Synthesis of Biologically Important Angiotensin-II and Angiotensin-IV Peptides by Using 4-(2',4'-Dimethoxyphenyl-Fmoc-Aminomethyl)phenoxy Resin

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In peptide chemistry, there is the goal to produce pure biologically active peptides with the highest possible efficiency, even its possible by chemical synthesis purity and yield as a major problem. In this work, we focus to overcome above difficulty by novel synthetic approach. Angiotensin an oligopeptide is a hormone and a powerful dipsogen, which exhibits a wide range of biological activities, the 8-amino acid sequence Angiotensin-II and 6-amino acid sequence. Angiotensin-IV were synthesized by using 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, the modified Fmoc-chemistry and effective hydroxybenzenetriaizole, N,N'-diisopropylcarbodiimide coupling and activation methods was used. The yield and chromatographic purities were compared.

Key Words: Peptide, Angiotensin, 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin.

INTRODUCTION

Nowadays, large biotechnology-based initiatives, like the Human Genome Project^{1,2}, as well as the improved understanding of fundamental biological processes, provides a huge number of new protein sequences. This leads to a rapid increase in the number of novel or important targets for drugs applications². Therefore, there is a high demand of these new targets in at least micro multimilligram quantities. Obviously, access to these proteins should be provided within the shortest possible time frame, mainly used to fulfill this requirement is chemical synthesis³. Synthetic peptides find application in all areas of biomedical research including immunology, neurobiology, pharmacology, enzymology and molecular biology⁴. The chemical synthesis of peptides with the naturally occurring structure is possible, it was used for the development of artificial vaccines and potent drugs that can substitute the conventional drugs having various side effects. Investigation of structure-activity relationship of biologically active peptides also demands the synthesis of many analogues of a given peptide. But in peptide chemistry there is a goal to synthesize a peptide with highest purity and yield, it should be a considerable factor^{5,6}. For biological research the recommended peptide purity should be needed. Nowadays advanced purification process are applicable to purify the peptide, but purification process will reduce the peptide yield^{3,7}.

Here in this proposed research work we successfully synthesized Angiotensin II and Angiotensin IV. Angiotensin was independently isolated in Indian apolis and Argentina in the late 1930s (as 'angiotonin' and 'hypertensin', respectively) and subsequently characterized and synthesized by groups at the Cleveland Clinic and Ciba laboratories in Basel, Switzerland. Angiotensin is a peptide hormone that causes vasoconstriction and a subsequent increase in blood pressure^{8,9}. It is part of the renin-angiotensin system, which is a major target for drugs that lower blood pressure. Angiotensin also stimulates the release of aldosterone, another hormone, from the adrenal cortex. Aldosterone promotes sodium retention in the distal nephron, in the kidney, which also drives blood pressure up. Angiotensin II acts on the adrenal cortex, causing it to release aldosterone, a hormone that causes the kidneys to retain sodium and lose potassium. Elevated plasma angiotensin II levels are responsible for the elevated aldosterone levels present during the luteal phase of the menstrual cycle^{8,9}. In this work we concerned about the purity (hygienic) for biological research and yield for further development.

The chemical structure of AngiotensinII and Angiotensin IV peptide were shown in Figs. 1 and 2.

EXPERIMENTAL

Amino acids: Fmoc-Asp(otBu)-OH, Fmoc-Arg(pbf)-OH, Fmoc-Tyr-OH and Fmoc-Phe-OH where purchased from Aldrich, Switzerland, Fmoc-His(Trt)-OH, Fmoc-Val-OH and

Fmoc-Ile-OH were purchased from Alfa-easer, England, Fmoc-Pro-OH were purchased from Novabiochem, Germany, all above amino acids are biochemical reagent were of the highest purity available and tested.

Solid support: 4-(2',4'-Dimethoxyphenyl)-Fmoc-aminomethyl)phenoxy resin 100-200 mesh (loading 0.71 mmol/g) were purchased from Novabiochem, Germany.

