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Synthesis of Modified Neurotensin on Poly(styrene-co-hexanediol diacrylate) Resin: Quantification of Native Neurotensin in Brain During Diabetics Using Synthetic Peptide

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Quantification of neurotensin isolated from brain stem, cerebral cortex and hypothalamus of controlled and diabetic rats were carried out. The neurotensin peptide has been synthesized on a 2 % hexanediol dimethacrylate cross-linked polystyrene (PS-HDODA) support. The PS-HDODA support was prepared by aqueous free radical suspension polymerization of styrene and 1,6-hexanediol dimethacrylate. The resin was functionalized with aminomethyl group under controlled conditions. The resin with 0.2 mmol/g amino functionality was used for the synthesis of peptide. The acid labile linker, HMPB was incorporated to the resin and the peptide synthesis proceeds following the Fmoc-synthetic strategy. The peptide was purified by HPLC and the identity was exposed by amino acid analysis and mass spectroscopy. The pure peptide was employed for the construction of a standard working curve and the concentration of neurotensin in brain stem, cerebral cortex and hypothalamus of controlled and diabetic rats were determined from the HPLC peak area of the isolated peptide and the working curve. The neurotensin concentration decreases in brain stem and hypothalamus where as it increases in cerebral cortex during diabetics.

Key Words: Solid-phase peptide synthesis, Neurotensin, Diabetics, PS-HDODA.

INTRODUCTION

Neurotensin is a peptide comprising 13 amino acid residues, originally isolated from calf hypothalamus[1]. Neurotensin has a dual function of neurotransmitter or neuromodulator in the nervous system and of local hormone in the periphery. The biochemical and pharmacological properties of neurotensin in the brain and in peripheral organs have been well documented[2-6]. In the last decade, several studies with synthetic analogues of neurotensin have been reported for investigating structure-activity relationship, resulting in some clear conclusions that includes the sequence

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could be truncated at the N-terminus without considerable decrease of biological activity[7] and the C-terminal residue of the peptide could not be removed, amidated[8] or N-methylamidated[9] without a complete loss of biological activity. Junod *et al.*[9] after synthesizing a series of neurotensin analogues in which each residue was systematically replaced by its D-isomer, found that the important amino acids for binding to mast cells were located in the C-terminal part of the molecule. In order to elucidate the broad spectrum of results and determine the smallest chain length required for full activity, new well-defined truncated sequences of neurotensin were synthesized by solid phase technology.

The success and prospective of solid-phase peptide synthesis mainly depends on the appropriate choice of polymeric support and the immobilization of the C-terminal amino acid to the support. Classical Merrifield's solid-phase peptide synthesis[10] performed on low cross-linked hydrophobic polystyrene-divinylbenzene beads have sequence dependent coupling difficulties[11]. In polymeric supports used for solid-phase synthesis, more than 90 % of reactive functional groups are buried interior of the beads[12]. The highly solvated polymeric supports facilitates the squeezing of the reactants into the interior of the resin which improves the reaction between the reactants and the reactive functional groups in the support. The high swelling and solvation capability of the resin enables all excess reactants to be removed by simple wash procedures. The shortcomings of Merrifield resin are overwhelmed by the introduction of various hydrophilic-hydrophobic, polar and non-polar, grafted and immobilized and natural carbohydrate resins. The various resins presented the last two decades includes derivatized PEG grafted polystyrene-divinylbenzene, monomethyloxy PEG grafted polystyrene-divinylbenzene (PEG-PS), PEGA, PS-TTEGDA, PS-BDODMA, PS-TEGDMA, PEO-PEPS, CLEAR, POEPOP, POEPS, POEPS-3, SPOCC, CLPSER, HYDRA and GDMA-PMMA[13-27]. The carbohydrate resins include cotton and chitin was also used as supports for solid phase synthesis[28,29]. The hydrophilic and hydrophobic balance is an essential factor that determines the mechanical stability and swelling capability of the solid support. The styrene-based supports exhibited high mechanical stability and making use of this property, a resin was developed by the co-polymerization of styrene with 1,6-hexanediol diacrylate (PS-HDODA)[30]. The presence of hydrophilic HDODA improves the hydrophilic property of the polystyrene matrix. The solid support 2 % PS-HDODA polymer was prepared by aqueous suspension polymerization of the monomers in suspension stabilizer polyvinyl alcohol. This polymer support has low cross-linking and showed very high chemical stability[31]. Functionalized resin is highly solvated in different solvents resulting in a gel-like appearance. This property of the polymer makes it an efficient support as it allows effective diffusion of solution phase reagents to polymer bound functional groups and thus enhances the various organic reactions performed on the resin to completion. A modified procedure for the PS-HDODA resin synthesis, neurotensin peptide synthesis on PS-HDODA resin and the quantification of neurotensin during diabetics are described in this report.

