Journal of Peptide Science
J. Peptide Sci. **5**: 577–581 (1999)

Solid Phase Synthesis of Hydrophobic Peptides on 1,6-Hexanediol Diacrylate Cross-linked Polystyrene Resin

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Received 23 July 1999
Accepted 31 July 1999

Abstract: The synthesis of three hydrophobic peptides, which are partial sequences of thioredoxin, on a newly developed, flexible 1,6-hexanediol diacrylate cross-linked polystyrene, in good yield and purity, is described. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: flexible support; hydrophobic peptides; PS-HDODA resin; solid phase peptide synthesis

INTRODUCTION

The synthesis of hydrophobic peptides is a difficult process because of the non-polar side-chains and because of the coiling nature of the peptides [1]. Peptides that have substantial hydrophobic character also tend to aggregate with increasing concentration [2]. Another related factor is the low solubility of these peptides, which may be a limiting factor in some biological test systems [2].

The design and development of polymer supports having optimum hydrophobic–hydrophilic balance for solid phase peptide synthesis is difficult. Innumerable investigations dealing with the quantitative aspects of polymer supported reactions during the last two decades have shown that the insoluble support has a significant influence on the bound substrates. The success of solid phase synthesis depends on the swelling characteristics of the polymer and the solvation of the peptidyl resin in different solvents [3]. Hence, polymeric systems that swell and solvate to a high degree in solvents used for peptide synthesis have high reactivities. Based on this principle, polystyrene (PS) cross-linked with 1,6-hexanediol diacrylate (HDODA), with a hydrophobic–hydrophilic balance optimized based on the extent of cross-linking, has been developed for the synthesis of peptides [4]. In the present study, three hydrophobic partial sequences of thioredoxin were synthesized on a 2% cross-linked polymeric system.

Thioredoxin is a naturally occurring sulphur reducing protein containing 108 amino acid residues [5]. It has a lot of secondary structure (approximately 38% α-helix and 28% β-structure) [6]. It contains sequences of varying hydrophobic–hydrophilic patterns. The following hydrophobic partial sequences of thioredoxin (T) were synthesized:

(A) Ala-Ile-Leu-Val-Asp-Phe-Trp-Ala (T 22–29)
(B) Ile-Gly-Arg-Gly-Ile-Pro-Thr-Leu-Le-Leu-Phe (T 71–81)
(C) Thr-Leu-Leu-Leu-Phe (T 77–81)

These peptides were synthesized by following standard solid phase techniques. The synthesized peptides were purified using high performance liquid chromatography (HPLC), fast protein liquid chromatography (FPLC) and subjected to amino acid analysis.

EXPERIMENTAL

Materials and Methods

Styrene, HDODA, side-chain protected amino acids, dicyclohexyl carbodiimide (DCC), trifluoroacetic

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CCC 1075–2617/99/120577-05$17.50

acid (TFA) and thioanisol were purchased from Sigma. Simple Boc-amino acids were prepared according reported procedures [7,8]. Chloromethyl methylether (CMME) was prepared using the literature procedure [9].

