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Comparative Stepwise Polypeptide Synthesis Of ACYL Carrier Protein Fragment On Ps-Nvp-Hdoda Resin With Commerically Available Resins

Dr. SUNIL JACOB
Associate Professor ,Catholicate College Pathanamthitta, Kerala

Abstract
Acyl carrier protein was synthesised on novel PS-NVP-HDODA resin the protein was grown in a stepwise method from the resin . High efficiency of the resin in protein synthesis was proved beyond doubt by the estimation of purity of peptide synthesised.

I. Introduction

Merrifield's 1963 contribution to science served to foment, decades later, an explosion in the field of polymer supported organic synthesis. He envisaged that covalent anchoring of the peptide chain to an insoluble and inert polymer should greatly improve the quality and quantity of the target peptide. Merrifield introduced a lightly crosslinked polystyrene- divinyl benzene polymer having a pendant chloromethyl group as the point of attachment, which has come to be known simply as the Merrifield resin. The Merrifield resin showed good swelling behaviour in non polar solvents but, the hydrophilic peptide chain growing from the support got aggregated in non polar solvents. So, the purity and homogeneity of medium to large size peptide synthesised on this resin is still a challenging problem. A new terpolymer was developed by the radical aqueous suspension polymerisation of 1,6-hexanediol diacrylate, N-vinylpyrrolidone (NVP) and styrene(PS-NVP-HDODA). The resin had a very good hydrophobic-hydrophilic balance and showed high mechanical stability, and swelling properties. The utility of the new polymer for the stepwise synthesis of medium to large peptides was studied by synthesising few test peptides and the results were compared by synthesising the same peptide sequences on commercially available supports like the Merrifield resin and Sheppard resin under identical experimental conditions.

II. Materials and Methods

Styrene, 4-(Dimethylamino) pyridine (DMAP), cesium carbonate, Sheppard resins (Novasyn® KA 125), dicyclohexyl carbodiimide (DCC), 2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU), Boc and Fmoc-amino acids, HMPA, HOBt, and MSNT were purchased from Novabiochem Ltd., UK. Thioanisole, 1, 6-hexanediol diacrylate (HDODA), ethanedithiol, diisopropylethylamine (DIEA), TFA, 4-(hydroxymethyl) 3-(methoxy) phenoxy butyric acid (HMPB), piperidine were purchased from Sigma-Aldrich Corp., USA. N-Vinylpyrrolidone and 1,6-Hexanediol diacrylate were purchased from E.Merck, Germany. Chloromethylmethyl ether (CMME) was prepared using literature procedure. Solvents (HPLC grade) used were purchased from E. Merck (India) and BDH (India). IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C NMR measurements were conducted on a Bruker 300 MSL instrument operating at 75.47MHz. HPLC was done on a Pharmacia instrument using C-18 reverse phase semi- preparative HPLC column. Amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyser. For this, the peptide was hydrolysed using 6N HCl in a pyrex glass tube fused under N_2 for 15 hours at 130^{o}C.

PS-NVP-HDODA support

Inhibitors were removed from styrene by washing with 1% NaOH solution (2 x 30 ml) followed by distilled water (3 x 30 ml) and drying over anhydrous calcium chloride. NVP was purified by vacuum distillation. Four-necked reaction vessel equipped with a thermostat, teflon-bladed stirrer, water condenser and nitrogen inlet was used as the reaction vessel. A mixture of styrene (10.54 ml), NVP (0.54 ml), HDODA (0.67 ml) and AIBN(200 mg) were added to a solution containing sodium sulphate(10g), magnesium hydroxide (1g) and disodiumhydrogen phosphate (10 mg) in water (100 ml) by stirring the solution at 1600rpm. Temperature of the solution mixture was maintained at 70^{0}C under a slow stream of nitrogen. After 6 hours the copolymer was obtained as beads of 200-400μ size. Polymer was washed thoroughly with hot water (to remove the stabiliser), acetone (3 x 50 ml), and methanol (3 x 50 ml). The polymer was further purified by soxhlet

extraction with acetone methanol and dried under vacuum. IR (KBr):1724, 1686 cm^{-1} (ester), 690 and 755 cm^{-1} (aromatic).