Special reagents: N,N'-Diisopropylcarbodiimide (DIC), DIPEA, hydroxybenzenetriazole (HOBt), triisopropyl silane (TIS), trifluoroacetic acid (TFA), *m*-cresol, acetic anhydride, piperidine, pyridine, ninhydrin and dichlorodimethylsilane were purchased from Sigma-Aldrich China, Alfa-Asser England and Novabiochem Germany.

Solvents: N-methyl-2-pyrrolidone (NMP), dimethyl formamide (DMF), dichloromethane (DCM), diethyl ether, methanol and acetonitrile are of HPLC grade and were purchased from E. Merck (India) and Lobachemi, Mumbai. Solvents were purified by standard procedures.

The HPLC system specially made for peptide and biological samples (Make: M/s Shimadzu Corporation, Japan) RP-C₁₈ column diameter 250 mm × 4.6 mm, 25 cm length, 5 μm particle size, run time 0.5 h, injection volume: 20 μL, linear gradient 5 % acetonitrile: 95 % water at 0 min, 100 % acetonitrile : 0 % water at 0.5 h, flow rate 1 mL/min.

Reagents were prepared(freshly) under hygienic condition: 20 % piperidine in dimethylformamide for Fmoc deprotection.

Ninhydrin solutions to check free amino group: A. 0.5 g of ninhydrin in 10 mL of ethanol, B. 10 g of phenol in 10 mL

of ethanol. C. 1 mL of 0.001 N aqueous KCN in 15 mL of pyridine.

Fmoc deprotection: 3 mL of the mixture added to the resin and shake it for 10 min × 2 times filtrate were removed and washed.

Washing: Minimum amount of DMF × 4 times and NMP × 2 times shake well and filtrate were removed.

Ninhydrin test for free amino group: A small pinch of resin beads with one drop of A, B and C were taken in a small fusion tube and heated to 100 °C blue colour shows presence of free amino group^{7,10,11}.

TLC plates were coated with silica gel G, basic solvent system suspended CHCl₃-MeOH were prepared and iodine vapours were used as visualizing agent for monitoring the coupling process and purity¹⁰.

Swelling: 150 mg of a 4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl)phenoxy resin were transferred to well clean sililated and sterilized peptide synthesizer, to that sufficient amount of N-methyl pyrrolidone were added and swelled for 1 h and removed the filtrate under vacuum. Fmoc group from resin were removed and washed^{7,10,11}.

Coupling: Fmoc amino acid dissolved in minimum quantity of NMP in well closed 25 mL round bottom flask to that hydroxybenzenetriazole were added and dissolved and N,N'-diisopropylcarbodiimide were added and shake it well for 3 min and immediately the content was transferred in to the resin with moisture free atmosphere and shake it well for 5 min, to that N,N'-diisopropylethylamine were added and shake well for 45 min. Reaction progress were monitored by

Fig. 1. Chemical composition and structure of Angiotensin II contains 8 amino acid residue (m.wt :1046 g/mol)

Fig. 2. Chemical composition and structure of Angiotensin IV contains 6 amino acid residue (m.wt: 775 g/mol)

TLC. Small pinch of the resin were taken and washed, ninhydrin were tested in case positive means same amino acid coupling was repeated, in case negative means washed, deprotected, remaining amino acids coupling were done by above method.

The detailed synthetic strategy, time duration of reaction process and conditions are given in Tables 1 and 2.

Cleavage of crude peptide from resin: After synthesis the resin were washed with hexane, DCM, CHCl_3 and MeOH and dried. The cleavage were done with 95 % TFA: 2.5 % TIS: 2 % water: 0.5 % *m*-crosal at 3 h under nitrogen atmosphere and the resin were washed 4 times with TFA, the filtrate were collected in 50 mL RB-Flash and all the traces of TFA was evaporated by using Rota vacuum evaporator^{10,12}.

The peptide were isolated with excess of peroxide free pure cold diethyl ether and the peptide were washed 7 times with diethyl ether and centrifuged. The clear white powder form of peptide were collected in a small tubes and sealed⁵.

