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Development of solid phase synthesis method of cardioactive peptide and its analogue based on fmoc-amino acids

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«The discovery of neurosectretion of the hypothalamus peptides that govern the release of pituitary hormones represents a major milestone in our understanding of endocrine control; It is of great importance both for endocrinology and development of therapy based on influencing the hormone action. Few recent discoveries have had wide implications in the problem and have led to the formation of such a vast new field of science. To make the study definitive, the peptides must be isolated, their structure determined, and their activity compared to that of their synthetic analogs. This will be a difficult task, involving a great deal of work, but Dr. Galoyan A.'s group, with others, are well on their way to accomplishing this work»
Professor Abel Lajtha
Director of the Center for Neurochemistry, New York, USA
Editor-in-Chief of Neurochemical Research
Editor-in-Chief of the Handbook of Neurochemistry

During many years prof. Galoyan A. with co-workers studied the chemical pathways by which hypothalamic neurohormones regulate the functions of the organism. The initial hypothesis stated that many biologically active substances of the brain, including the releasing factors have peptidic nature [3]. The investigation of neurosecretion [1958-1961] under the influence of neurohumoral agents had revealed interesting data, which became one of the premises in the discovery of new hormones of the mammal hypothalamo-neuroendocrine system. Galoyan A. with co-workers have shown that the coronodilatory neurohormones, when going out of the brain into general circulation get bound to the albumins and gamma-globulins of the serum and are transported to the heart with subsequent binding to the heart proteins [1]. Later, a neurohormonal feedback mechanism between heart and hypothalamus

has been discovered [6] and for 24 peptides of the hypothalamic fraction, the amino acid sequence has been determined by means of HPLC.

The discovered Proline-Rich Peptides (PRP, 1962) represent a new family of hypothalamic neuropeptides which are synthesized in the form of a common precursor protein, NVAG (neurophysin-vasopressin-associated glycoprotein) by genetically determined mechanisms and released from the precursor by proteolysis during axonal transport [2]. For the PRP the biochemical, immunological and physiological studies indicated the universal and neuroregulatory characteristics together with anti-stress function [4]. It has been shown and described the formation of PRP by proteolysis of the C terminal glycoprotein neurophisine2 [2]. Biological properties of PRP were studied in the aspect of metabolic activity, antibacterial and antiviral action, mielogenesis, lymphopoesis, immunomodulatory actions, neurohormonal and neuroprotective properties. All data highlighted the polyfunctionality of PRP and engagement of this neuropeptide in many biologically important processes such as inflammation,coronary dilation, vessels pressure regulation in both normal and pathological conditions [4]. So far four analogues of proline-rich peptide have been isolated from bovine hypothalamus (Fig.1).

PRP1	Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro-Gly-Val-Tyr
PRP2	Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro-Gly-Val
PRP3	Ala-Gly-Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro-Gly
PRP4	Ala-Pro-Glu-Pro-Ala-Glu-Pro-Ala-Gln-Pro

Fig. 1. The amino acids sequences of PRP analogues (amino acids are indicated in the standard three-letter code).

Since the neuropeptides are present in bone marrow, atria in small amounts, and their isolation and identification requires the most modern technical equipment and materials, we aimed to develop new analytical methods for isolation, purification, identification, determination, as well as synthesis of PRP1 and its analogue that contains L-Ala to D-Ala replacement of PRP1 amino acids. Considering the characteristics of PRPs structure we decided to use the solid phase peptide synthesis method (SPPS) by R.B. Merrifield [5].

When considering the general coupling reaction in SPPS process, its rate and yield is limited by a set of variables such as the choice of resin and choice of the solvent. These variables will in part determine the swelling of the peptide-resin and influence the accessibility to the reactive sites and it will also have a direct effect on the kinetics of the coupling groups. We have studied the technique of solid-phase peptide synthesis (SPPS) in collaboration with the Dept. of Natural Compounds of St. Petersburg State University. Synthesis of

PRP1 and PRP1(D-Ala) has been carried out in the analytical laboratory of the Institute of Biochemistry by Buniatian (NAS RA).

