical interest in peptide synthesis. Studies on the structure-reactivity and structure-property correlations helped us to design a novel 1,6-hexanediol diacrylate-crosslinked polystyrene (HDODA-PS) support with optimum hydrophilic-hydrophobic balance, mechanical stability and other essential requirements for an efficient support for the synthesis of highly hydrophobic peptides. Though many works have been done on the syntheses of short fragments of hydrophobic peptide chains[9], the solid phase peptide synthesis of REF has not been reported yet. As far as our survey, this is the pioneer report on the solid phase peptide synthesis of the highly hydrophobic fragments of REF. The supports were characterized using IR spectroscopy. Certain highly hydrophobic fragments of REF were prepared using this support following usual solid phase strategies. The synthesized peptides were purified and characterized by various analytical techniques.

EXPERIMENTAL

Materials and methods

Styrene, 1,6-hexanediol diacrylate and polyvinyl alcohol were purchased from Sigma Chemical Company, U.S.A. Benzoyl peroxide from Sisco, Bombay was recrystallised before use. Boc-Gly and Boc-Pro were prepared in the laboratory following Schnabel's procedure[10] and Boc-ON method.[11] All solvents were of reagent grade and were obtained from E.Merck, India. All side chain protected L-amino acids, DCC, t-butyloxycarbonyloximino-2-phenylacetonitrile (Boc-ON), thioanisole, 1,2-ethanedithiol and cesium carbonate were purchased from Sigma Chemical Company, USA. IR spectra were recorded on a Shimadzu IR-470 spectrophotometer using KBr pellets. Reversed phase HPLC was done on a preparative column (Biorad, HI-pore RP-318, 25 x 2.15 cm) using a gradient system consisting of 0.1% (w/v) TFA in water and acetonitrile at a flow rate of 2mL/min. Detections were done at 230 and 280nm. Characterization of the peptides was done using an amino acid analyzer (Shimadzu column, CTU 10A, temp. 55°C, Spectroflourimetric detector, Styrene-DVB cation exchange resin). A micromass Quattro II triple quadrupole mass spectrometer was used for recording electrospray ionization mass spectrum.

Preparation of 2% HDODA-PS

The copolymer was prepared as microporous beads by suspension polymerization under nitrogen atmosphere. 22.4mL of destabilized styrene, 0.9mL of HDODA and 1g benzoyl peroxide were dissolved in 20mL toluene. The mixture was added to 345mL of 1% PVA solution in a reaction vessel equipped with a stirrer, water condenser, nitrogen inlet and kept at 80°C on a water bath. The polymerization was allowed to proceed for 6h. The obtained white shiny beads were collected by filtration through a sintered funnel (G-2) and washed thoroughly with hot water (20ml x 3, 3 min), methanol (20ml x 3, 3min) and drained. The resin obtained was Soxhlet extracted using acetone to remove all the low molecular weight impurities and linear polymers and

dried in the oven at 50°C. Beads were sieved into different sizes using standard sieves.

Chloromethylation of HDODA-PS

The polymer beads (200-400 mesh, 2g) was allowed to swell in dry dichloromethane (20ml). Chloromethyl methyl ether (12mL) and 0.04mL catalyst (an. ZnCl2/THF) was added to the swollen polymer under anhydrous conditions slowly with stirring. The mixture was refluxed at 50°C for 5h. It was then cooled and filtered through a sintered glass funnel (G-2), washed with THF (20ml x 3, 3min), finally with methanol and drained. The polymer was Soxhlet extracted with THF and dried under vacuum. The chlorine capacity was estimated by pyridine fusion method and was found to be 1.7 and 1.9 mmol/g.

Synthesis of Short Fragments of Rubber Elongation Factor (REF) Protein

Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro (44-55)

Attachment of Boc-Pro to the chloromethyl polystyrene

To Boc-Pro in ethanol, a saturated solution of cesium carbonate was added till the pH of the solution reached 7.0. The solvents were rotary evaporated by azeotropic distillation with dry benzene. The Cs-salt thus obtained was dissolved in NMP and chloromethylated polystyrene (1.9mmol/g) was added. The reaction mixture was kept at 50°C for 48h. The polymer was washed with NMP, NMP/water (1:1 v/v), methanol, DCM and dried under vacuum.