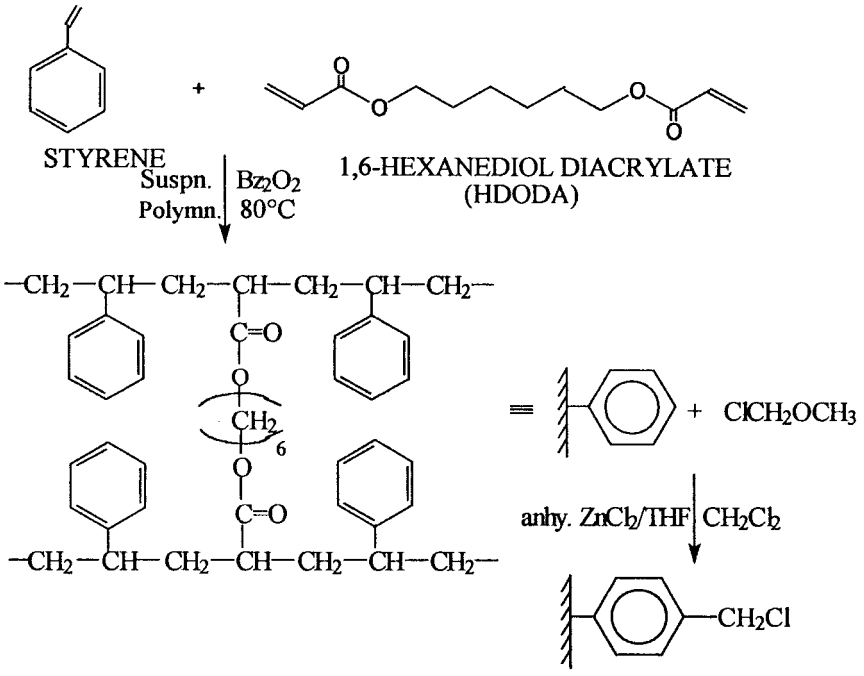
Polymer Synthesis

Preparation of HDODA cross-linked polystyrene. The polymer was prepared using the usual suspension polymerization method [10]. Styrene was destabilized by washing with 1% sodium hydroxide solution (3 × 20 ml) and then washing with distilled water (3 × 20 ml). It was then dried over anhydrous calcium chloride. A 1% solution of polyvinyl alcohol (PVA; molecular weight 75000; 1.1 g) in water (110 ml) was prepared and kept mechanically stirred at 80°C. A mixture of styrene (11.2 ml), HDODA (0.44 ml), toluene (as inert diluent; 8 ml) and benzoyl peroxide (500 mg) was prepared and added to the PVA solution being kept at 80°C with stirring. The polymerization was complete within 6 h. The white shining beads obtained were collected by filtration and washed thoroughly with hot water (20 ml, 3 × 3 min), acetone (20 ml, 3 times × 3 min), methanol (20 ml, 3 × 3 min) and dried. The resin obtained was soxhleted using acetone to remove all low molecular weight impurities and linear polymers and then dried. The yield of the polymer was 10 g. The beads were sieved into different sizes using standard sieves; 200–400 mesh size beads were used for the synthesis of the peptides (Scheme 1).

Functionalization of PS-HDODA support with chloromethyl groups (11). The dry resin beads (2 g, 200–400 mesh size) were pre-swollen in a dry 2-necked round bottomed flask using dry dichloromethane (DCM) (12 ml). A solution of anhydrous zinc chloride (1 M, 0.2 ml), dissolved in tetrahydrofuran (THF), was added to chloromethyl methylether (12 ml) and this solution was slowly added to the resin under anhydrous conditions, with shaking. The mixture was refluxed at 50°C with a calcium chloride guard tube for 5 h. The mixture was cooled, filtered and washed with THF (30 ml; 3 × 10 min), THF/4 N HCl (30 ml; 3 × 3 min), THF/water (30 ml; 3 × 3 min), THF (30 ml; 3 × 3 min), DCM (30 ml; 3 × 3 min) and finally methanol (30 ml; 3 × 3 min) drained and dried (Scheme 1). The chlorine capacity was determined by the pyridine fusion method [12].

Swelling and solvation studies. Swelling studies conducted using the PS-HDODA resins and the corresponding chloromethyl resins show that they have better solvation properties than the Merrifield (DVB-PS) resin (Table 1).

These results indicate that PS-HDODA resin has almost double the swelling capacity in all the



Scheme 1 Preparation of HDODA cross-linked polystyrene by suspension polymerization and functionalization.

Table 1 Swelling capacity of DVB-PS and HDODA-PS resins in solvents used for peptide synthesis

Solvent	DVB-PS (ml/g)	HDODA-PS (ml/g)
DCM	5.2	10.0
DMF	3.5	7.2
MeOH	0.95	2.0
NMP	4.0	9.1

solvents used for peptide synthesis. High values of swelling capacity mean easy accessibility of functional groups by reagents and chemicals, which could eventually lead to enhanced reactivity.

General procedure for solid phase peptide synthesis. Solid phase peptide synthesis (SPPS) was carried out manually in a glass reaction vessel. Peptides were synthesized using this polymer support by following the conventional Boc-benzyl ester strategy of Merrifield [13]. The first amino acid was attached to the chloromethyl resin by Gisin's [14] cesium salt method and the amino capacity was determined by the picric acid method [15]. Subsequent amino acids were assembled in a stepwise manner to form the desired sequence by the DCC method [16]. The Boc group was deprotected using 30% TFA in DCM and neutralization was carried out by using 5% triethylamine (TEA). *N*-methyl pyrrolidone (NMP) was used as the solvent and the coupling time was 1 h. The same procedure was adopted for the coupling of all the remaining amino acids. Progress of the coupling was monitored at every stage using the Kaiser test [17]. In all the couplings a 3-fold molar excess of Boc-amino acid was used and the precipitated dicyclohexyl urea (DCU) was removed by washing with 33% MeOH in DCM. The final cleavage of the peptide from the support was effected by the TFA/thioanisol methods [18]. The benzyl side-chain protecting group was removed by hydrogenation of the crude peptide using activated palladium charcoal in methanol under hydrogen for 24 h [19].

