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SYNTHESIS, CIRCUILAR DICHROISM AND BIOLOGICAL ACTIVITIES OF BRADYKININ AND SOME ANALOGS

Somaya E. Abdel Rahman
Chemistry Department, Faculty of Science, Zagazig University, Zagazig, Egypt.

ABSTRACT

Bradykinin (BK) and three of its analogs, $(DAla)^7$ BK, $(Gly)^7$ BK and $(Pro)^6$ BK were synthesized by the continuous flow method. Polystyrene-polyethylene glycol graft copolymer was employed as modified polymeric support. The effect of the replacement of Pro^7 by DAla, Gly and Ser^6 by Pro on the 3--->1 intramolecular hydrogen bond present in BK chain, between the carbonyl oxygen of Ser^6 and the amide proton of Phe^8 bridging Pro^7, was investigated. Although there are remarkable changes in the CD spectral features of these analogs compared to that of BK, they exhibited comparable biological activities.

INTRODUCTION

Bradykinin (BK) was discovered by Rocha et al.[1] and isolated later by Elliott et al.[2]. This peptide shows apparently wide variety of activity on uterus, smooth muscles contraction, pain, and blood pressure etc. Since the determination of its primary structure, a large number of bradykinin analogs have been synthesized [3] in order to examine the structure activity relationship .The secondary structure of BK in solution has been previously studied by several research groups [4-6].

On the basis of comparison of the CD spectra of several BK analogs, the presence of an intramolecular hydrogen 3--->1 bond between the carbonyl oxygen of Ser^6 and the amide proton of Phe^8, an intramolecular 4--->1 hydrogen bond between the carbonyl oxygen of Pro^2 and the amide proton of Phe^5, and a salt bridge between the guanidino group of Arg^1 and the carboxyl group of Arg^9 was emphasized [7,8]. $A^{13}CNMR$ study by London et al.[9] ,provided an evidence for the existence of the 3--->1 hydrogen bond. Chipens et al. [10] implied a certain similarity in the secondary structure between BK and cyclo BK analogs from studies of their CD spectra.

The present work was carried out to further investigate the presence of the intramolecular 3--->1 hydrogen bond in the secondary structure of BK in solution. Moreover, the sequences and specific amino acid residues required for its stability were studied. The synthesis, the CD spectra and the biological activities of BK, $(DAla)^7$BK,$(Gly)^7$BK and $(Pro)^6$BK are described.

EXPERIMENTAL

HPLC measurements :

Column, nucleosil 120 C_{18}, 25 x 4.5 mm solvent system A (0.1% TFA in H_20), B (0.1% TFA in acetonitrile), gradient : 0 - 50%, B in 30 min. Detection by 220 nm and 245 nm.

Amino acid analysis :

0.1 (Mm) of the peptide hydrolysed by 3 - 4 ml 6NHCl in evacuated sealed tube for 24 h. at 110 C^o. Then the solvent was removed under vacuum to give a residue which analyzed after several washing and dryness. Amino acid analyzer is (Biotronik system LC 6000 E) with integrator system 1.

Ion spray mass spectroscopy :

The ion spray mass spectroscopy were measured using sciex mass spectrometer.

Circular dichroism spectra :

The CD spectra were measured on a JASCOJ - 20CD spectrophotometer using quartz cell with bath length of 0.50 mm. The measurements were carried out at room temperature (24^oC) and at concentration of 3.2×10^{-4} M.

Kaiser test and UV test for coupling capacity :

Kaiser test was carried out as described by Kaiser et al.[13], while UV test was done as represented by Meinhofer et al., [14].

Peptide synthesis :

The synthesis was carried out on polystyrene - polyethylene glycol graft copolymer. The polymer was synthesized as described by Rapp et al.,[15]. Polystyrene - polyethylene glycol was coupled to the acid labile anchor group, (4-hydroxymethyl - 3-methoxy phenoxy acetic acid) through the DIC/HOBT activation method [16].