Chloromethyl PS-NVP-HDODA support

PS-NVP-HDODA support (4g) was swollen in DCM (50 ml). After 1 hour excess DCM was filtered off. The swollen resin was shaken with CMME (24 ml) and 1M $ZnCl_2$ in THF (0.6 ml) for 2 hours at 50°C. The resin was filtered using a sintered glass funnel, washed with THF (4 x 30 ml), THF/water (1:1) (3 x 30 ml), THF (3 x 30 ml), methanol (3 x 30 ml) and then soxhletted with THF and methanol.

Estimation of halogen content in functionalised PS-NVP-HDODA resin (Volhard's method)

Chloromethyl PS-NVP-HDODA support (100 g) was digested with pyridine (3 ml) in a Kjeldahl digestion flask for 3 hours at 100-110°C. It was quantitatively transferred to a 125 ml conical flask using 50% acetic acid (30 ml). Con. HCl was added followed by slow addition of standard $AgNO_3$(0.1N) solution (10 ml) with magnetic stirring. Water (50 ml) was added to the mixture. The excess $AgNO_3$was determined by back titration with standard ammonium thiocyanate solution (0.1N) using ferric alum as indicator till a dark brown colour was obtained. A calibration titration was carried out with standard NaCl solution. From the titre values the halogen capacity of the resin was calculated. Capacity of the resin = 0.24 mmol Cl/g as estimated by Volhard's method.[25] IR (KBr): 1724, 1686 cm^{-1} (ester) and 1256 cm^{-1} (CH_2-Cl).

Aminomethylation

PS-NVP-HDODA (0.24 mmol Cl, 1 g) was made to swell in DMF for 1 hour. Excess DMF was removed. Potassium phthalimide (0.44 g. 2.4 mmol) was dissolved in DMF (1 ml), added to the resin and the mixture was stirred at 120 °C for 12 hours. The resin was filtered and washed with DMF (5 × 15 ml), DCM (5× 15 ml), THF (5 × 15 ml) and ether (5 × 15 ml). It was then dried under vacuum. The dried resin was swollen in distilled ethanol (20 ml) for 1 hour. 5% hydrazine hydrate (0.02 ml) in ethanol was added and the reaction mixture was refluxed at 80 °C for 8 hours. The resin was collected by filtration, washed with hot ethanol (5 × 15 ml), methanol (5 × 15 ml), ether (5 × 15 ml) and dried under vacuum.

e) PS-NVP-HDODA-HMPA support

4-Hydroxymethyl phenoxyacetic acid (1.89 g, 10 mmol), HOBt (2.2 g, 20 mmol) and DCC (2 g, 10 mmol) were dissolved in DCM (10 ml) and shaken for 1 hour. DCU precipitated was filtered off. From the filtrate DCM was removed in vacuum and the HOBt active ester of 4-hydroxymethyl phenoxyacetic acid obtained was dried in vacuum. Aminomethyl resin (5 g, 0.24 mmol NH/g) was swelled in NMP (100 ml) for 1 hour. Excess NMP was removed by filtration. HOBt active ester of 4-hydroxymethylphenoxyacetic acid was added to swelled aminomethyl resin. After 1 hour resin was filtered, washed with NMP (3 × 30 ml), dioxane (3 × 30 ml), dioxane: H_2O (1:1)(3 × 30 ml), MeOH (3 × 30 ml) and dried in vacuum. The resin showed hydroxyl capacity of 0.16 mmol OH/g. IR (KBr):3380 cm^{-1} (OH), 1164 cm^{-1} (ether), 3400 cm^{-1} (NH), 1643 cm^{-1} (NHCO).

Fmoc-Gly- HMPA-PS-NVP-HDODA

Fmoc-Gly (475.6 mg, 1.6 mmol) dissolved in DCM (5 ml) was shaken with DCC (165 mg, 0.8 mmol) dissolved in DCM (5 ml). After 1 hour, DCU was filtered off. Solvent was evaporated from the filtrate under vacuum to obtain the anhydride which was further dried in vacuum. HMPA-resin (1.00 g, 0.16 mmol OH/g) was swelled in DMF (100 ml) for 1 hour. Excess DMF was removed from the resin. Fmoc-Gly anhydride in DMF (5 ml) was added to the swollen resin followed by DMAP (19.5 mg, 0.16 mmol) and the reaction mixture was shaken for 1 hour at room temperature. Resin was filtered, washed with DMF (3 x 40 ml), isoamyl alcohol (3 x 20 ml), acetic acid (3 x 20 ml), and dimethyl ether (3 x 40 ml), and then dried in vacuum. Remaining free hydroxyl functional groups of the HMPA resin was masked by shaking it with acetic anhydride (1 ml) and DMAP (19.5 mg, 0.16 mmol) for 1 hour. Resin was filtered and purified by the above washing procedure. Resin was found to have a capacity of 0.14 mmol Gly/g as estimated by measuring OD at 290 nm of a 10 mg resin suspended in 3 ml of 20% piperidine solution in DMF for 30 minutes.

