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COMPARABLE STUDY BETWEEN THE APPLICATION OF MICROWAVE IRRADIATION TECHNIQUE AND CONVENTIONAL METHOD IN THE SYNTHESIS OF NONAPEPTIDE (B_{22}-B_{30}) OF INSULIN B-CHAIN

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This work embraced a systematic search for potentiated methodology of peptide synthesis through an approach for convenient synthesis of the nonapeptide (B_{22}-B_{30}) of insulin B-chain. The modified solid phase peptide synthesis (SPPS) with and without the application of microwave irradiation technique was used for synthesis of nonapeptide (B_{22}-B_{30}) of insulin B-chain with comparable study between the results of two methods. The produced target peptide was characterized by means of their amino acid analysis; Electrospray ionization mass spectroscopy (ESI-MS) and FT-IR data. Additionally, thermal gravimetric analysis (TGA) and differential thermal analysis (DTA) were used in the conformational study for prepared peptides. The results showed and confirmed all the advantages and importance of microwave irradiation technique in the peptide chemistry field, purity of the products, reducing reaction time and higher yield without any effect on the chemical structures and peptide bonds of the target peptide.

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1. Introduction

The peptide chemistry is one of the major roles of research in bioorganic, and biochemistry especially in branch of solid phase peptide synthesis [1]. Peptides have numerous therapeutic potential as hormones, enzymes, antibiotics, antitumor agents and neurotransmitters. Compared with small molecule therapies, peptides have higher specificity and lower toxicity, no accumulation in organs and no side effects [2]. Insulin was the first peptide to be administered therapeutically [3]. The biological activity of insulin is known to be closely related to the C-terminal nonapeptide (B_{22}-B_{30}) fragment of its B-chain [4-6]. Microwave irradiation has gained popularity in the past decade as a powerful tool for rapid and efficient synthesis of a variety of compounds because of selective absorption of microwave energy by polar molecules [7]. A microwave-assisted solid phase peptide synthesis procedure has been reported [8]. The application of microwave heating to solid phase peptide synthesis is particularly advantageous as the acceleration of coupling and deprotection reactions should lead to shorter cycle times, higher repetitive yields, and ultimately purer peptides [9]. Unlike conventional solid phase peptide method, microwave energy directly activates any molecule with a dipole moment and allows for rapid heating at the molecular level [10-13].

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Microwave energy has also been successfully used to increase the rate of peptide coupling reactions and not generate appreciable racemization; in addition, intermolecular aggregation, β-sheet formation, and steric hindrance can be overcome with microwave energy [14]. Also, there are several successful publications of microwave-assisted solid phase peptide synthesis of various unnatural biopolymers such as peptoids, pseudo peptides, small peptides [15], phosphopeptides [16], difficult peptides [14], β-peptide libraries [17] and glycopeptides [18]. In view of these observations we became interested in the comparison study of peptide synthesis using conventional and microwave irradiation methods.

2. Experimental

The organic solvents and chemicals used in this part were obtained from Sigma (USA) and Fluka (Switzerland) chemical companies. All Fmoc amino acids were purchased from Novabiochem (Sweden); the used amino acids were of L-configuration. The side chain protecting groups were Pmc for Arg and t-But for Lys, Thr and Tyr. TentaGel-NH_2 polymer was kindly supplied by Rapp polymer company (Tübingen, Germany). Microwave irradiations were carried out using a domestic microwave oven Sanyo-EM-S3555G operating at 2450 MHz and 10% of the total power.

Synthesis of nonapeptide by modified SPPS (conventional method)

The nonapeptide (B_{22}-B_{30}) of insulin B-chain was prepared according to our reported method [19].