Fmoc amino acids were synthesized according to Carpino et al.,[17] . t. butyl group (t.Bu) and methoxytrimethylphenyl sulfonyl (Mtr) group were used to protect the side chains of Arg and Ser respectively. The frist amino acid Fmoc Arg (Mtr)-OH was coupled to the polymer manually, 6g of the polymer anchor (1.26 m equiv.) suspended in 25 ml DMF/CH_2 Cl_2 (1:1) was added to a mixture of 1.6 g DIC (12.6 mmol), (7.6g). Fmoc Arg (Mtr)-OH (12.6 mmol), 1.7g HOBt (1.26 mmol) and, 0.06g DMAP (0.5 mmol) dissolved in 10 ml DMF/CH_2 Cl_2 (1:1).

The mixture was shaked for 24 h. at RT, filtered, washed several times by DMF, CH_2 Cl_2, MeOH, DMF, CH_2 Cl_2 and dried under high vacuum. The capacity of coupling was checked by Kaiser test and U.V detection of Fmoc group. It was about 98%. The product was then packed in the synthesizer column. Continuous flow solid phase peptide synthesis was carried out using MilliGen 9050 pep. synthesizer, which consists of three modules, an IBM PC-At computer with its keyboard and monitor, the MilliGen 9050 cabiner, and the amino acid module. The standard cycle consists of four main steps usually requiring about 27 min to complete.

In the frist step the deprotection of Fmoc group of support bound C-terminal amino acid is accomplished with a 4 min wash with 20% piperidine in DMF. In the second step, a 7 min DMF wash removes excess piperidine from the column and fluid lines in preparation for amino acid addition. The third step consists in preparation of activated amino acid on the amino acid module. Where it dissolves in 0.33 M (HOBt/DMF) solution. This solution is transferred to the amino acid loop with the MilliGen 9050 cabinet and is swept out of the loop and recirculated for 12 min. to complete the coupling reaction.

The final step is 4 min. DMF wash. These four steps are necessary to complete all chemical processes required to lengthen the peptide chain by one amino acid. Once coupling is complete, the next cycle begins with deprotection of the -amino group of the N-terminal amino acid. The process of -amino group deprotection followed by coupling is repeated for as many cycles as necessary to assemble the desired peptide chain.

The cleavage of the polymeric support and isolation of the free peptides were carried out by adding 10 ml solution of TFA/ethandithiol mixture (4:1) to 1g of the polymer peptide. Then, the reaction mixture was shaked for 10 min, and left to stay for 1 hour at RT. Then, the solid was filtered, washed thoroughly with TFA and the filtrate was left for additional 4 hours with interval shakings. The solution was then concentrated under vacuum and the residue was dissolved in 2 ml acetic acid and added dropwise to 10 ml ice cold ether. The solid produced was filtered, washed with cold ether and redissolved in 2 ml acetic acid, reprecipitated by ice cold ether, filtered and lyophilized to obtain the free peptide. Using this method, all side chain protecting groups were removed with the polymeric support.

After HPLC separation the pure peptide chains were characterized using ion spray mass, spectroscopy, and amino acid analysis.

Characterization of the synthesizedpeptides :

peptide (1): H_2N-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (MS : m/z1060.4)

amino acid analysis :

	Arg	Pro	Gly	Phe	Ser
Calcd. :	2	3	1	2	1
Found :	1.9	2.7	1	2	0.9

peptide (2): H_2N-Arg-Pro-Pro-Gly-Phe-Ser-DAla-Phe-Arg-OH (MS : m/z 1034.2)

Amino acid analysis :

	Arg	Pro	Gly	Phe	Ser	DAla
Calcd. :	2	2	1	2	1	1
Found :	1.9	1.8	1	2	0.9	1

peptide (3): H_2N-Arg-Pro-Pro-Gly-Phe-Ser-Gly-Phe-Arg-OH (MS : m/z 1020.5)

Amino acid analysis :

	Arg	Pro	Gly	Phe	Ser
Calcd. :	2	2	2	2	1
Found :	1.9	1.8	2	2	0.9

peptide (4): H_2N-Arg-Pro-Pro-Gly-Phe-Pro-Pro-Phe-Arg-OH (MS : m/z 1070)