Synthesis of peptide using Fmoc- amino acids

Different peptides were synthesised on corresponding C-terminal amino acid attached swollen HMPA and HMPB resins in DMF using a manual peptide synthesiser. Fmoc protection was removed using 20% piperidine solution in DMF (25 ml x 20 min), followed by washing the resin with DMF (3 x 25 ml). Coupling reactions were carried using the respective amino acids (3.5 meq excess with respect to amino capacity of C-terminal amino acid attached HMPB resin) with a mixture of HBTU (3.5 meq), HOBt (7 meq) and DIEA (3.5 meq) in DMF for 50 minutes. The resin was washed with DMF (3 x 20 ml). Cleavage of Fmoc protection and

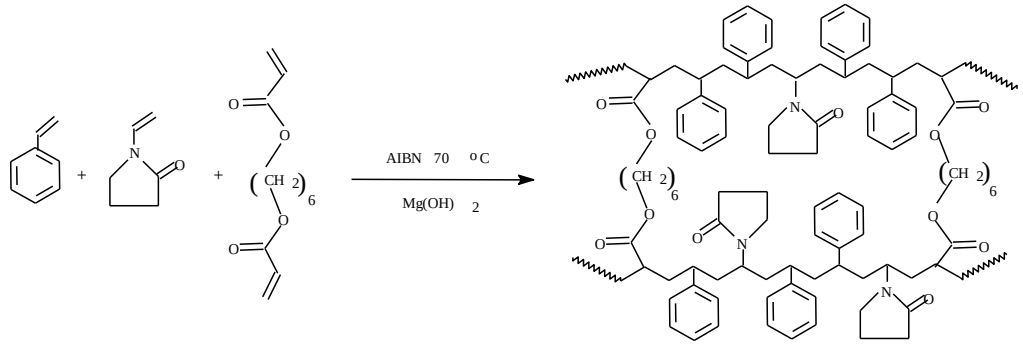
extent of coupling in each cycle was monitored by Kaiser semi-quantitative ninhydrin test. The following sequence of operations were carried out for the introduction of each amino acid residue: (a) washing with DMF (4 x 25 ml), (b) washing with 20% piperidine in DMF (1 x 25 ml), (c) deprotection with 20% piperidine in DMF (1 x 25 ml x 20 min), and (d) washing with DMF (4 x 25 ml). Acylation was carried out with 3.5 mmol excess of Fmoc-amino acid, HBTU, DIEA and 7 mmol excess of HOBt relative to amino capacity of C-terminal amino acid present in the HMPB resin. After the incorporation of all amino acids, Fmoc protection of N-terminal amino acid in peptide resin was removed using 20% piperidine solution in DMF (25 ml x 20 min). Finally, the peptidyl-resin was washed with DMF (5 x 25 ml), isopropanol (5 x 25 ml), ether (5 x 25 ml) and dried in vacuum.

Synthesis of acyl protein fragment (65-74)

PS-NVP-HDODA-HMPA-Gly-Fmoc(100mg,0.01mmol Gly), PS-DVB-HMPA-Gly-Fmoc resin (58g, 0.01 mmol Gly) and Sheppard resin (105 mg, 0.01 mmol Gly) were used for the comparative synthesis ofacyl carrier protein fragment (65-74). The resins were taken in a manual peptide synthesiser and swelled in DMF (50 ml) for 1 hour. The Fmoc protection was removed by treating the resins with 20% piperidine/DMF (10 ml) solution for 20 minutes. The resin was washed thoroughly with DMF (6× 10 ml). For each acylation cycle the respective Fmoc-amino acids (0.1 mmol each), Val (33.9 mg, 0.1 mmol), Gln (61.1 mg, 0.1 mmol), Ala (31.1 mg, 0.1 mmol), Ile (35.3mg, 0.1 mmol), Asp (41.15 mg, 0.1 mmol), Tyr (45.96 mg, 0.1 mmol), Asn (59.7 mg, 0.1 mmol), Gly (29.7 mg, 0.1 mmol), HBTU (30.76 mg, 0.1 mmol), HOBt (13.5 mg, 0.1 mmol), and DIEA (17.41 µl, 0.1 mmol), in minimum amount of DMF, were added to Fmoc-deprotected resins. Coupling solutions were made at triple and apportioned equally into the three synthesisers. The coupling reactions were carried out for 1 hour at room temperature. The coupling and deprotection steps were monitored by ninhydrin test. After incorporation of all the amino acids, Fmoc-protection of the N-terminal amino acid of the target peptidyl resins was removed and resins were washed thoroughly with DMF (5 × 5 ml), methanol (5 × 5 ml), ether (5 × 5 ml) and dried under vacuum. HPLC analysis of the peptides were carried out by injecting a small amount of peptide dissolved in water to C-18 RPC and eluted using a gradient of Buffer A: water containing 0.5% TFA and Buffer B: MeCN in water containing 0.5% TFA.