Synthesis of Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro (44-55)

Boc-Pro incorporated polystyrene was treated with 1mL of 33% TFA/DCM and kept for 30 min. for the removal of Boc group. The polymer was washed with DCM and neutralized with 5% DIEA in DCM. Filtered off the solution and again washed with DCM, NMP and then 0.38mmol of the HOBt active ester of the next amino acid (Gly) in NMP was added and shaken for 60 min. After the synthesis, the peptidyl polystyrene was washed with NMP, DCM, 33% MeOH /DCM, DCM and dried under vacuum. The progress of the coupling step was monitored by picking up a single bead from the reaction mixture and performing the Kaiser test. If positive, the next coupling was repeated in the same manner until the test was negative. The same coupling procedure was employed for all the couplings performed. This cycle of operations was repeated for the stepwise incorporation of the remaining amino acids.

Cleavage of peptide from the support

To 100 mg of polymer bound peptide in TFA (10mL), thioanisol (0.1mL) and 1,2-ethanedithiol (0.1mL) were added and kept at room temperature for 22 h. TFA was removed by rotary evaporation after filtration and the crude peptide was precipitated by the addition of ice-cold ether. It was washed repeatedly with ether and dried.

Purification of the peptide

Purification of crude peptide was done by reverse phase HPLC on a preparative column (Biorad, Hi-Pore RP318, 25x2.15cm) using a gradient system of 0.1% TFA (w/v) in water and acetonitrile. Homogeneity of the synthesised peptide was checked in analytical RP-HPLC using binary solvent system of (A) 0.1% TFA/water and (B) 50% CH3CN in 0.1% TFA. Distinct peak observed in HPLC profile was collected and rotory evaporated to remove solvents and was hydrolysed using 6N HCl at 110°C for 24h. This was then subjected to amino acid analysis.

Val-Lys-Asn-Val-Ala-Val-Pro (56-62)

Attachment of Boc-Pro to the chloromethyl polystyrene

Boc-Pro was dissolved in ethanol and the pH of the solution was brought to 7 by slow addition of saturated solution of cesium carbonate. The solvents were rotary evaporated by azeotropic distillation with dry benzene. The Cs-salt of Boc-Pro obtained was dissolved in NMP and chloromethylated polystyrene (1.9mmol/g) was added. The mixture was kept at 50°C for 48h with gentle stirring. The polymer was washed with NMP, NMP/water (1:1 v/v), methanol, DCM and dried under vacuum.

Synthesis of Val-Lys-Asn-Val-Ala-Val-Pro (56-62)

1mL of 33% TFA/DCM was used for Boc-

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deprotection. The polymer was washed with DCM and neutralized with 5% DIEA in DCM. It was again washed with DCM, NMP and then 0.34mmol of the HOBt active ester of the next amino acid (Val) in NMP was added and shaken for 60 min. After the synthesis of the desired sequence, the peptidyl polystyrene was washed with NMP, DCM, 33% MeOH/ DCM, DCM and dried under vacuum.

Cleavage and purification

100 mg of the peptidyl polymer was treated with a mixture of TFA (10mL), thioanisol (0.1mL) and 1,2-ethanedithiol (0.1mL) at room temperature for 22h. The mixture was filtered and the solution rotary evaporated to remove TFA. Ice-cold diethyl ether was then added to precipitate the peptide. It was washed repeatedly with ether and dried. The crude peptide was purified by RP-HPLC. The peptide obtained after HPLC was hydrolysed using 6N HCl at 110°C for 24h. This was then subjected to amino acid analysis on a Shimadzu column, CTU 10A (Styrene-DVB cation exchange resin, column temperature 55°C).

Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly (125-135)

Attachment of Boc-Gly to the chloromethyl polystyrene

Boc-Gly was dissolved in ethanol and a saturated solution of cesium carbonate was added till the solution is neutral. The solvents were rotary evaporated by azeotropic distillation with dry benzene. The Cs-salt of Boc-Gly thus obtained was dissolved in minimum volume of NMP and chloromethylated polymer (1.9mmol/g) was added. The reaction mixture was kept at 50°C for 48h. The polymer was filtered, washed with NMP, NMP/water (1:1 v/v), methanol, DCM and dried under vacuum.

Synthesis of Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly (125-135)

1mL of 33% TFA/DCM was used to remove Boc-protection. The polymer was washed with DCM and neutralized with 5% DIEA in DCM. The polymer was then washed well with DCM, NMP and then 0.76mmol of the HOBt active ester of the next amino acid (Tyr) in NMP was added and shaken for 60 min. After the synthesis, the peptidyl polymer was washed with NMP, DCM, 33% MeOH /DCM, DCM and dried under vacuum.

Cleavage and purification

To 100 mg of peptidyl polymer in TFA (10mL), 0.1mL thioanisol and 0.1mL 1,2-ethanediol were added and kept at room temperature for 20h. The polymer was removed by filtration and TFA by rotary evaporation. Ice-cold diethyl ether was then added to precipitate the peptide. It was washed repeatedly with ether and dried. The yield was 230mg. The purified sample from HPLC was hydrolyzed using 6N HCl at 110°C for 24h and diluted with buffer. The major peak collected from HPLC was dried and subjected to amino acid analysis for characterization (Shimadzu column, CTU 10A, Styrene-DVB cation exchange resin, column temperature 55°C).

RESULTS AND DISCUSSION

The concept of optimum hydrophobic-hydrophilic balance served as a guideline for the development of a support with styrene and 1,6-hexanediol diacrylate. This polymer showed better solvation and swelling in solvents used for the synthesis of peptides. Polymer support with uniform size, shape and mechanical stability were prepared by copolymer-