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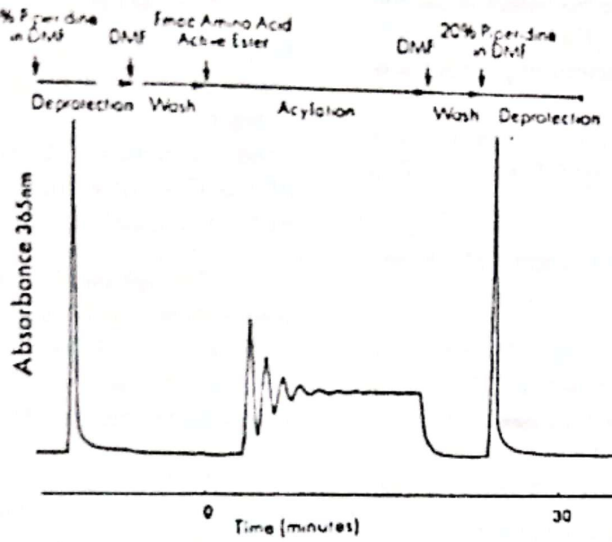

Fig. (1) Typical spectrophotometric monitoring of a synthetic cycle.

Amino acid analysis :

	Arg	Pro	Gly	Phe
Calcd. :	2	4	1	2
Found :	1.9	3.7	1	2

Biological activity measurements :-

The effect of the peptides on the arterial pressure and heart rate of rats was measured in adult made albino rats, bred in Zagazig University Laboratories. The weights of animals ranged between 18 - 200 gram each. All rats were fasted for 24h. befor the experiments. A 25% solution of urethane injected intraperitoneally [1-1.5 g/kg of body weight] was used for anaesthesia. A dose of 20 ug/kg of each peptide was dissolved in 0.1 ml of 0.9 NaCl solution. Before the experiment the left carotid artery was catheterizd with a polyethylene tube [PE 50] under complete anaesthesia. Mean arterial pressure [MAP] and heart rate [HR] were measured using the bioscience 400 series [18].

RESULTS AND DISCUSSION

The following peptide chains were synthesized using the rapid continuous flow method.

(1) H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH

(2) H-Arg-Pro-Pro-Gly-Phe-Ser-DAla-Phe-Arg-OH

(3) H-Arg-Pro-Pro-Gly-Phe-Ser-Gly-Phe-Arg-OH

(4) H-Arg-Pro-Pro-Gly-Phe-Pro-Pro-Phe-Arg-OH

The side chains of Arg and Ser were protected by methoxytrimethyl phenyl sulfonyl (Mtr) group and t.Butyl (tBu) group, respectively. Polystyrene - Polyethylene glycol graft copolymer was employed as polymeric support. 9 Fluorenyl methoxycarbonyl (Fmoc) group was used as N-terminal protecting group. It enables the UV spectroscopic monitoring of the coupling and deprotection reactions at the inlet and outlet of the column. This protocol is strongly recommended for rapid flow synthesis[11].

An essential advantage of the continuous flow method is that the excess of coupling components and solvents normally used in the solid phase method can be drastically reduced. This is a result of carrying out the reaction in a continuous flow. When a relatively high concentrated solution of the coupling components is allowed to pass through the column, a concentration profile develops. Since only a relatively small region of the functional polymer comes in contact with the coupling components while the reagent flows through the column, the excess of reagents in every column segment is larger than would have been the case if the reagents had been in contact with the total polymer . The narrower the coupling components band, the larger the excess of reagents in a given column segment. Hence, the reaction can proceed more completely, and the content of the peptides with statistical failure sequences will be lowered (Fig. 1).

When the desired sequence is achieved the polymeric support was cleaved using TFA/ethandithiol mixture (4:1). The side chain protecting groups were deprotected simultaneously.