Results and Discussion

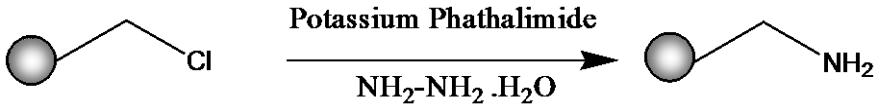
The crosslinked polymer was synthesised by free radical aqueous suspension copolymerisation of the monomers styrene, N-vinylpyrrolidone and 1,6-hexanediol diacrylate. The amount of these monomers was selected according to the mole ratios required to make a definite percentage polymer. Magnesium hydroxide and sodium sulphate were added to the suspension medium .Mechanical stirring was provided to form small uniform droplets of the dispersed monomer mixture suspended in the non solvent phase. The polymerisation reaction was initiated by adding radical initiator AIBN. It got solubilised in the monomer droplets and promoted the thermally induced polymerisation reaction. The temperature of the medium was raised to 70^{o}C to initiate the polymerisation process and the medium was kept at this temperature till the polymerisation was completed. The bead size distribution of the polymer was found to be affected by the stirring rate, geometry of the reaction vessel and amount of the stabiliser.



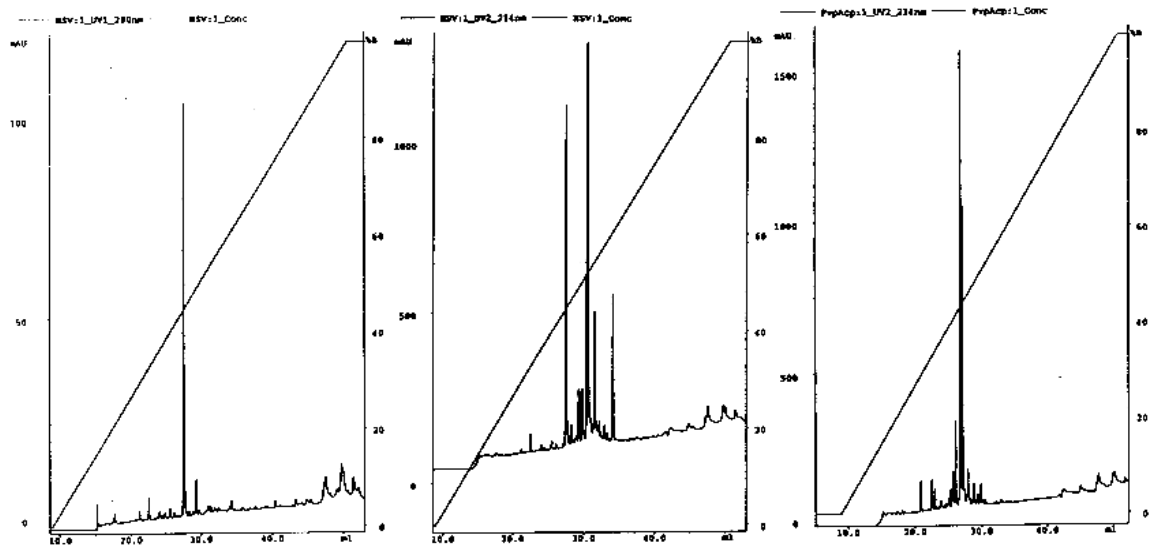
Synthesis of PS-NVP-HDODA polymer

The shape and speed of the paddle together with the shape of the polymerisation vessel influences the formation of droplets where the polymerisation takes place which in turn determine the size and quality of beads. The non solvent phase may be thought of as providing millions of tiny 'spherical moulds' in which the polymer beads formed are constrained by surface tension. Stabilisers helps to reduce the surface tension of the droplets and this prevent droplet aggregation which leads to distorted beads. The indentation force resulting from geometry of the vessel, forces the suspended mixture towards the rotating stirrer blade. This can lead to a homogeneous shearing environment for the monomer droplets that create droplets of uniform size. Reproducible droplet of the monomers with 200-400 µ size was