EXPERIMENTAL

Styrene, HDODA, polyvinyl alcohol (PVA, MW~75000), trifluoro acetic acid (TFA), chloromethyl phathalimide, piperidine, thioanisole, ethanedithiol, diisopropyl ethylamine (DIEA) and phenol were obtained from Aldrich Chemical Co., USA. 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluoro phosphate (HBTU), 1-hydroxybenzotriazole (HOBt), Dhbt-OH, Fmoc-amino acid-OPfp esters and 4-hydroxymethyl 3-(methoxy)phenoxy butyric acid (HMPB) were purchased from Novabiochem Ltd., UK. All solvents used were of HPLC grade.

IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C CP-MAS solid-state NMR measurements were conducted on a Bruker 300 MSL CP-MAS instrument operating at 75.47 MHz. HPLC was done on a Pharmacia Akta purifier instrument using C-18 reverse phase HPLC column. The amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyzer. For this the peptide was hydrolyzed using 6 N HCl in a Pyrex glass tube fused under nitrogen for 15 h at 130 ºC. Mass spectra of peptides were obtained with a Kratos PC-Kompact MALDI TOF MS instrument.

Polymer synthesis and functionalization: The styrene and HDODA were washed with 1 % NaOH in distilled water to remove the inhibitors. A four-necked reaction vessel equipped with a thermostat, Teflon stirrer, water condenser and argon inlet was used for the polymerization process. A 0.5 % polyvinyl alcohol (PVA, MW~75000) in nanopure water (0.55 g PVA in 110 mL water) along with calcium phosphate (50 mg) and calcium sulphate (50 mg) was used as the suspension stabilizer. The solution was added to the reaction vessel and deoxygenated by purging argon gas. The monomer mixture (styrene, 10.41 g and HDODA, 0.46 g) in toluene (9 mL) was added to the reaction vessel. The aqueous-organic medium in the vessel was stirred at a constant rate of 1000 rpm with a mechanical stirrer. The free radical initiator benzoyl peroxide (0.25 g) in toluene (1 mL) was added to the reaction mixture after 2 min. The reaction vessel was sealed with rubber septum and the system was kept under argon atmosphere. The temperature of the reaction mixture was kept at 70 ºC and the reaction was allowed to continue for 5 h. The copolymer was obtained as white shiny beads. The beads were washed thoroughly with hot water (to remove the stabilser), acetone (6 × 30 mL), benzene (6 × 30 mL), toluene (6 × 30 mL) and methanol. The copolymer was further purified by Soxhlet extraction with acetone and methanol and dried under vacuum and kept over P_2O_5 to yield 8.3 g of dry resin (87 %). IR (KBr, cm^{-1}): 1720, 1484, 752 and 699. ^{13}C CP-MAS NMR: 40.83, 76.21, 128.33, 146.6 and 180.04 ppm.