Coupling of the first amino acid (Fmoc-Ala) to the polymer-anchor

To a solution of polymer-anchor (0.15 gm, 0.22 mmol) in 2 mL DMF/CH_2Cl_2, (1:1) a solution of Fmoc-Ala-OH (0.082 gm, 1.76 mmol), *N*-hydroxybenzotriazole (HOBt), (0.036 gm, 1.76 mmol), *N,N*-diisoprobylcarbodiimide (DIC), (0.033 gm, 1.76 mmol) and dimethylaminopyridine (DMAP), (20 mg, 0.3 mmol) in 5 mL DMF/CH_2Cl_2, (1:1) was added. The mixture was shacked at room temperature until Kaiser test [20] become negative. The solution was then filtered off and washed three times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH and ether.

Deprotection of Fmoc- N^{α}-protecting group

Fmoc-Ala- anchor-polymer (0.22 mmol) was suspended in least amount of DMF. Then a solution of 25% piperidine/DMF was added and the mixture shacked at room temperature for 45 min until Kaiser test become positive. The reaction mixture was then filtered off and washed three times with DMF, CH_2Cl_2, MeOH, DMF, CH_2Cl_2 and ether.

Coupling of next amino acid (Fmoc-Lys) to the peptide chain

To a solution of NH_2-Ala-anchor-polymer (0.15 gm, 0.22 mmol) in 2 mL DMF/CH_2Cl_2, (1:1), a solution of Fmoc-Lys (0.17 gm, 1.76 mmol), HOBt (0.036 gm, 1.76 mmol) and DIC (0.033 gm, 8 mmol) in 4 mL DMF/CH_2Cl_2, (1:1) was added. The mixture was shacked at room temperature until Kaiser test become negative. The solution was then filtered and washed three times with DMF, CH_2Cl_2, DMF, CH_2Cl_2, MeOH and ether. Nonapeptide Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-Anchor-Resin was synthesized according to the above steps.

Synthesis of nonapeptide by modified SPPS (microwave method)

The nonapeptide (B_{22}-B_{30}) of insulin B-chain was synthesized by the use of modified SPPS strategy under the application of microwave irradiation. The coupling and deprotection reactions were carried out inside the domestic microwave oven at 2450 MHz using 10% of full power. Cleavage of the peptide linker was carried out using (10 mL) a mixture of TFA/ethanedithiol (4/1) for each gram of resin [19].

Amino acid analysis

The amino acid composition of peptides was determined by amino acid analysis using a LC3000 Eppendrof with an integrator system 1. Prior to analysis samples were hydrolyzed in 6 N HCl in sealed and evacuated tubes at 110 ^{o}C for 24 hours.

ESI-MS were run on Finnigan LCQ-DECA-XP Spectrometer

In ESI-MS, the sample solution is sprayed in a fine mist of charged droplets containing sample ions by application of a large negative or positive voltage (typically ± 4.5 to ± 5 kV). A flow of nitrogen drying gas is directed at droplets and individual positive or negative ions are produced. ESI accommodates a liquid flow of 0.1 mL/min to 1 mL/min. This ionization technique is very suitable for the analysis of polar, thermally labile molecules such as DNA, RNA, peptides, drugs and protein.

Fourier-transform infrared (FT-IR) spectrometry

Infra-red spectra were recorded on FT-IR 1650 Perkin- Elmer (Germany) spectrometer. 5 mg of sample was mixed with 100 mg of Potassium bromide (KBr) by trituration. This triturated mixture was filled in dye press and then compressed to prepare a disc. The prepared disc was put in sample holder and scanned from wave no. 4000 to 400 cm^{-1} using spectrum software of Perkin-Elmer spectrometer.

Thermal Analysis (TGA/DTA)

Thermal gravimetric analysis (TGA) and differential thermal analysis (DTA) were carried out in dynamic nitrogen atmosphere (30 mL/min) with a heating rate of 10 ^{o}C/ min from ambient temperature to 600 ^{o}C using a Shimadzu TGA-50H thermal analyzer.

3. Results and discussion

On seeking in the scientific literatures for those interested in the applications of microwave irradiation techniques with chemistry field, we found that all the talk about the importance of the microwave technique with multiple advantages and benefits without get into the subject of its effect on the chemical structures and chemical bonds of the prepared compounds. Hence our study was closely tied to this subject, especially in the peptide chemistry field.