obtained by adjusting the stirring speed between 1500-2000rpm. When the speed increased beyond 3000 rpm polymer yield was found to decrease considerably. This may be due to the excessive shearing of the polymer bead. After each round of polymerisation, it was observed that the next reaction proceeds smoothly only if the reaction vessel was silanised. By the careful adjustment of the above discussed parameters, yield and size of the new PS-NVP-HDODA polymer can be reproduced. Chloromethylation was found to proceed smoothly when anhydrous $ZnCl_2$/THF was used as the catalyst. The degree of functionalisation was estimated by Volhardt's method.Chloromethyl resin was converted to aminomethyl resin by Gabriel's phthalimide reaction using potassium phthalimide followed by hydrazinolysis. The extent of conversion was determined by picric acid method.The amino capacity measurement showed that the conversion was almost quantitative. The IR (KBr) spectrum of the polymer showed absorption at 3400 cm^{-1} corresponding to the amino group. The functionalised supports were then well suited for polypeptide synthesis.



The efficiency of the new PS-NVP-HDODA-HMPA-OH resin as a solid support for polypeptide synthesis was further tested by the synthesis of ACP (65-74), H-VQAAIDYING-OH, fragment with commercially available PS-DVB-HMPA-OH and Sheppard resins (Novasyn® KA 125) using Fmoc chemistry under the same experimental conditions. This sequence exemplified many of the sequence dependant problems encountered in the course of solid phase synthesis. These problems caused poor synthetic results that could be traced back to the internal development of secondary structure which were competing with the desired amide bond formation. All the coupling reactions were carried out using three equivalent excess (with respect to Gly load) of respective Fmoc-amino acid, HOBt, HBTU and DIEA. The total amount of these reagents required for the incorporation of an amino acid in these resins were weighed together, dissolved in DMF and then distributed equally to each synthesiser. After the synthesis, N-terminal Fmoc protection was removed and then the peptide was cleaved from the corresponding resin under the same cleavage conditions. The ACP fragment obtained was dissolved in equal volume of HPLC buffer A and the purity was analysed by injecting it to reverse phase HPLC Sephasil Peptide C-18 column. HPLC analysis of the crude ACP-peptides obtained from different resins are shown in fig-a, b & c.



a **b** **c**

HPLC elution profile of ACP fragment peptide. The resins are (a) PS-NVP-HDODA-HMPA-OH, (b) PS-DVB-HMPA-OH and (c) Sheppard resin Buffer A: 0.5 ml TFA in 100 ml water; Buffer B: 0.5 ml TFA in 100 ml acetonitrile: water (3:1). Flow rate: 1 ml/min. Gradient: 0% B to 100% B in 40 minutes

It is evident that the average purity of ACP synthesised vary only marginally in PS-NVP-HDODA and Sheppard resin compared to Merrifield resin, showing the effectiveness of the new PS-NVP-HDODA resin in solid supported polypeptide synthesis. The eluting fractions corresponding to different peaks of each peptide, obtained from the different resins were collected and analysed by amino acid analysis. From the HPLC profile,

the peak area corresponding to ACP fraction showed that the yield of the pure peptide was only about 59% in PS-DVB resin, about 80% in Sheppard resin whereas PS-NVP-HDODA yielded over 90% under the same synthetic conditions. The poor swelling of PS-DVB resin in the reaction medium and aggregation of the peptide chain during the synthesis led to incomplete acylation and deprotection reactions resulting in the contamination of the target peptide with truncation and deletion peptide sequences. Yield of crude peptide from PS-NVP-HDODA resin = 40.1 mg (90%). Amino acid analysis: Gly, 1.06 (1); Ile, 1.98 (2); Tyr, 0.68 (1); Asp, 2.03 (2); Ala, 2.07 (2); Glu, 0.96 (1); Val, 1.0 (1). Low value of Tyr is due its partial degradation. Gln and Asn were hydrolysed to Glu and Asp. Yield of crude peptide from PS-DVB resin=27 mg (59%) and Sheppard resin=33.2 mg (80%).

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