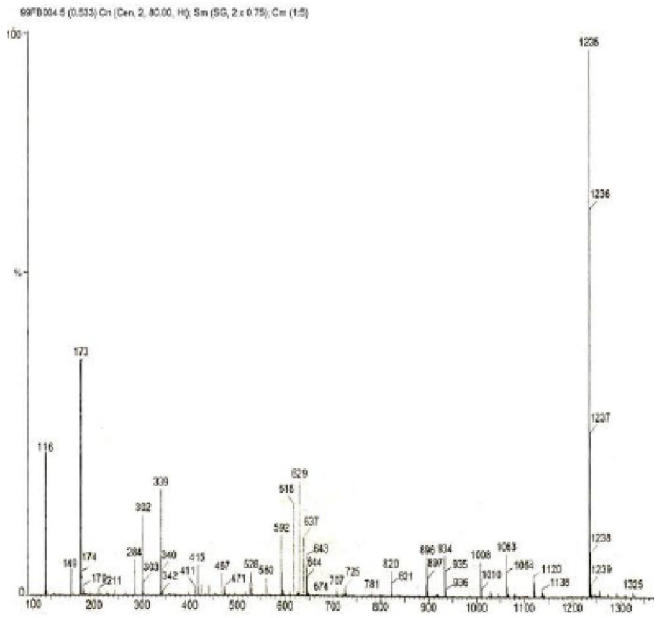
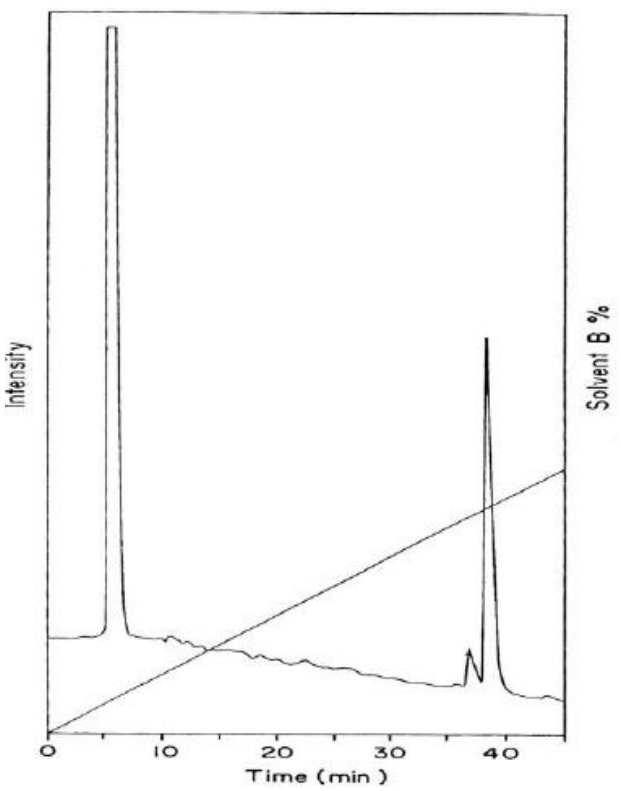
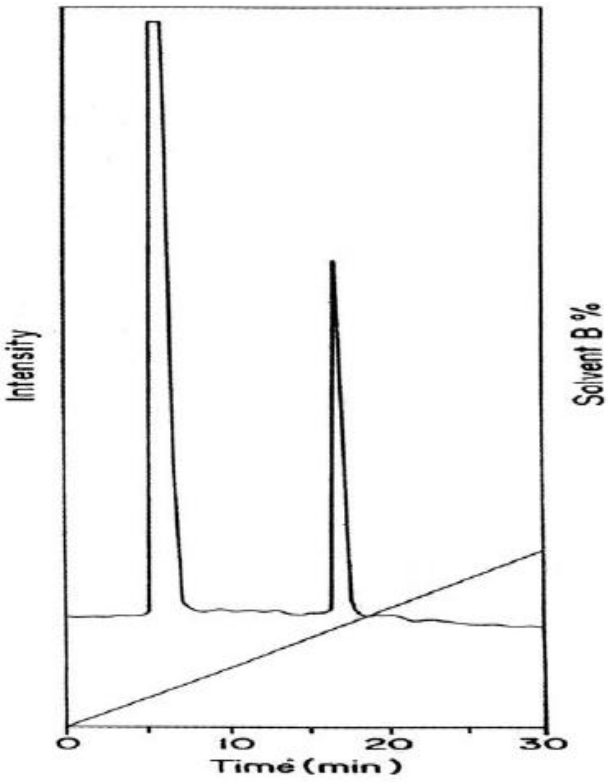


Figure 1 : ESI-MS of Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro





Conditions: Solvent A (0.1% TFA in water), Solvent B (50% acetonitrile in A), 230nm

Figure 2(A) : HPLC trace of crude Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro

Figure 2(B) : HPLC trace of pure Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro

TABLE 1 : Details of the aminoacid analysis of Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro

Amino acid	Actual value	Calculated value
Pro	3	2.6
Leu	1	0.9
Glu	2	1.7
Gly	2	1.5
Val	1	0.82
Asp	1	1.1
Ile	2	1.6