In the present work, the model peptide chain nonapeptide (B_{22}-B_{30}) of insulin B-chain HO-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-NH_2 was prepared by the use of modified solid phase method with and without microwave energy [19] Figure 1. It is evident that, the correct choice of the synthetic strategy, the protecting groups and the polymeric support could contribute most fundamentally to the improvement of the used methods. The crude peptide chain after polymer cleavage was obtained by centrifugation, dissolved in distilled water, lyophilized, and subjected to HPLC. The two target peptides that were synthesized using modified SPPS with and without microwave energy were characterized and compared with each other by spectral analysis.

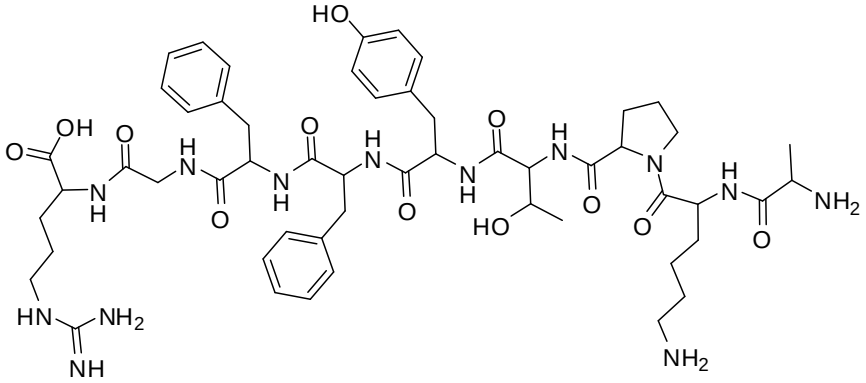

Fig. 1. Chemical structure of nonapeptide (B_{22}-B_{30}) of insulin B-chain

Amino acid analysis

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins and peptides. It is necessary to hydrolyze a peptide to its individual amino acid constituents before amino acid analysis. Following peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations**.** Table 1 shows the sequencing of most amino acids equal of the theoretical and calculated, and all amino acids that were used in the synthesis of target peptides were existed in the chemical structure [19].

Table 1. Amino acid analysis of target peptide using SPPS with and without microwave irradiation

SPPS Methods		Amino Acids Sequencing							
		Ala	Lys	Pro	Thr	Tyr	Phe	Gly	Arg
With microwave	Calculated	1	1	1	1	1	2	1	1
	Found	1	1	0.96	0.93	0.88	1.98	1	0.99
Without microwave	Calculated	1	1	1	1	1	2	1	1
	Found	1	0.97	0.95	0.82	0.82	1.85	1	0.89

Electrospray ionization mass spectra (ESI-MS)

The target peptides were analyzed by HPLC and positive-mode electrospray ionization mass spectroscopy ($^{+}$P ESI-MS). Figure 2 shows the ESI-MS of the peptide synthesized using modified SPPS without microwave energy. The target peptide is represented by the peak at *m*/*z* of 1298.66. Figure 3 shows the ESI-MS of the peptide synthesized using modified SPPS with microwave energy. The target peptide is represented by the peak at *m*/*z* of 1298.67. From two figures we can see the peptide that prepared under application of microwave irradiation indicates the presence of single major product and more pure than conventional method [19].