The target peptide chains (1-4) were purified from any impureties and failure sequences by the HPLC chromatography. The pure chains were separated at retension times 20.32, 20.87, 19.93, and 19.11 respectively . The purity of the obtained peptide chains was proved by correct amino acid

analysis (exp. section) and the mz values of the ion spray mass spectroscopy Fig. (2)a,b. & Fig. (3)a,b. The conformation of the characterized peptides was investigated using the CD spectroscopy. The measurements were carried out in aqueous solutions at RT. The wave length is ranged from 200 - 250 nm.

Fig. (4) a-c shows the CD spectra of BK and three of its analogs.

The spectrum of BK Fig. (4)a shows a negative band with low intensity at 234 nm, a positive peak at 221 nm and a deep negative trough at 200 nm. The negative band at 234 nm is attributed to the intramolecular 3--->1 hydrogen bond between the carbonyl oxygen of Ser^6 and the amide proton of Phe^8 bridging Pro^7. The peak at 221 nm was attributed to the intramolecular 4--->1 hydrogen bond between the carbonyl oxygen of Pro^2 and the amide proton of Phe^5, as well as, the salt bridge between the guanidino group of Arg^1 and the carboxy group of Arg^9. It was also suggested that the band at 200 nm arises from the sequences Arg^1 Pro^2 Pro^3 Phe^4 and Phe^5 Ser^6 Pro^7.(8).

Fig. (4b) represents the CD spectrum of peptide chain (2) in which Pro^7 is replaced by D Ala residue which is known to stabilize BII turn according to the semiemperical conformational energy calculations reported by N'emethy et al., (12). The spectrum shows a common feature like that of BK with a relative intensification of the negative band at 234 nm and diminution of the positive peak at 221 nm.

The increment in the intensity of the negative band at 234 nm, which is characterstic for 3--->1 hydrogen bond, may attributed to the presence of D Ala which stabilizes this intramolecular hydrogen bond and/or decreases the intramolecular interaction between the peptide and the water molecules.

Fig. (4c) shows the spectrum of $(Gly)^7$BK in which Pro^7 is replaced by Gly residue. It indicates that the negative band at 234 nm is nearly disappeared while the positive peak at 221 nm in the BK spectrum is replaced by two broad bands at 217 nm and 224 nm. The absence of the negative band at 234 nm may indicates that the presence of Gly at position 7 may decrease the tendency to form the intramolecular 3--->1 hydrogen bond between Ser^6 and Phe^8.

Fig. (4c) represents the spectrum of $(Pro)^6$ BK, in which Ser^6 was replaced by Pro residue. The spectrum shows that the negative band at 234 nm characterstic for intramolecular 3--->1 hydrogen bond is completely disappeared. The overall shape of the CD curve may indicate that the peptide exihibits a disordered structure, under this condition.

Through out the foregoing presentation of the results stress has been placed on the changes in the intramolecular 3--->1 hydrogen bond, because it considered one of the features stabilizing the secondary structure of BK.

It appears that Ser residue at position 6 is essential for the presence of the intramolecular 3--->1 hydrogen bond, while the replacement of Pro^7 by DAla enhences its stability. On the other hand, Gly at position 7 greatly reduces the tendency to form this bond.

The biological activity measurements showed that BK and its analogs under investigation exhibit a prolonged hypotensive action in the anaesthetized rats without affecting heart rate. Arterial pressure returned to normal after 20 min (Fig. 5).

The results imply that the contribution of the secondary structure for biological activity is ambiguous. We could not find any appreciable relationship between the CD spectra and the biological activities.

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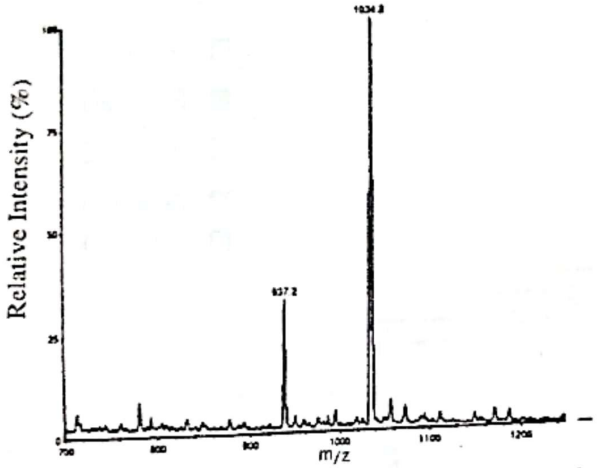

Fig. (2a) ion spray mass spectroscopy of peptide (2) before HPLC separation.