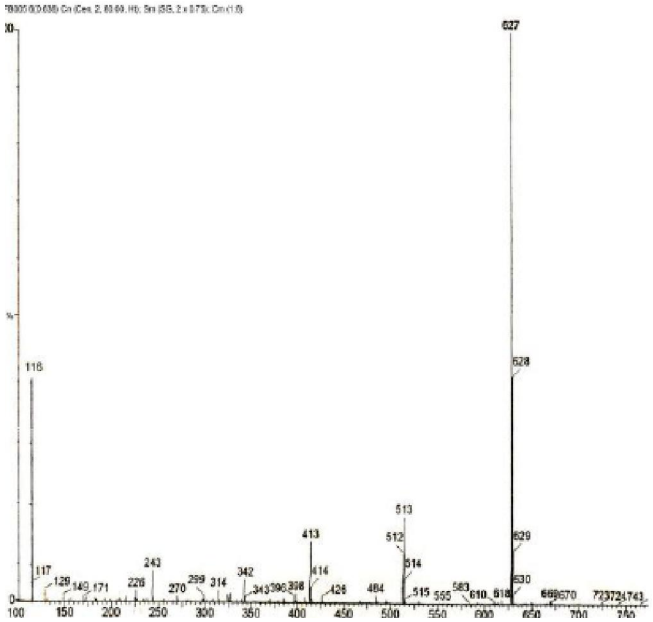


Figure 3 : ESI-MS of Val-Lys-Asn-Val-Ala-Val-Pro

izing the hydrophobic styrene and hydrophilic and flexible hexanediol diacrylate (HDODA).[12] The support synthesized by the suspension polymerization of styrene and 1,6-hexanediol diacrylate has been found to exhibit an amphiphilic nature and flexibility required to use as a support for the synthesis of highly hydrophobic peptides. The polymer was obtained in bead form with high yield and showed sufficient physical and chemical stability. IR (KBr): 1720, 1490cm-1 (ester C=O); 2910, 2850 cm-1 (CH2 str. of HDODA and polystyrene); 3020, 700 cm-1 (C-H of benzene).

The polymer was functionalized by chloromethylation using chloromethyl methyl ether (CMME) and ZnCl2 (Lewis acid catalyst) in THF.[13] HDODA-PS polymer having chlorine capacities 1.7

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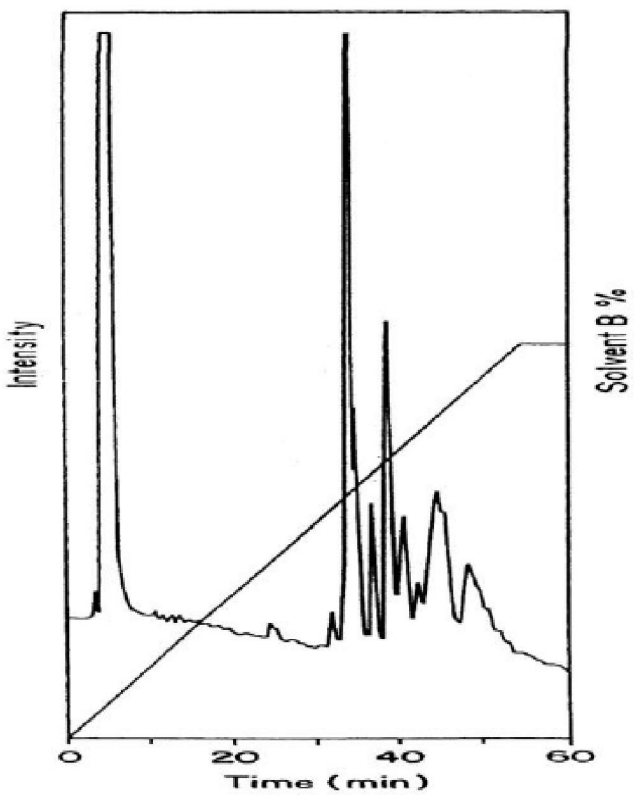
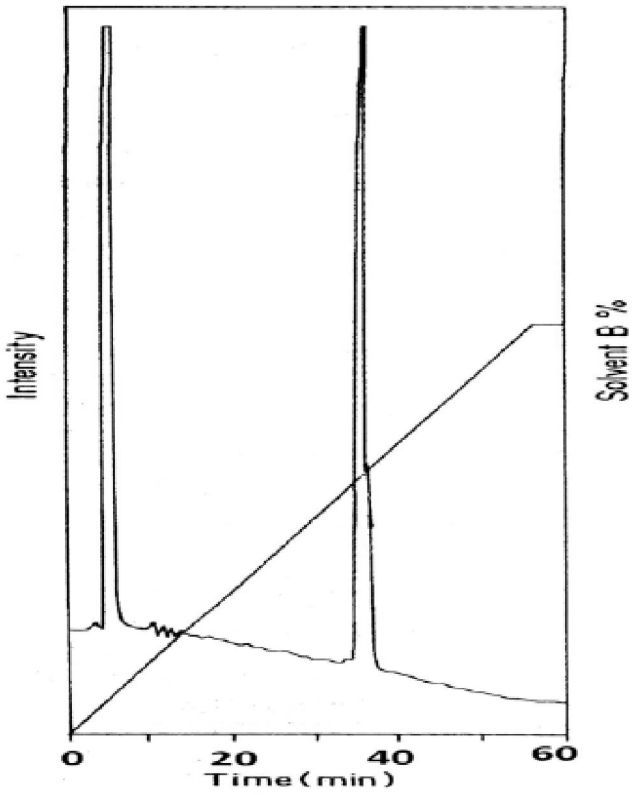