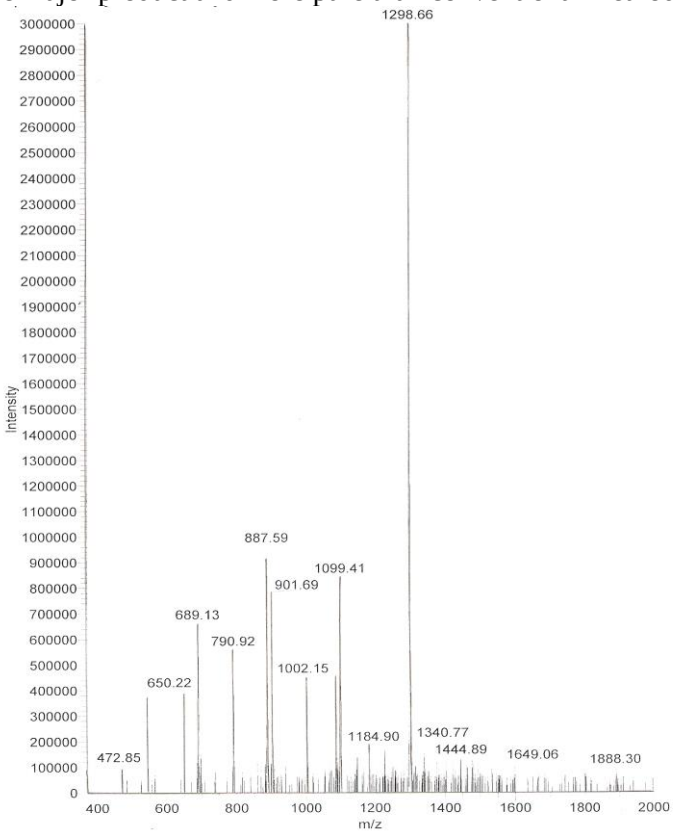

Fig.2. ESI-MS data for the target peptide without microwave energy

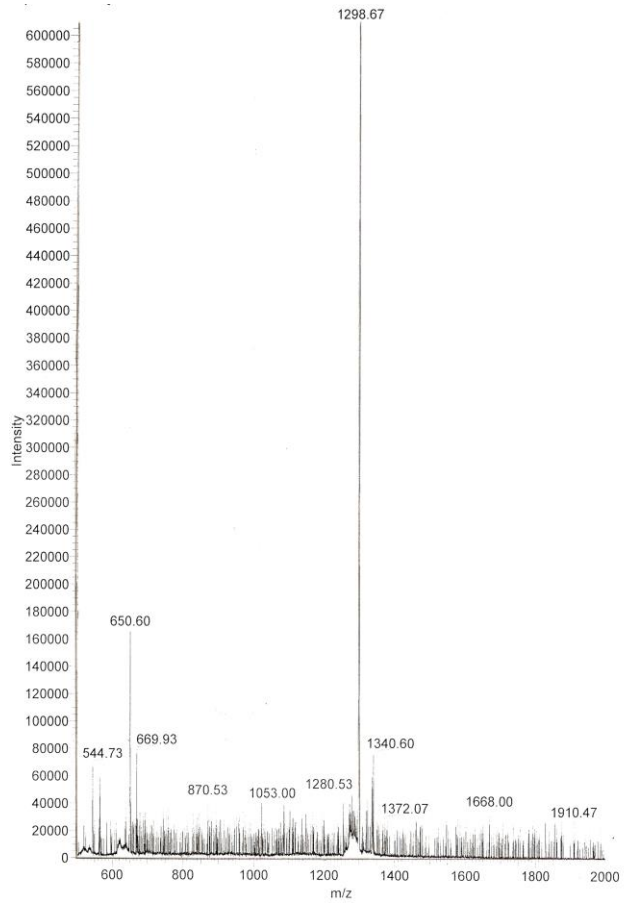

Fig.3. ESI-MS data for the target peptide with microwave energy

Fourier-transform infrared (FT-IR) spectrometry

The infrared spectra of nonapeptide (B_{22}-B_{30}) of insulin B chain, which were synthesized by two different methods [19], have the same characteristic bands of the main functional groups and matched to each other. Therefore, microwave excitation of molecules does not affect the chemical structure of an organic molecule, and the interaction is purely kinetic and illustrated in Figure 4.