The PS-HDODA resin (5 g) was swelled in DMF (50 mL). After 1 h, excess DMF was removed and the swollen polymer was transferred to a three-necked 100 mL round bottom flask equipped with argon inlet, addition funnel, mechanical stirrer and reflux condenser and heating mantle. Chloromethyl phthalimide (195.6 mg, 1 mmol) and anhydrous $ZnCl_2$ (0.1 M in dry THF, 1 mL) were dissolved in DMF (30 mL) and the mixture was added to the resin. The suspension was refluxed

for 4 h under argon atmosphere. The reaction mixture was cooled and the resin was filtered and washed with dichloromethane (5 × 30 mL), dioxane (5 × 30 mL), ethanol (5 × 30 mL) and methanol (5 × 30 mL). The dried resin was suspended in ethanol (20 mL) and hydrazine hydrate (100 mL, 2 mmol) was added. The reaction mixture was refluxed for 8 h. The resin was filtered and washed with ethanol (5 × 30 mL) and methanol (5 × 30 mL). The product resin was dried in vacuum. Amino capacity of the resin measured as 0.19 mmol/g by the picric acid titration method[32]. IR (KBr, cm^{-1}): 1720, 1523, 1484, 753 and 699. ^{13}C CP-MAS NMR: 40.83, 46.09, 76.21, 128.33, 146.33, 146.6 and 180.04 ppm.

HMPB linker attachment: Amino resin (1 g, 0.19 mmol) were pre-swelled in 10 mL anhydrous dichloromethane for 0.5 h at room temperature. HMPB linker (137 mg, 0.57 mmol) was dissolved in dichloromethane (5 mL). Mixed DIEA (99.3 μL, 0.57 mmol), HBTU (216 mg, 0.57 mmol) and HOBt (77.6 mg, 0.57 mmol) with the linker and kept for 5 min. The activated HMPB linker was added to the swollen resin, shaken well and kept at room temperature for 1 h. The quantitative reaction was estimated by Kaiser test[33]. The resin was filtered and washed with DMF (6 × 30 mL), dioxane/water (1:1, 6 × 30 mL), MeOH (6 × 30 mL) and ether (6 × 30 mL). The resin was collected and dried in a vacuum.

Attachment of Fmoc-Leu to HMPB resin: Fmoc-Leu (102 mg, 0.33 mmol), HBTU (125 mg, 0.33 mmol), HOBt (45 mg, 0.33 mmol) and DIEA (5 μL, 0.33 mmol) were added to the pre-swollen HMPB resin (600 mg, 0.11 mmol) in DMF. The reaction mixture was kept at room temperature for 1 h. The resin was filtered, washed thoroughly with DMF (6 × 30 mL), dichloromethane (6 × 30 mL), methanol (6 × 30 mL) and ether (6 × 30 mL) and dried in a vacuum. The extent of attachment of amino acid was estimated by adding a 20 % piperidine in DMF (3 mL) to 10 mg of the resin. After 20 min optical density (OD) of the solution was measured at 290 nm. From the OD value the amino capacity of the resin was calculated.

Synthesis of neurotensin (1-13) (H-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH): The peptide was synthesized manually in a disposable plastic syringe fitted with a Teflon filter and connected to a filter pump through a two-way Teflon valve. The peptide synthesis was carried out using fully protected Fmoc-amino acid OPfp esters (3 equiv; Fmoc-Ile-OPfp (156 mg), Fmoc-Tyr(tBu)-OPfp (188 mg), Fmoc-Pro-OPfp (151 mg), Fmoc-Arg(Pmc)-OPfp (249 mg), Fmoc-Lys(Boc)-OPfp (191 mg), Fmoc-Asn(Trt)-OPfp (229 mg), Fmoc-Glu(tBu)-OPfp (178 mg), Fmoc-Leu-OPfp (156 mg)) in DMF with the addition of Dhbt-OH (16.3 mg, 0.1 mmol, 1 equiv) as an acylation catalyst and the indicator of the end point of acylation. The reaction was quantitative in 2 h. The Fmoc group was removed by using 20 % piperidine in DMF. The PS-HDODA-HMPB-Leu-Fmoc resin (600 mg, 0.1 mmol) was swollen in DMF for 0.5 h. The solvent was drained off from the syringe and the N-terminal Fmoc-protection was cleaved off using 20 % piperidine in DMF. After the incorporation of all the amino acids in the sequence, the terminal Fmoc protection removed and the peptidyl resin was washed with DMF (6 ×),

DCM (6 ×) and dried under vacuum. The peptide was cleaved from the resin by suspending in TFA (2.6 mL), water (150 µL), phenol (150 µL), thioanisole (150 µL) and ethanedithiol (75 µL) for 2 h at room temperature. The resin was filtered, washed with TFA and dichloromethane and the combined filtrate was evaporated. The peptide was precipitated by adding ice-cold ether and washed thoroughly with ether to remove the scavengers added. This was then dissolved in 1-2 % acetic acid in water and passed through a Sephadex G-15 column. The peptidyl fractions were collected and lyophilized.