Conditions: Solvent A (0.1% TFA in water), Solvent B (50% acetonitrile in A), 230nm

Figure 4(A) : HPLC trace of crude Val-Lys-Asn-Val-Ala-Val-Pro

Figure 4(B) : HPLC trace of pure Val-Lys-Asn-Val-Ala-Val-Pro

TABLE 2 : Details of the aminoacid analysis of Val-Lys-Asn-Val-Ala-Val-Pro

Amino acid	Actual value	Calculated value
Val	3	3.040
Lys	1	1.084
Asn	1	1.057
Ala	1	1.212
Pro	1	1.435

and 1.9 mmol/g were prepared by this method. Characterization of the functionalized support was done by IR spectroscopy. IR (KBr): 668, 1420 cm-1 (C-Cl). Some highly hydrophobic fragments of rubber elongation factor protein were synthesized using this support. The synthesized peptides were purified and characterized by various analytical methods.

The following fragments of REF were synthesized on chloromethyl HDODA-crosslinked polystyrene using the system of benzyl derived side chain blocking group in conjunction with Boc-amino acids with some modifications. For the efficient coupling, active esters of amino acids with HOBt were used. NMP was used as solvent for coupling reaction. Each step was monitored by Kaiser test[14,15].

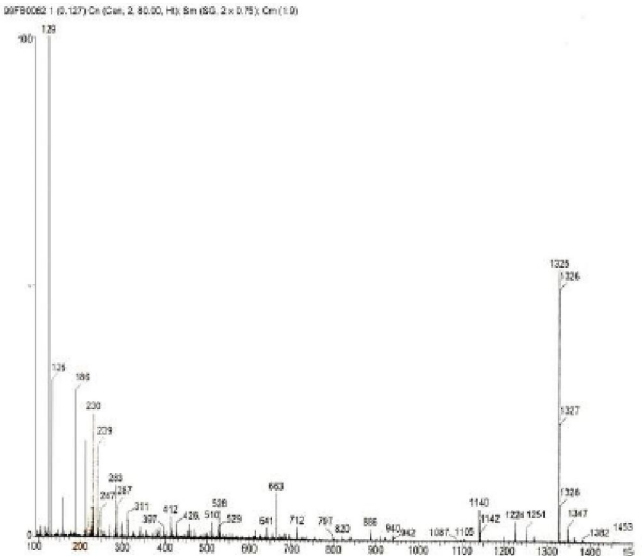
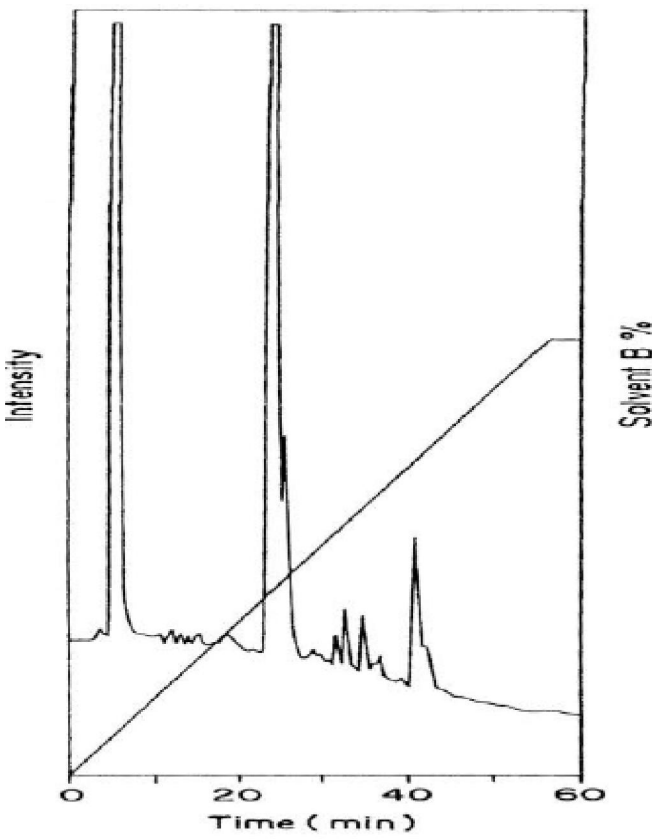
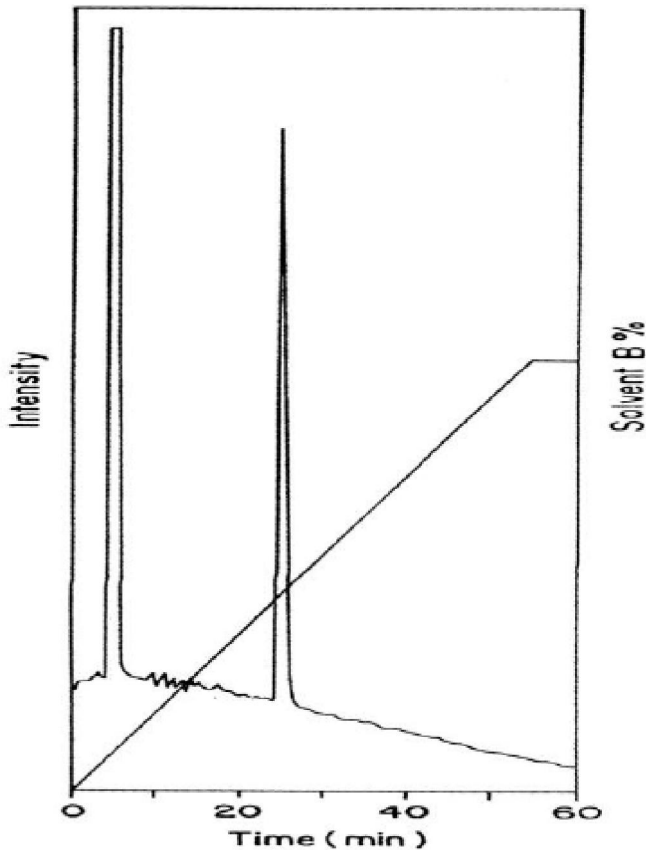


Figure 5 : ESI-MS of Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly

a. Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro (44-55)
b. Val-Lys-Asn-Val-Ala-Val-Pro (56-62)
c. Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly (125-135)

After the synthesis of desired sequences, peptides were separated from the polymer using anhy-





Conditions: Solvent A (0.1% TFA in water), Solvent B (50% acetonitrile in A), 230nm

Figure 6(A) : HPLC trace of crude Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly

Figure 6(B) : HPLC trace of pure Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly

TABLE 3 : Details of the aminoacid analysis of Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly

Amino acid	Actual value	Calculated value
Gln	1	1.37
Thr	1	1.23
Lys	2	2.32
Ile	1	1.05
Leu	1	1.32
Ala	1	1.26
Val	1	0.91
Phe	1	1.00
Tyr	1	1.27
Gly	1	1.29

drous TFA, thioanisole and 1,2-ethanedithiol. The purity of these peptides were confirmed by HPLC and characterized by amino acid analysis and ESI-MS.