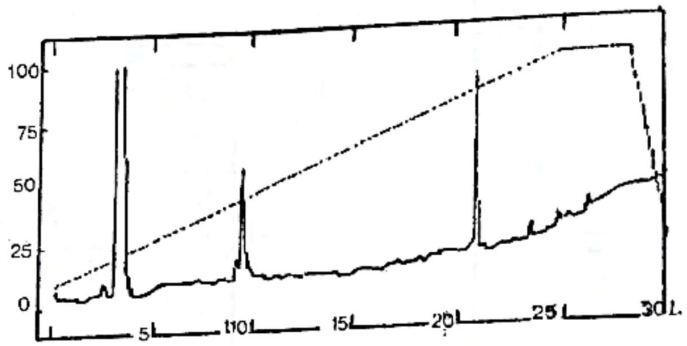

Fig. (2b) HPLC profile of crude peptide (2).

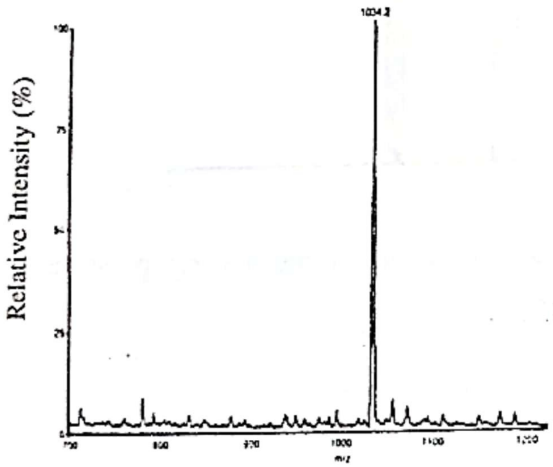

Fig. (3a) ion spray mass spectroscopy of peptide (2) after HPLC separation.

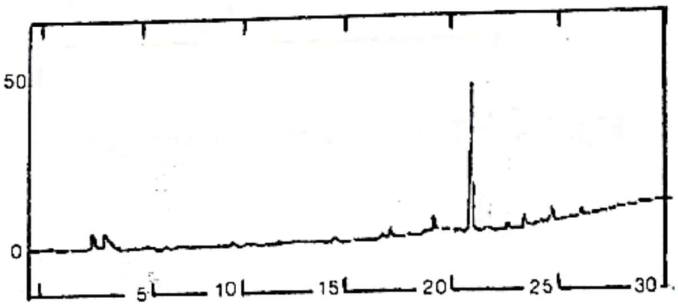

Fig. (3b) HPLC profile of pure peptide.