RESULTS AND DISCUSSION

In this present work, three valuable factors are focused. The hygienic purity of peptide were recorded, expected yield should be achieved and the observations of this work compared with earlier methods. A very important part of this research was that the biologically important crude peptide with > 90 % purity (hygienic) should be achieved. In earlier methods the main factor is purification process reduce the peptide yield, so crude peptide with maximum purity should be needed for further development. The most essential biologically important Angiotensin II and Angiotensin IV crude peptide with > 90 % purity were achieved.

The accuracy of HPLC assay method was assessed by standard method (the known peptide sample purity were recorded and compared with standard reference). The purity of both crude peptides were shown in Figs. 3 and 4.

Fig. 3. HPLC of Angiotensin-2 (NH_2)Asp-Arg-Val-Tyr-Ile-His-Pro-Phe(CONH_2). The single sharp peak at ret. time 7.24 min shows our target peptide purity. Crude peptide yield: 83.67 %

Fig. 4. HPLC of (NH_2)Val-Tyr-Ile-His-Pro-Phe(CONH_2), the single sharp peak at ret. time 7.14 min shows our target peptide purity. Crude peptide yield: 85.78 %

The given HPLC analysis report gives clear evidence for the purity of the crude Angiotensin II and Angiotensin IV peptide.

Comparison: The success of this modified synthetic approach was compared with other known methods (Fig. 5). Here maximum crude peptide purity and yield was obtained in new modified fmoc synthetic approach when compared to other approach, the result and observations of this work is compared with earlier reported peptide synthetic procedures. The following graphical evidence clearly reveals the success of this methods.

TABLE-1
SYNTHESIS OF ANGIOTENSIN-II (NH_2)Asp-Arg-Val-Tyr-Ile-His-Pro-Phe(CONH_2)

Amino acid (Fmoc-)	Coupling(min)			Ninhydrin	Washing	Deprotection 15 min ($\times 2$)	Washing
	1st	2nd	3rd				
Fmoc-Phe-OH	45	40	–	-ve	Done	Done	Done
Fmoc-Pro-OH	30	35	–	-ve	Done	Done	Done
Fmoc-His(trt)-OH	30	30	–	-ve	Done	Done	Done
Fmoc-Ile-OH	30	30	45	-ve	Done	Done	Done
Fmoc-Tyr(tbu)-OH	30	40	–	-ve	Done	Done	Done
Fmoc-Val-OH	30	35	40	+ve*	Done	Done	Done
Fmoc-Arg(pbf)-OH	30	35	–	-ve	Done	Done	Done
Boc-Asp(tbu)-OH	30	40	–	-ve	Done	–	–

*In case ninhydrin is +ve after 3rd coupling acetylation were done.

TABLE-2
SYNTHESIS OF ANGIOTENSIN-IV (NH_2)Val-Tyr-Ile-His-Pro-Phe(CONH_2)

Amino acid (Fmoc-)	Coupling (min)			Ninhydrin	Washing	Deprotection 15 min ($\times 2$)	Washing
	1st	2nd	3rd				
Fmoc-Phe-OH	45	40	–	-ve	Done	Done	Done
Fmoc-Pro-OH	30	30	–	-ve	Done	Done	Done
Fmoc-His(trt)-OH	30	30	–	-ve	Done	Done	Done
Fmoc-Ile-OH	30	40	45	-ve	Done	Done	Done
Fmoc-Tyr(tbu)-OH	30	35	–	-ve	Done	Done	Done
Boc-Val-OH	30	35	40	+ve	Done	–	–

Fig. 5. Purity and yield of the biologically active peptide achieved from a modified Fmoc solid phase synthesis method were compared with standard reference of solution phase and earlier solid phase synthetic methods. A. Solution phase peptide synthesis. B. Fmoc peptide chemistry. B*. Modified Fmoc peptide chemistry

Conclusion

The present modified fmoc chemistry method has been found to enhance the biologically active peptide purity and yield. Here biologically active Angiotensin II and Angiotensin IV peptide synthesized and hygienic purity was absorbed and the success of this method were compared. The main goal of the proposed modified fmoc peptide synthetic method was enhance the biologically important peptide purity and yield. The coupling method is also relatively fast as compared with previously reported procedures. It will helpful for further research and improvement.

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