Materials and Methods

All the used chemicals, reagents, solvents, resins were obtained from Advanced ChemTech company. We used the fmoc-protective group for orthogonal N terminal protection of amino acids and dimethylformamide (DMF) as solvent for all processes during synthesis. A wide range of linkers is available for use with the fmoc strategy, facilitating the synthesis of a large variety of peptide types, e.g. aminoacids, protected fragments, etc. We used 2ChlTrt resin taking into account the features of our PRP. Deprotection of the N-terminal protecting group was normally achieved by piperidine in DMF.

PRP1

Purification and monitoring of the synthesized PRP1 was carried out on preparative two-component system of high performance liquid chromatography (HPLC) by Waters (USA). Injector Rheodyne was used to enter the sample, volumes of the loop for a sample are of 200 mcl. Detection was performed at the detector Lambda with a full scan in the range 190-360 nm. To record the results of chromatography it was used a system of storage, retrieval and mathematical data processing program of Lambda. We used the column «Symmetry Si-100 C18» (4,6h250 mm) for reversed phase HPLC. Eluent flow rate was 50 ml/min. Separation was carried out in gradient mode H2O/ACN/TFA (98/2/0.1) / (0.100.0.1) for 15 min. Rechromatography was done on Shimadzu LC-10 HPLC system. Rechromatography was performed on an analytical system Knauer HPLC (column X-bridge C18 (2.6x150 mm). Detection was carried out in similar conditions with full scan in the UV range 190 - 360 nm (Fig 2). The synthesized peptide PRP was subjected to mass spectral analysis on the CSU "Analytical spectrometry" in St. Petersburg State University.

PRP1 (D-Ala)

The analogue PRP1 (D-Ala) contains L-Ala to D-Ala replacement of PRP1 amino acids. The purification and monitoring of the PRP1 (D-Ala) were carried out on preparative two-component system of high performance liquid chromatography (HPLC) by Shimadzu (USA). We used the sample injector Shimadzu, volumes of the loop for a sample of 250 mcl. Detection was performed at the detector Shimadzu LC-10. To record the results of chromatography we used a system of storage, retrieval and mathematical data processing program Shimadzu LC-10. We used the column «Biosphere Si-100 C8» (4,6 h250 mm) for reversed phase HPLC. Eluent flow rate - 50ml/min. Separation was performed in gradient mode H2O/ACN/TFA (98/2/0.1) / (0.100.0.1) for 15 min. Rechromatography was done with the analytical system Knauer HPLC (column X-bridge C18 (2.6x150 mm). The synthesized peptide

PRP1 (D-Ala) was subjected to mass spectral analysis on the CSU "Analytical spectrometry".

Results and Discussion

Processing the results of synthesized PRP1 and PRP1(D-Ala) obtained by HPLC has confirmed that the peptides are in homogeneous form. (Fig. 2, 3)

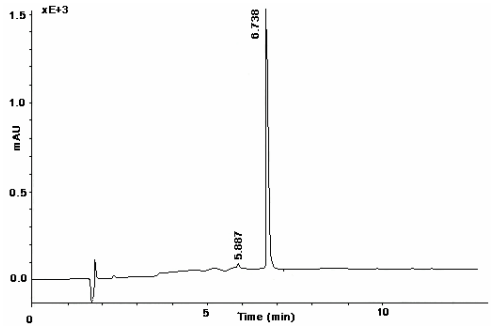

Fig. 2 Rechromatogram of synthesized PRP-1 (L-Ala) peptide by Knauer HPLC system *X-bridge Si300 C18 (2.6x150 mm).* Eluents: acetonitrile/water 0.1% TFA. Linear gradient with flow 8ml/min. Duration of experiment - 15 min, detection - on 214nm.

After purification, the obtained synthetic PRP and PRP (D-Ala) analogue peptides have reached a purity of 99.6% and 99.2% accordingly. Mass spectral analysis has proved the homogeneity of synthesized peptides (Fig. 4, 5). The results will let us continue studying the ways by which PRP regulates various processes in the organism.

PRP 1-15 (D-ALA)

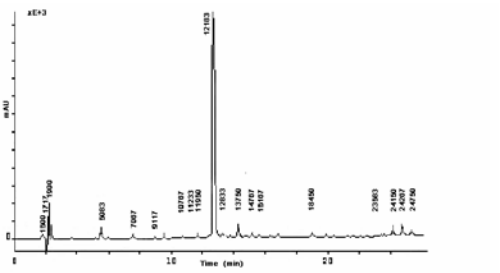

Fig. 3. Rechromatogram of synthesized PRP-1 (D-Ala) peptide by Knauer HPLC system *X-bridge Si100 C8 (2.6x150 mm).* Eluents: acetonitrile/water 0.1% TFA. Linear gradient with flow 8ml/min. Duration of experiment - 15 min, detection - on 214nm.