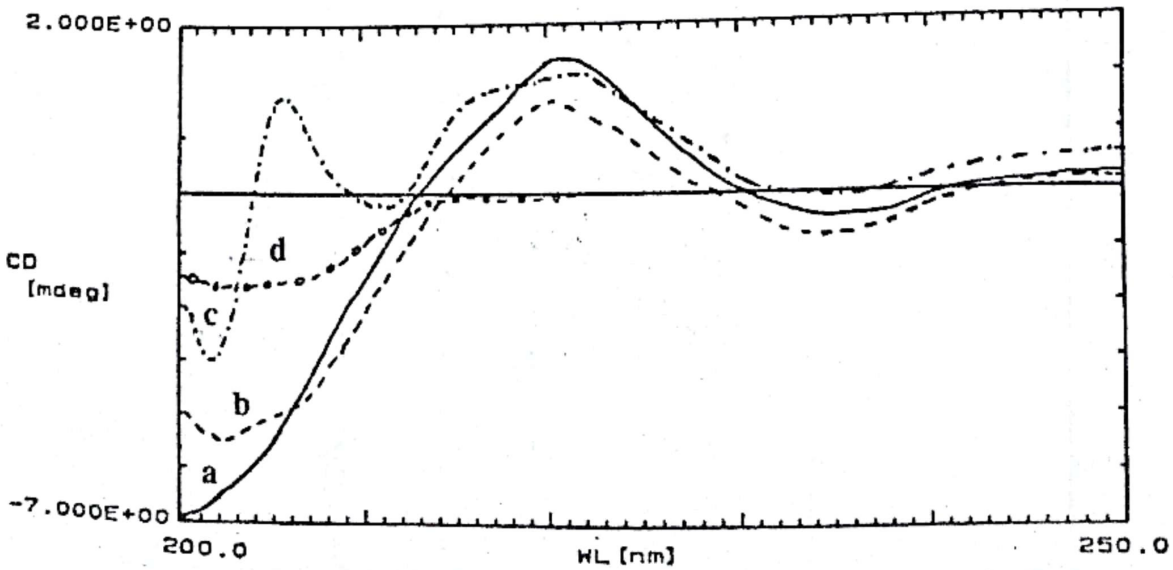

Fig. (4) CD spectra of BK (——), $(DAla)^7$ BK (------), $(Gly)^7$ BK (- - - - - -) and (Pro)6 BK (-o-o-o-)