The amino acid analysis: Glu, 2.18 (2); Leu, 2.11 (2); Tyr, 1.89 (2); Asp, 0.93 (1); Lys, 0.95 (1); Pro, 1.92 (2); Arg, 1.83 (2); Ile, 1.05 (1) (Asn residue is hydrolyzed to Asp). MALDI TOF MS $(M+H)^+$, 1690.41 and the required mass is 1689.90.

Isolation of native neurotensin from controlled and diabetic rats: Wistar rats of *ca.* 200 g body weight were used for all experiments. The rats were housed in separate cages for 12 h dark periods. The animals were maintained on standard food pellets and water *ad libitum*. Diabetes was induced by a single intra-femoral dose of streptozotocin (STZ) prepared in citrate buffer, pH 4.5[9,34]. Animals were sacrificed by decapitation and the brain regions were dissected out according to reported procedure[34]. Tissues were homogenized in perchloric acid (0.4 N), centrifuged at 2500 rpm for 10 min and the supernatant was filtered and lyophilized.

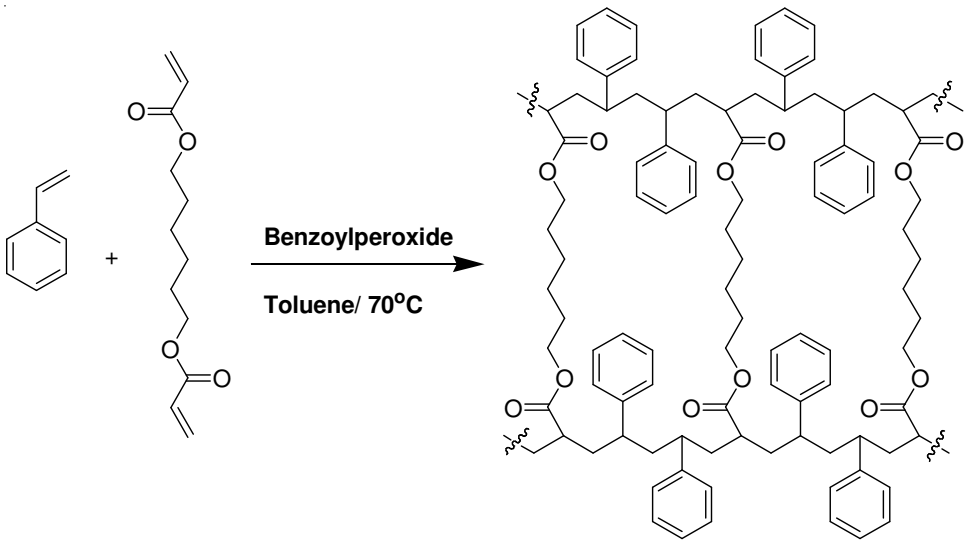
Purification of isolated lyophilized crude native neurotensin: Lyophilized crude peptides were subjected to gel filtration on Sephadex G-15 (200 × 4 cm column: eluted with 0.1 M ammonium acetate, pH 8.6). The peptides were again purified by ion-exchange chromatography. Ammonium acetate elution gradients were used with initial and final buffer concentrations chosen as a function of the calculated electrical charge of the peptide at neutral pH. Column dimensions were 25 × 2.5 cm and a three vessel gradient forming device was used. Elution profiles were monitored by measuring absorbance at 230 nm, with a Beckman DB-GT spectrophotometer connected to a Gilson sampler.