L - Ala 1-15 M=1453.6

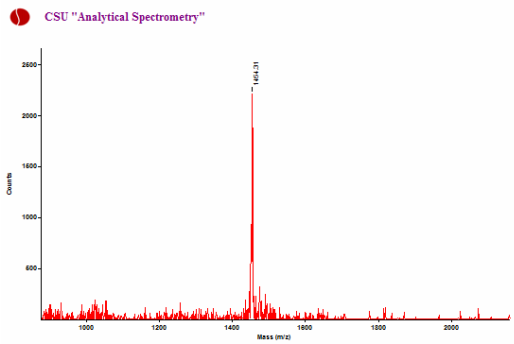

Fig. 4. Mass spectral analysis of synthesized PRP-1(L-Ala) on CSU "Analytical spectrometry"

PRP 1-15 (D-ALA)

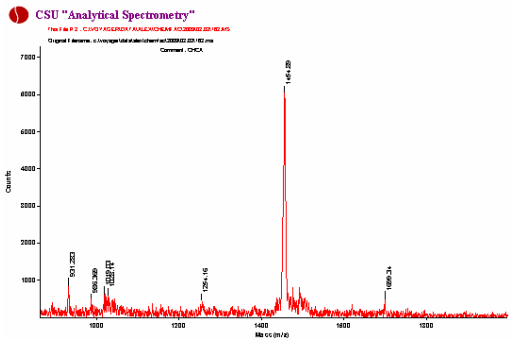

Fig. 5. Mass spectral analysis of synthesized PRP-1(D-Ala) on CSU "Analytical spectrometry"

Comparison of PRPs structure shows the presence of PRPs similarity to protein 2Dvj, which is associated with tyrosine kinase receptors. There is an idea to synthesize PRP with phosphorylated amino acids and check their biological activity.

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Կարդիոակտիվ պեպտիդի և նրա համանմանի պինդ ֆազային սինթեզի մեթոդի մշակումը fmoc -ամինաթթուների հիման վրա

Գ.Ս. Չաիլյան

Ակադեմիկոս Ա.Ա. Գալոյանի և իր աշխատակիցների կողմից 1963 թվականին առաջին անգամ ցլի հիպոթալամուսից առանձնացվել է պրոլինով հարուստ պեպտիդը (PRP)։ PRP-ի կենսաբանական ուղղվածության հետագա հետազոտությունների նպատակով խիստ անհրաժեշտ դարձավ սինթետիկ PRP -ի ստացումը։ Պեպտիդների սինթեզի համար մեր կողմից օգտագործվել է պինդ ֆազային սինթեզի մեթոդն իր հետագա ձևափոխություններով։ Որպես սուբստրատ օգտագործել ենք 2-քլորտրիտիլային խեժը, իսկ որպես ամինաթթուների պաշտպանիչ՝ fmoc-խումբը։ Մեզ հաջողվել է սինթեզել ոչ միայն PRP, այլև նրա համանմանը՝ D-Ala տեղակալումով։ Սինթեզված նյութերի մաքրությունն ու համապատասխանությունը ստուգվել է հեղուկ քրոմատոգրաֆիայի մեթոդով և մասս-սպեկտրալ անալիզով։

Разработка метода твердофазного синтеза кардиоактивного пептида и его аналога на основе fmoc-аминокислот

Г.С. Чаилян

Академиком Галояном А.А. и его сотрудниками из бычьего гипоталамуса впервые в 1963 году был выделен пролином богатый пептид (ПБП). В целях дальнейших исследований биологической направленности ПБП стало крайне необходимо получение синтетических аналогов ПБП. Для синтеза пептидов нами был использован метод твердофазного синтеза с его дальнейшей модификацией. В качестве подложки нами была использована 2-хлортритильная смола. В качестве защиты аминокислот использовалась fmoc-группа. Нам удалось синтезировать не только ПБП, но и его аналог с заменой L-Ala на D-Ala. Чистота и идентичность синтезированных препаратов проверялись методами высокоэффективной жидкостной хроматографии и масс-спектрального анализа.

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