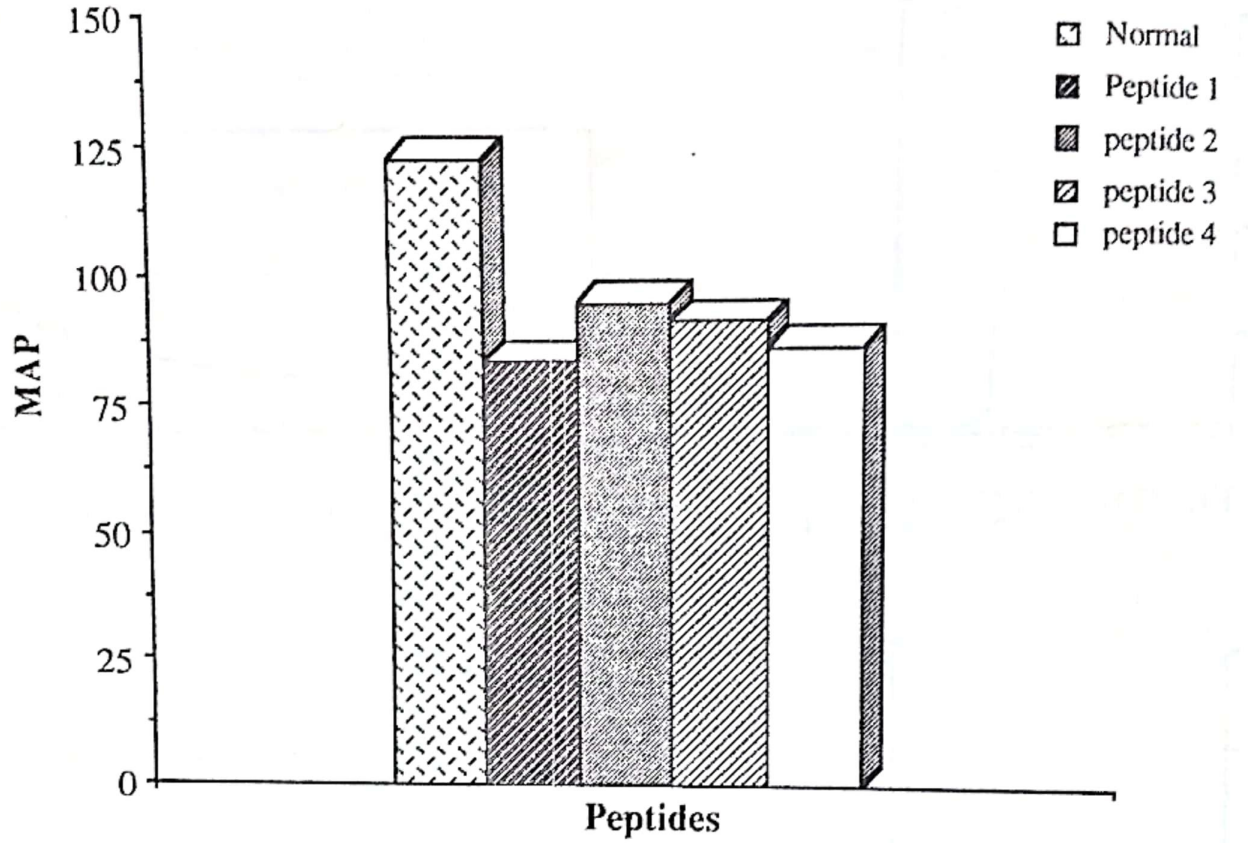

Fig. (5a) : The effect of the investigated peptide chains (1-4) on the mean artery pressure (MAP) of the rat .