Quantities of peptides were calculated from the mean residue value of stable amino acids after acid hydrolysis (6 M HCl, 15 h, 130 ºC) of an aliquot followed by amino acid analysis. The peptides were again purified by reverse-phase HPLC using C-18 column. Two homologous columns were fitted in series and run with 10 mM triethyl ammonium phosphate (pH 3)/methanol (50/50, v/v) at acetonitrile (74/26, v/v) at 1.5 mL/min. Peptides was dissolved in the eluting buffer and about 20 nmol were injected. Column effluents were continuously monitored by absorbance at 230 nm.

Construction of working curve: The synthetic neurotensin (1-13) peptide (1.0 mg) dissolved in water (1.0 mL). The solution was diluted to 100 µL. 0.25 mL (0.148 nmol), 0.5 µL (0.296 nmol), 0.75 µL (0.444 nmol), 1.0 µL (0.592 nmol) and 1.4 µL (0.823 nmol) of the standard solution of peptide were taken and the corresponding HPLC peak area was determined. A working curve was created from the HPLC peak area and the concentration of peptide.

RESULTS AND DISCUSSION

The 1,6-hexanediol diacrylate cross-linked polystyrene polymer support was synthesized by a modified procedure described in the literature[30]. A well-defined polymer with narrow distribution in size was prepared by the aqueous suspension polymerization technique (**Scheme-I**). The respective amount of monomers, styrene and 1,6-hexanediol diacrylate in a measured volume of diluent toluene were added to the suspension medium containing 0.5 % PVA in nanopure water containing calcium sulphate and calcium phosphate. Small uniform droplets of the monomer mixture was formed by the mechanical stirring of the reaction medium and after 2 min the free-radical initiator in toluene was added. The initiator becomes solubilized in the organic droplets and endorses the thermally induced polymerization. The polymer was obtained as spherical uniform beads and the surface is even and smooth (Fig. 1). Polymer beads of 100-200 μm sizes were obtained by adjusting the stirring speed at 1000 rpm. The polymer was characterized by IR and ^{13}C CP-MAS NMR spectroscopy. The IR spectrum of the polymer showed an intense sharp peak at 1720 cm^{-1} corresponding to the ester carbonyl of the cross-linker along with the normal peaks of polystyrene. Solid-state ^{13}C CP MAS NMR spectrum showed a sharp intense peak at 128.33 ppm corresponding to the aromatic polystyrene carbons and a small peak at 146.6 ppm corresponds to C-3 of styrene. The methylene carbon of the cross-linker shows a peak at 76.21 ppm and the peak at 40.83 ppm represents the backbone methylene carbon of the polymer. The ester carbon represents the peak at 180.04 ppm.