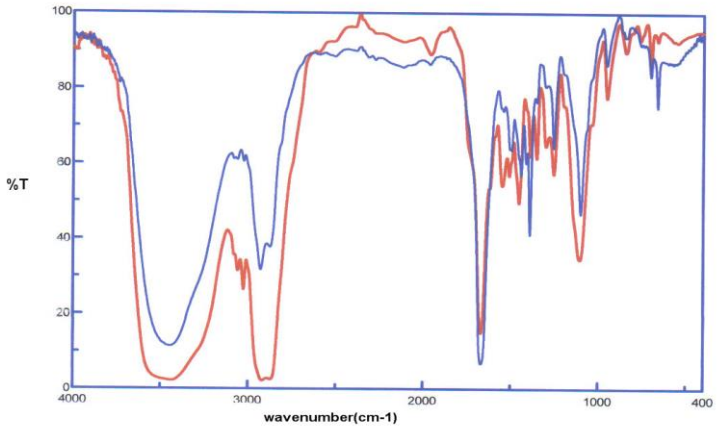

Fig.4. FT-IR of the target peptides: blue color with microwave, and red color without microwave

Thermal Analysis (TGA/DTA)

Thermal analysis is a branch of material science where the properties of materials are studied as they change with temperature, and is considered a new approach in peptide chemistry field. In our work, we used thermal gravimetric analysis (TGA) and differential thermal analysis (DTA) in the conformational study for prepared peptide. The nonapeptide (B_{22}-B_{30}) of insulin B-chain was studied by thermo gravimetric analysis from ambient temperature to 600 ^{o}C in nitrogen atmosphere. The TGA curves were redrawn as mass loss *vs*. temperature. Typical TGA curves are presented in Figure 5.

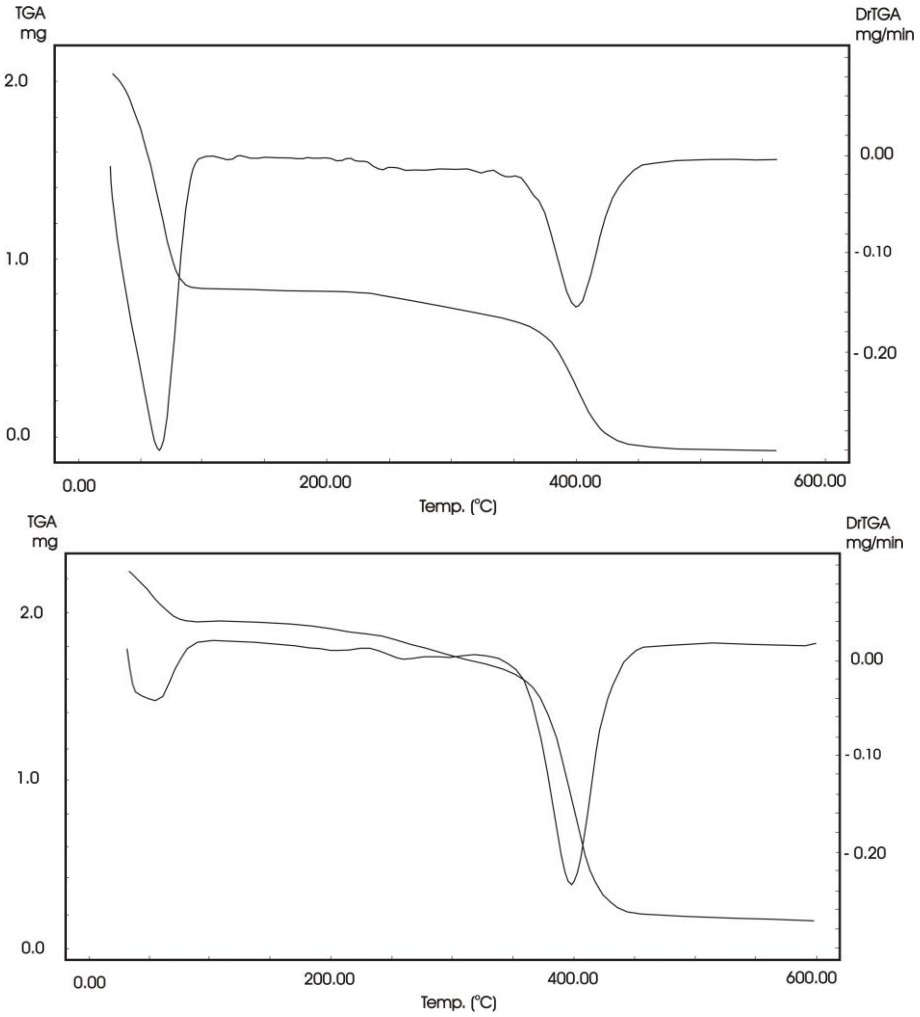

Fig.5. Thermo gravimetric analysis (TGA) of the target peptide using SPPS without microwave energy, top and SPPS with microwave energy, bottom