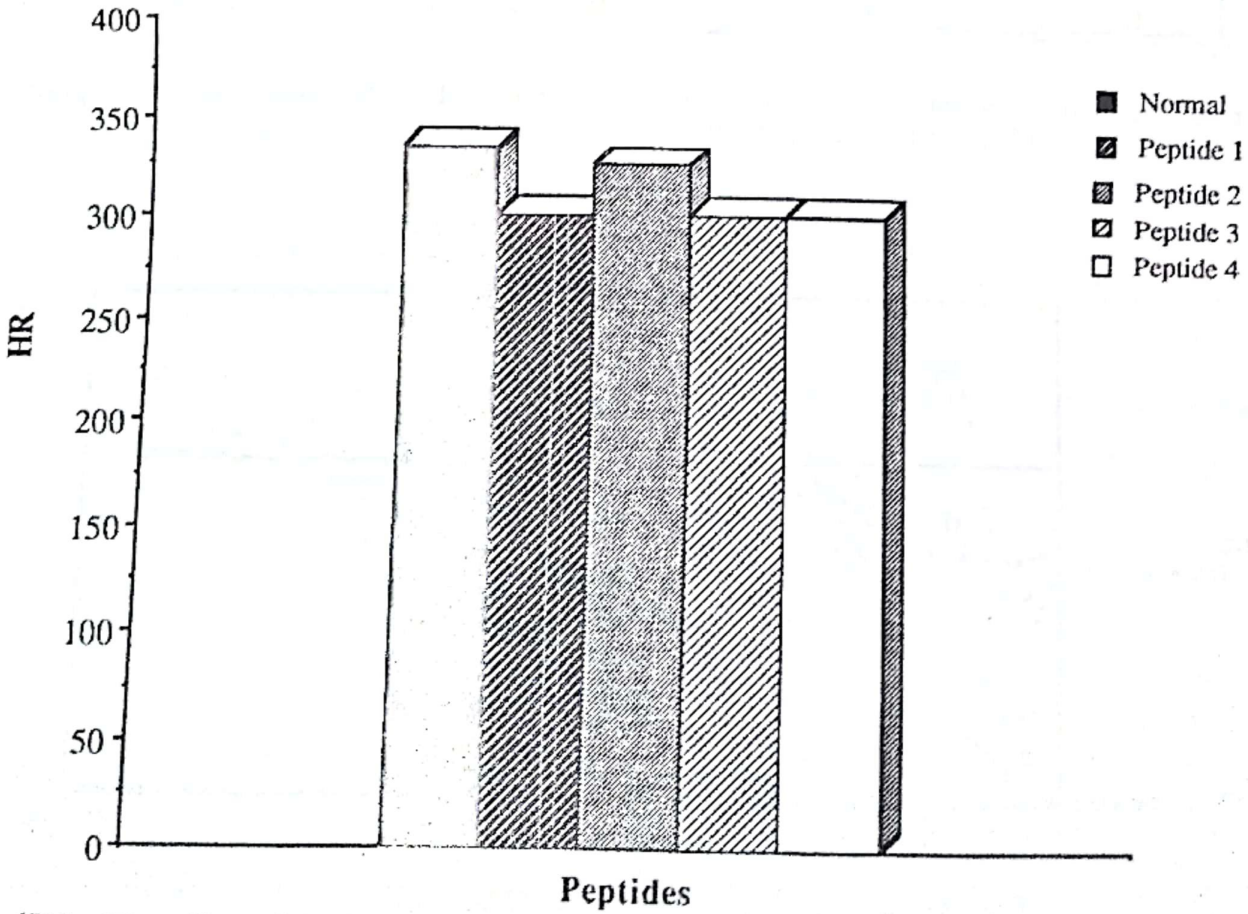

Fig. (5b) : The effect of the investigated peptide chains (1-4) on the heart rat (HR) of the rat .

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تحضير ودراسة طيف الدوران والنشاط البيولوجي للبريدكينين وبعض أشباهه

سميه الشيخ محمد عبد الرحمن

قسم الكيمياء – كلية العلوم – جامعة الزقازيق – مصر

نظرا لأهمية البريدكينين البيولوجيه وتأثيره على إنقباض العضلات اللاإرادية وتشمل عضلات الرحم وكذلك فى خفض ضغط الدم وغير ذلك من العمليات الحيوية داخل الجسم فقد تم عزله ودراسة تركيبه الكيميائى والفراغى.

وفى هذا البحث تم تحضير البريدكينين وهو سلسلة مكونه من ٨ أحماض أمينية وكذلك ثلاثة أشباه له مع استبدال الحمض الأمينى رقم ٧ البرولين بواسطة الجلايسين مرة والـ D ألانين مرة أخرى كما تم استبدال الحمض الأمينى السادس السيرين بالبرولين وذلك لدراسة تأثير ذلك على الرابطة الهيدروجينية بين كربونيل السيرين رقم ٦ فى السلسلة وهيدروجين مجموعة أميد حمض الفينيل ألانين رقم ٨ فى السلسلة والتى يقع البرولين رقم ٦ فى السلسلة بينهما.

وذلك لدراسة التركيب الثانوى (الفراغى) للبريدكينين والذى يثبت بواسطة هذا النوع من الروابط الهيدروجينية.

وتم التحضير فى الوسط الصلب بواسطة جهاز تحضير البيتيدات أوتوماتيكيا. وقد تم ربط الحمض الأول (الأرجنين) بالبولى ستيرين بولى إثيلين جليكول يدويا قبل ملئ عمود الجهاز به واستخدمت طريقة التدفق المستمر للمواد المتفاعلة على عمود التفاعل توفيرا للوقت والمواد المستخدمة حيث تستغرق دورة ربط الحمض الأمينى أقل من ٣٠ دقيقة وقد تم تنقية البيتيدات المحضرة كروماتوجرافيا وفصلها بواسطة HPIC كما تم تحديد كتلتها بواسطة مطياف الكتلة الأيونى وتم تحليل الأحماض الأمينية المكونة لكل سلسلة كميا.

وبدراسة التركيب الفراغى بواسطة طيف الدوران(C.D.) اتضح أن وجود الـ D ألانين بدلا من البرولين رقم ٧ يثبت الرابطة الهيدروجينية ويدعم التركيب الثانوى للبريدكينين بينما يقلل وجود الجلايسين فرص تكون هذه الرابطة بشكل ملحوظ.

كما ثبت أهمية وجود السيرين فى السلسلة لتكوين هذه الرابطة حيث اختفت نهائيا باستبداله بالبرولين. وبدراسة التأثير البيولوجي للبتيدات المحضرة وجد أنها مشابه للبريدكينين فى تأثيره على ضربات القلب وخفض ضغط الدم بالرغم من اختلاف تركيبها الفراغى وكذلك ترتيب وأنواع الأحماض الأمينيه المشتركة فى السلسلة البيتيديه كما لم يثبت وجود علاقة بين التركيب الفراغى للبيتيدات وتأثيرها البيولوجى.