Scheme-I: Synthesis of PS-HDODA resin

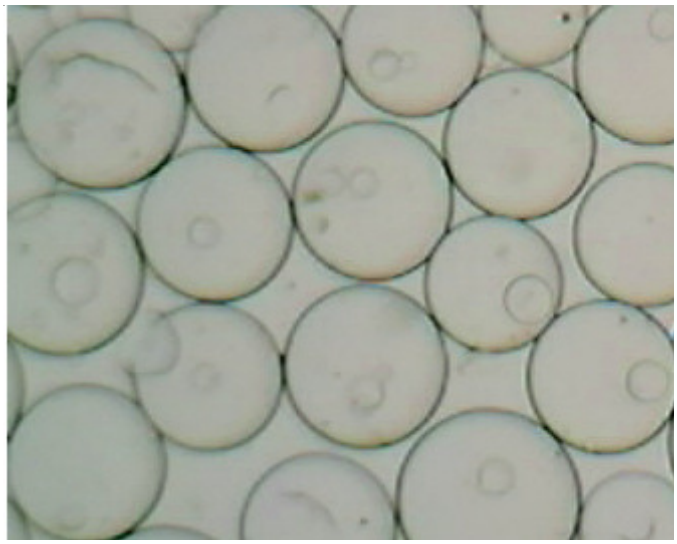
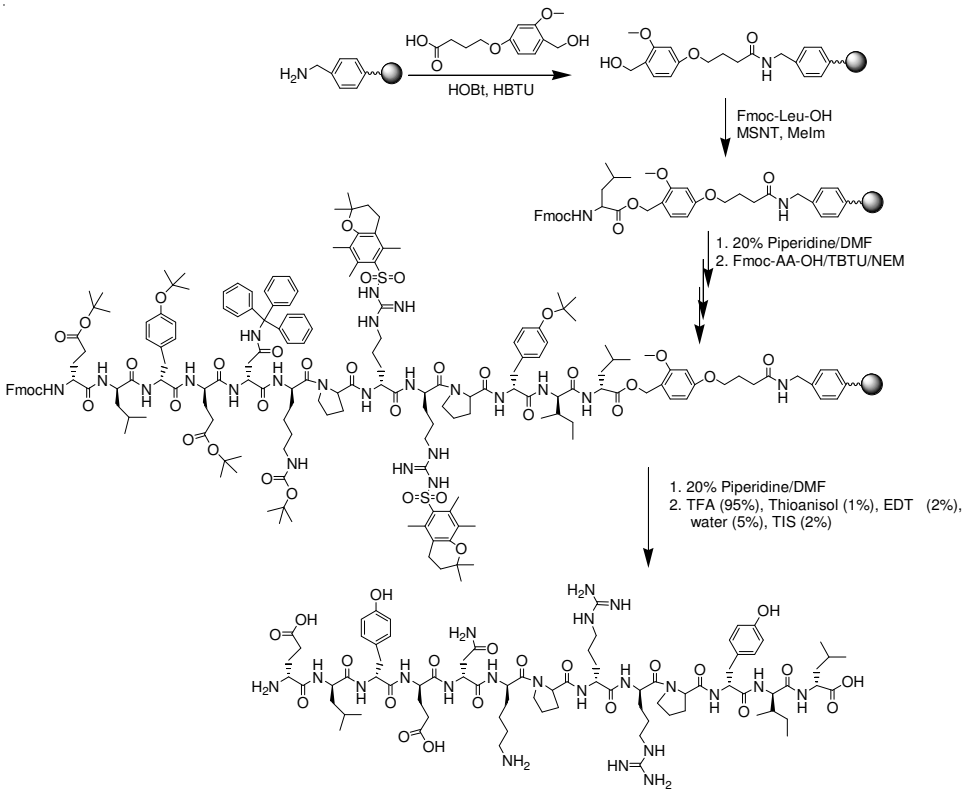


Fig. 1. Micrograph of PS-HDODA resin

The aminomethyl functionalized PS-HDODA support was prepared by the derivatization of preformed resin. The aminomethyl group was introduced to the resin by treating with chloromethyl phthalimide in presence of $ZnCl_2$. Amino functionality of the resin showed positive Kaiser test[32]. The amino capacity of the resin was estimated by picric acid titration method[31]. IR and ^{13}C CP-MAS NMR spectroscopic methods are used for the characterization of the resin. The amino-PS-HDODA resin shows a characteristic IR absorption band at 1720, 1484 (ester) and 1523 cm^{-1} (amino). ^{13}C CP-MAS NMR shows a peak at 46.09 ppm for methylene carbon of aminomethyl group and a small peak in the region 146.33 ppm for C-6 carbon of polystyrene ring. The HMPB linker was attached to the aminomethyl resin using the HOBt/HBTU coupling method. The quantitative reaction was estimated by negative Kaiser test[32]. The IR spectrum of the resin gave a peak at 1620 cm^{-1} corresponding to the amide bond and a broad peak at 3400 cm^{-1} corresponding to the hydroxyl group.