Purification of Peptides

The crude peptides were purified by HPLC and FPLC. FPLC was performed on a Pharmacia instrument on a C-18 reverse phase, semi-prepared FPLC column, using solvent system A: 0.1% TFA in water and B: 0.1% TFA in CH_3CN, and detection was at 214 nm. HPLC was performed on a C-18 (10 μ) column using acetonitrile–water (CH_3CN–H_2O) gradient elution. The solvent system used was 80% CH_3CN–20% H_2O (0.1% TFA) and 100% H_2O (0.1% TFA). The gradient used was 5–45% CH_3CN–H_2O in 40 min. The major peak was collected, the solvent evaporated from it and lyophilized to get the pure peptide as a white powder.

Amino Acid Analysis

Amino acid analysis was performed on a pharmacia LKB Alpha plus amino acid analyser after hydrolysing the samples. For this the free peptide (1 mg) was taken in a sample tube and a mixture of TFA:6N HCl (1:2) was added to it. The tube was sealed under vacuum and heated at 110°C for 24 h. The residue was dried and dissolved in the amino acid loading buffer. This was then applied to the amino acid analyser.

RESULTS AND DISCUSSION

Synthesis of (A)

Ala-Ile-Leu-Val-Asp-Phe-Trp-Ala (T 22-29)
| |
cHex CHO
(cyclohexyl ester)

This peptide was synthesized on a 2% HDODA-PS resin. Boc-Ala was attached to the chloromethylated resin (capacity 1.91 mmol/g) by the cesium salt method. The first amino acid capacity was determined by the picric acid test and was found to be 1.8 mmol/g. A further 200 mg of Boc-Ala resin was used for later synthesis, DCC was the coupling agent and NMP was the solvent medium used for all the attachments. The completion of coupling was checked by the Kaiser test. Most of the couplings were completed by the first coupling. However, in all cases, a double coupling was performed to ensure complete coupling. The finished peptide was cleaved from the support by the TFA/thioanisol method.

Homogeneity of the crude peptide was checked on FPLC and got only one major peak. This was further purified by FPLC and the crude and purified profiles are shown in Figure 1. Purity: $>90\%$, yield: 85%. The pure peptide was subjected to amino acid analysis.

Amino acid analysis:

Ala 2.20 (2.00), Ile 0.9 (1.0), Leu 0.8 (1.0), Val 1.0 (1.0), Asp 1.2 (1.0), Phe 1.1 (1.0).

Trp was lost during hydrolysis.

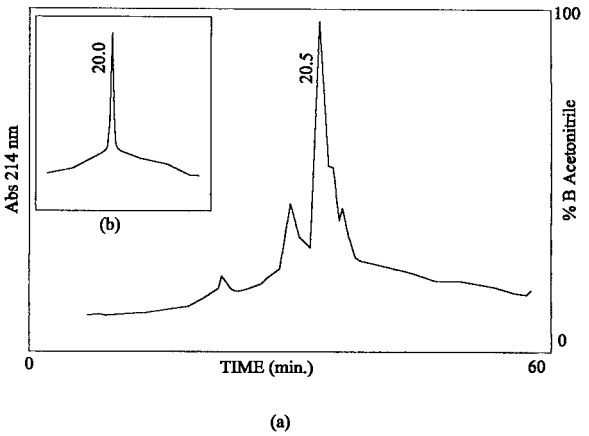


Figure 1 FPLC profiles of Ala-Ile-Leu-Val-Asp-Phe-Trp-Ala; (a) crude (b) purified. Conditions: solvent A, water containing 0.1% TFA; solvent B, CH_3CN containing 0.1% TFA; flow rate, 0.5 ml/min; detection, 214 nm.

Synthesis of (B)

Ile-Gly-Arg-Gly-Ile-Pro-Thr-Leu-Leu-Leu-Phe (T 71-81)
Mts
(4-Methoxy-2,3,6 -
Trimethylbenzenesulfonyl)
OBzl (benzyl ester)

Chloromethylated 2% HDODA cross-linked PS resin with a chlorine capacity of 2.01 mmol/g was used for the synthesis of this hydrophobic sequence. After attaching Boc-Phe to the resin the substitution level obtained was 1.9 mmol/g. The DCC method was used for the coupling of all the remaining amino acids and NMP was the solvent. Here, as for (A), most of the couplings were completed by the first coupling. However, in all cases, a second coupling was performed to ensure complete coupling. The finished peptide was obtained by the TFA/thioanisol method.