When comparing the two TGA/DTA figures shows that both co-existence of three maxima peaks at around 50-70 ^{o}C, 240-260 ^{o}C and around 400 ^{o}C. This means that the component of the two different procedures for the synthesis of nonapeptide (B_{22}-B_{30}) of insulin B-chain is the same. But there is one essential difference in the 50-70^{o}C regions, this region assigned to the content of moisture in the synthetic peptide with conventional method. This difference shows how important method (microwave method) developed in the preparation of nonapeptide (B_{22}-B_{30}) of insulin B-chain and how to decreasing the level of moisture. Thus, this new method gives real value of the weight composite which can be trusted to conduct critical tests correctly.

Kinetic parameters for the main stages around 400 ^{o}C, calculated by employing Coats-Redfern [21] and Horowitz-Metzger [22] equations are summarized in Table 2. The results show that the values obtained by various methods are comparable.

Table 2. Kinetic and thermodynamic data of the target peptide by two methods, (I) SPPS with microwave and (II) SPPS without microwave

No.	stage	method	Parameter					r
			E (kJ mol^{-1})	A (s^{-1})	ΔS (J mol^{-1} K^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	
I	1st	CR HM average	3.99×10^{4}	2.54×10^{4}	-164	3.72×10^{4}	9.02×10^{4}	0.9996
	2nd	CR HM average	5.01×10^{4}	5.70×10^{3}	-181	4.63×10^{4}	1.28×10^{5}	0.9998
II	1st	CR HM average	3.24×10^{4}	3.61×10^{3}	-181	2.94×10^{4}	9.33×10^{4}	0.9949
	2nd	CR HM average	4.28×10^{4}	7.75×10^{3}	-180	3.89×10^{5}	1.22×10^{5}	0.9998

The kinetic data obtained with the two methods are in harmony with each other. The activation energy of the two prepared compounds is nearly. The *E** values calculated using the Coats-Redfern and Horowitz-Metzger methods for the definite decomposition stages of the nonapeptide (B_{22}-B_{30}) of insulin B-chain which synthesized using modified SPPS with microwave, method (I) and modified SPPS without microwave, method (II). Reached through the higher value of activation energy in case of using microwave method rather than the conventional method to the result which is supports the data deduced from thermal analysis. Furthermore, the thermal stability of the nonapeptide (B_{22}-B_{30}) of insulin B-chain compound synthesized by microwave procedure is better than the conventional method.

Interestingly, in thermo gravimetric analysis (TGA), the energy in a microwave photon is very low, relative to the typical energy required to break a molecular bond. Therefore, microwave excitation of molecules does not affect the chemical structure of an organic molecule, and the interaction is purely kinetic. Further, the target peptide chains (method I & II) were then subjected to IR-spectroscopy and thermal analysis with the main goal of revealing the microwave energy does not affect the chemical structures and peptide bonds of nonapeptide (B_{22}-B_{30}) of insulin B-chain.

4. Conclusion

The aim of the present study was to synthesize, and compare between the produced peptides that prepared using two different methods, solid phase peptide synthesis with and without the application of microwave irradiation technique. Amino acid analysis, Electrospray ionization mass spectroscopy (ESI-MS) and FT-IR data were used in the characterization of products. Additionally, thermal gravimetric analysis (TGA) and dynamic thermal analysis (DTA) were measured for the comparative study of two methods. The results showed that microwave excitation of molecules did not affect the peptide bonds and chemical structures of our target peptide.

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