The peptide was synthesized manually on PS-HDODA-HMPB resin using a disposable syringe fitted with a Teflon filter at the bottom (**Scheme-II**). Fmoc-amino acid-OPfp esters (3 molar excess) along with Dhbt-OH were used for the coupling reaction. A 2h coupling was required for the quantitative reaction on the resin. The Fmoc group from the resin was removed by 20 % piperidine in DMF and the all amino acids in the desired sequence were incorporated step by step. After the synthesis the Fmoc protection was removed and the resin was washed with DMF and lyophilized. The peptide resin was treated with TFA in presence of scavengers to cleave the peptide from the resin. The crude peptides obtained in 169.4 mg (91 %), yield as revealed by the amino acid analysis of the residual resin after cleavage of



Scheme-II: Synthesis of neurotensin (1-13) on PS-HDODA resin

the peptide. The HPLC profile (Fig. 2) showed a single major peak and the purity estimated from the chromatogram was 98 %. The pure peptides were characterized by amino acid analysis and MALDI TOF MS. The amino acid analysis results and the mass spectra of the peptide confirm the identity of the peptide.

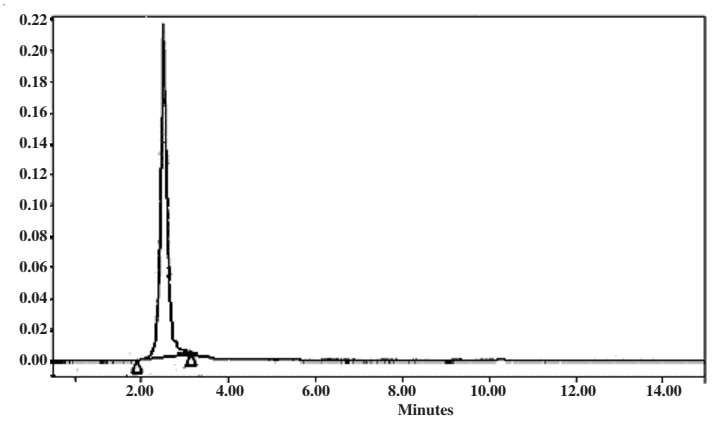


Fig. 2. HPLC of crude neurotensin (1-13) synthesized on PS-HDODA resin

Quantification of neurotensin: The synthesized pure neurotensin peptide was made in to different concentrations (nmol) and the peak area corresponding to each concentration was determined by HPLC. A standard working curve was created using the peak area and concentration (Fig. 3). Neurotensin from brain stem, cerebral cortex and hypothalamus of controlled and diabetic rats were isolated and purified as described in experimental section.

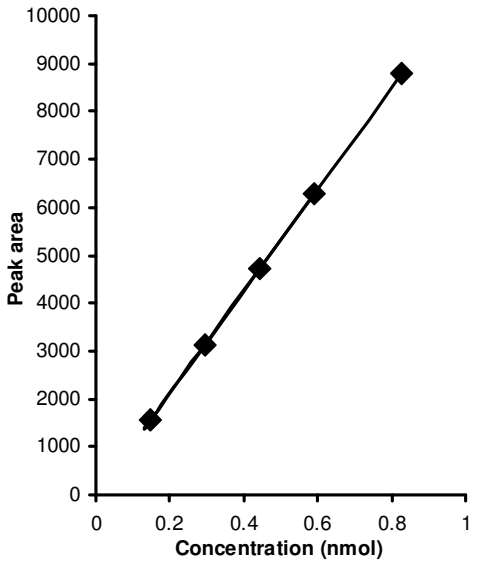


Fig. 3. Working curve created from different concentration of synthetic neurotensin (1-13) peptide and the corresponding HPLC peak area

Concentration of neurotensin isolated from brainstem, cerebral cortex and hypothalamus: Neurotensin was isolated from 10 mg of brain stem, cerebral cortex and hypothalamus of controlled and diabetic rat and the HPLC profiles are shown in the Fig. 4a and 4b, respectively. The peak area and the corresponding concentration of neurotensin are presented in Table-1. The results indicate that the neurotensin concentration decreases in brain stem and hypothalamus, while increases in cerebral cortex during diabetics.