The purity of the crude peptide was checked with HPLC and was found to be $>90\%$ pure. The product was again purified and the HPLC profiles are shown in Figure 2. Yield: 90%. Homogeneity of the peptide was confirmed by amino acid analysis.

Amino acid analysis:

Ile 2.30 (2.00), Gly 1.67 (2.00), Arg 0.90 (1.00), Pro 0.98 (1.00), Thr* 0.40 (1.00), Leu 2.89 (3.00), Phe 1.00 (1.00).

* Thr was lost during hydrolysis.

Synthesis of (C)

Thr-Leu-Leu-Leu-Phe (T 77-81)
OBzl
(benzyl ester)

For the synthesis of this pentapeptide, the same Boc-Phe resin used for the synthesis of (B) was

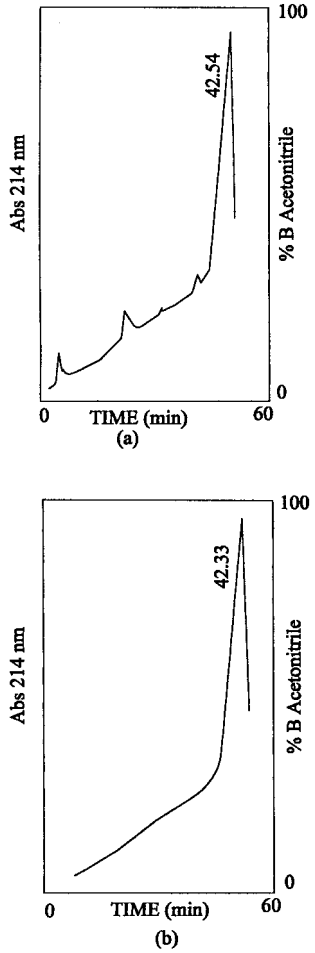


Figure 2 HPLC profiles of Ile-Gly-Arg-Gly-Ile-Pro-Thr-Leu-Leu-Leu-Phe; (a) crude (b) purified. Gradient used: 5–45% CH_3CN water in 40 min.

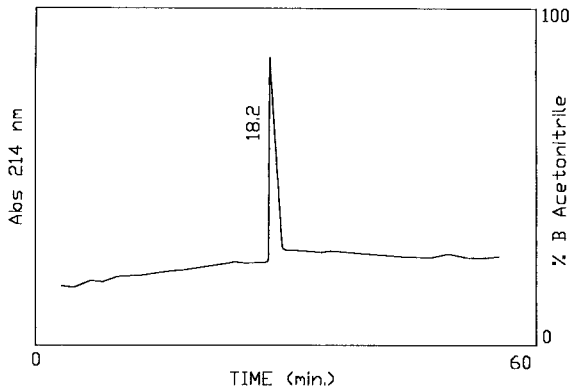


Figure 3 FPLC profile (crude) of Thr-Leu-Leu-Leu-Phe. Conditions: solvent A, water containing 0.1% TFA; solvent B, CH_3CN containing 0.1% TFA; flow rate, 0.5 ml/min; detection, 214 nm.

used. For all the attachments, the DCC method of coupling in NMP was used. Here also all the couplings were completed by the first coupling. The final peptide was cleaved from the support by TFA/thioanisol.

Purity of the crude peptide was checked on FPLC and was found to be 100% pure. The FPLC profile of the crude peptide is shown in Figure 3. Yield 90%. This peptide was subjected to amino acid analysis and the values are in good agreement with theoretical values.

Amino acid analysis:

Thr* 0.51 (1.00), Leu 2.85 (3.00), Phe 0.90 (1.00)

Thr* was lost during hydrolysis.

CONCLUSION

Three hydrophobic peptides were synthesized in good yield and purity on a 2% PS-HDODA resin. The synthesis of hydrophobic peptides is a difficult process because of the non-polar side-chains and because of the coiling nature of the peptides. All the peptides were synthesized using the standard solid phase technique. Homogeneity of the peptides was confirmed by HPLC and FPLC and was characterized by amino acid analysis. The polymer support exhibits good swelling in all the solvents used for synthesis and the flexible nature of the support enhances the reactivity. From the high yield and purity of the peptides it is inferred that PS-HDODA resin is suitable for the synthesis of hydrophobic peptides.

Acknowledgements

The authors are grateful to Professor P. Balaram, Indian Institute of Science, Bangalore, for providing analytical facilities and them (JTV) is grateful to the CSIR for financial support.

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