Synthesis of Short Fragments of Rubber Elongation Factor (REF) Protein

Pro-Leu-Gln-Pro-Gly-Val-Asp-Ile-Ile-Glu-Gly-Pro (44-55)

Boc-Pro was anchored to 2% HDODA-crosslinked chloromethyl polymer by Gisin's cesium salt method[16]. DCC/HOBt in NMP was used for the coupling of all the remaining amino acids. Kaiser test was used to monitor the progress of the coupling reactions. The first coupling time was 45 min and for each amino acid, increment of 5 min was given. The DCU precipitated was washed off with 33% MeOH/DCM followed by washing with DCM. Double coupling was done at every stage for maximum completion of reaction. The coupling of Ile to Ile was incomplete even after a second coupling. Hence a third coupling was also carried out. This is due to the bulk nature of the side chain. When the desired sequence was attained the peptide was cleaved from the support using neat TFA, thioanisole and EDT. The synthesized peptide was analysed by

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ESI-MS (Figure 1). The observed molecular weight was in good agreement with the calculated value.

Analytical HPLC of the crude peptide showed only one major peak (Figure 2(A)). This was further purified on reverse phase column (Biorad, Hi-Pore RP318, 25x2.15 cm) using a solvent system of 0.1% TFA in water and acetonitrile (Figure 2(B)). The fraction collected from HPLC was evaporated, hydrolysed with 6N HCl at 100°C for 24h and amino acid analysis was used for the characterization of the purified peptide. The experimental values were found to agree with theoretical values (TABLE 1).

Val-Lys-Asn-Val-Ala-Val-Pro

The synthesis of this heptapeptide was carried out on a chloromethylated 2% HDODA-PS polymer with a chlorine capacity of 1.9 mmol/g. After attachment of amino acid, the substitution level was found to be 1.7 mmol/g. All amino acids were attached by DCC/HOBt coupling in NMP. Double coupling was performed for each amino acid to ensure maximum reaction. When the assembly of amino acids were completed, the peptide was cleaved from the support using the neat TFA in presence of thioanisole and 1,2-ethanedithiol. The synthesized peptide was analysed by ESI-MS (Figure 3). The observed molecular weight was in good agreement with calculated value. The purity of the peptide was checked on analytical HPLC using binary solvent system (0.1% TFA in water and 50% CH_3CN in 0.1% TFA) (Figure 4(A) and 4(B)). Aminoacid analysis gave values which agreed with the accepted values (TABLE 2).

Gln-Thr-Lys-Ile-Leu-Ala-Lys-Val-Phe-Tyr-Gly (125-135)

Boc-Gly was anchored to 2% HDODA-crosslinked chloromethyl polystyrene polymer by cesium salt method and the amino acid capacity was found to be 1.8 mmol/g by picric acid method. Boc group was removed by 33% TFA in DCM followed by neutralization. The remaining amino acids were attached to the amino acyl resin by HOBt active ester method. The coupling solvent used was NMP. A second coupling was carried out to ensure completion of reaction. When the desired sequence was attained, the peptide was cleaved from the support using TFA/thioanisole/EDT.

The synthesized peptide was analysed by ESI-MS (Figure 5). The observed molecular weight was in good agreement with the calculated value. The cleaved peptide was checked on analytical column (Figure 6(A)) and was further purified by reverse phase HPLC (Figure 6(B)). The peptide was also characterized by amino acid analysis (TABLE 3).

CONCLUSION

Fragments of REF protein which are highly hydrophobic, insoluble even in acetonitrile were synthesized in nearly quantitative yield using 2% HDODA-crosslinked polystyrene. This point to the fact that HDODA-PS can be an efficient support for the synthesis of peptides, especially hydrophobic peptides, the synthesis of which remains still a challenge to peptide chemists. Removal of REF from rubber particles and reconstitution of a rubber biosynthetic system has not been successful to date. The work reported here and its future refinements may contribute to the synthesis of complete sequences of REF protein and its introduction to synthetic polymer chemistry. Fragments of REF which suffer recovery problems due to its hydrophobic nature were synthesized in high purity using HDODA-PS support. The peptide chain assembly was almost quantitative in coupling, deprotection and neutralization steps. It is clear that by the employment of HDODA-PS and the right choice of synthetic strategy, the synthesis of hydrophobic peptides can be effectively achieved.

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