TABLE-1
CONCENTRATION OF NATIVE NEUROTENSIN ISOLATED FROM BRAIN STEM, CEREBRAL CORTEX AND HYPOTHALAMUS OF CONTROLLED AND DIABETIC RAT

	Concentration of native neurotensin			
	Controlled		Diabetic	
	HPLC peak area	Concentration (nmol)	HPLC peak area	Concentration (nmol)
Brain stem	3623	0.341	2520	0.237
Cerebral cortex	3237	0.301	4939	0.465
Hypothalamus	7720	0.727	2994	0.282

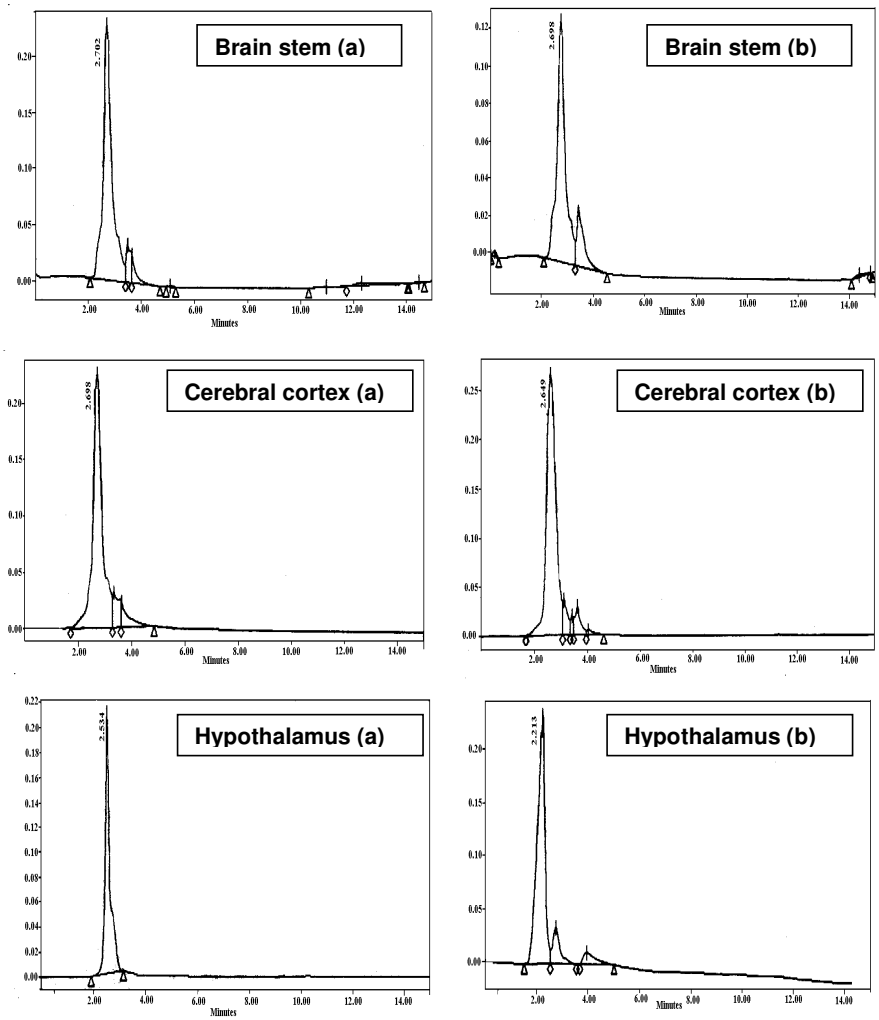


Fig. 4. HPLC of native neurotensin isolated from (a) controlled rat and (b) diabetic rat

Conclusion

An efficient method for the synthesis of well-defined PS-HDODA resin was developed. A narrow size distribution of the beads was achieved by the use of calcium salts along with the suspension stabilizer. The utility of the resin in peptide synthesis was demonstrated by the synthesis of a 13-residue neurotensin peptide. The high yield and purity of the peptide supports the efficiency of PS-HDODA resin in solid phase peptide synthesis. The synthetic neurotensin (1-13) peptide was used for quantifying native neurotensin peptide present in the central nervous system of controlled and diabetic rat and the results revealed that the neurotensin concentration decreases in brain stem and hypothalamus, while increases in cerebral cortex during diabetics.

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