Synthesis of nonapeptide (B_{22}–B_{30}) of insulin B-chain using modified solid-phase methods with and without microwave energy

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Background and objectives
This work embraced a systematic search for potentiated methodology for peptide synthesis through an approach for convenient synthesis of the nonapeptide B_{22}–B_{30} of human insulin B chain. The modified solid-phase method with and without microwave technique was used for synthesis of this active part.

Materials and methods
Nonapeptide Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OH corresponding to B_{22}–B_{30} of human insulin B chain was synthesized using modified solid-phase peptide synthesis. Time consumption, yields, and purity of all products using the different methods were compared with each other.

Results and conclusion
We can interpret that these results provide a bird's eye view of the benefits of microwave energy with solid phase peptide synthesis (SPPS) in the enhancement of coupling and deprotection reactions in peptide synthesis. In addition, the application of microwave energy with SPPS provides an efficient tool for peptide synthesis, as microwave energy can effectively disrupt intermolecular aggregation and prevent β-sheet formation. Microwave technique proved to be the better approach used for this purpose in the present work.
In addition, the advantages of microwave energy include reduced side reactions that result in racemization, cyclization, or premature peptide formation. Microwave energy produces peptides with yields and purity better than the conventional method. It can accelerate the rate of coupling and deprotection reactions, reducing the reaction time from hours to minutes.

Keywords:
Fmoc amino acids, insulin, microwave energy, modified solid phase, peptide

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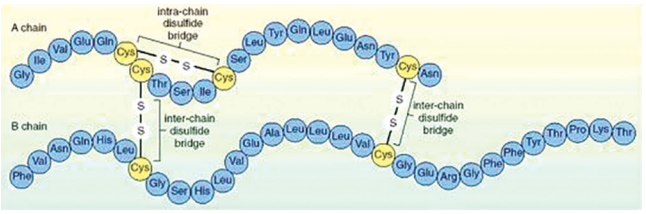
Introduction

Human insulin monomer is a peptide hormone produced by the pancreatic β cells of the islets of Langerhans. It regulates the uptake of glucose from the blood into the cells for use as an energy source. In addition, it also regulates the formation of glycogen for storage in cells and regulates many anabolic processes such as cell growth and cellular protein synthesis [1]. When control of insulin levels fails, high levels of glucose are produced in the blood and cause hyperglycemia. This disorder is known as diabetes mellitus. There are two types of diabetes; type I (insulin-dependent diabetes mellitus) is caused by a decrease or a complete cease in the production of insulin due to the destruction of β cells and type II (noninsulin-dependent diabetes mellitus) occurs when the body produces an insufficient amount of insulin or the insulin produced does not function properly [2]. Insulin hormone consists of two main chains called A chain and B chain. The A chain has 21 amino acids, whereas the B chain has 30 amino acids [3]. These two chains are linked together by disulfide bonds formed between A7–B7 and A20–B19. In addition, the A chain contains an intrachain disulfide bridge between A7 and A11 (Fig. 1). 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].

Merrifield's ingenious idea was to use an insoluble and filterable polymeric support such as cross-linked polystyrene, which functions at the same time as the carboxyl-protecting group for the C-terminal amino acid. Thus, the N^{α}-protected C-terminal amino acid is attached to the chloromethylated polystyrene-

Figure 1



Simplified chemical structure of insulin hormone.

divinylbenzene [7–9]. After removal of the N^{α}-protection, the next N^{α}-protected amino acid is coupled and the process is repeated until the entire desired peptide is assembled on the polymeric support [10].

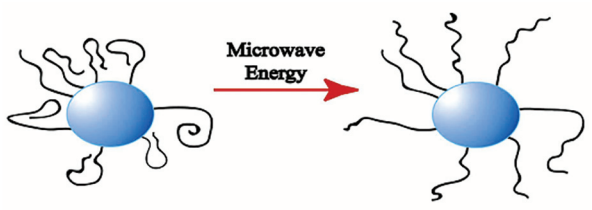
9-Fluorenylmethoxycarbonyl (Fmoc) amino acids were first used for solid-phase peptide synthesis a little more than a decade ago. Since that time, Fmoc-solid-phase peptide synthesis methodology has been greatly enhanced by the introduction of a variety of solid supports, linkages, and side chain-protecting groups, as well as by increased understanding of solvation conditions. These advances have led to much impressive synthesis, such as those of biologically active and isotopically labeled peptides and small proteins. The great variety of conditions under which Fmoc-solid-phase and liquid-phase peptide synthesis may be carried out represent a truly 'orthogonal' scheme, and thus offers many unique opportunities for bioorganic chemistry [11–13]. The graft copolymers of cross-linked polystyrene and polyethylene glycol show pressure stability, similar swelling behavior in different solvents, chemical stability, spherical form, and equal bead size [14,15]. All of these properties are prerequisite for a rapid peptide synthesis, thus combining the advantages of liquid and solid-phase methods (modified solid-phase peptide synthesis).

In the last few years, microwave synthesis has become widely accepted to increase the reaction rates in organic synthesis up to 1000-fold. Unlike conventional heating, microwave energy directly activates any molecule with a dipole moment and allows for rapid heating at the molecular level [16–19]. 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 [20] as shown in Fig. 2.

Materials and methods

The organic solvents and the chemicals used in this part were obtained from Sigma (Louis, Mo, USA) and Fluka (Louis, Mo, USA) Chemical Companies. All Fmoc amino acids were purchased from Novabiochem (Opsala, Swiden); the used amino acids were of l-configuration. The side chain-protecting groups were Fmoc for Arg and t-But for Cys, Thr, and Tyr. TentaGel-NH_2 polymer was kindly supplied by Rapp polymere Company (Tübingen, Germany). Infrared spectra (KBr) were recorded on FT-IR 1650 Perkin-Elmer (Germany) spectrometer. Amino acid analysis of the peptide sequence was carried out using the amino acid analyzer LC3000 Eppendrof. Electrospray ionization mass spectra were run on Finnigan LCQ-DECA-XP Spectrometer. The synthesis was carried out using assemble microwave peptide synthesizer (homemade) and domestic microwave oven Sanyo – EM-S3555G operating at 2450 MHz and 10% of the total power.

Figure 2



Disruption of aggregation due to microwave absorption.

Synthesis of nonapeptide by modified solid-phase method without microwave technique (stepwise)

Coupling of the first amino acid to the polymer anchor

To a solution of polymer anchor (0.15 g, 0.22 mmol) in 2 ml DMF/CH_2Cl_2 (1 : 1), a solution of Fmoc-Ala-OH (0.082 g, 1.76 mmol), *N*-hydroxybenzotriazole (HOBt) (0.036 g, 1.76 mmol), *N,N*-diisopropylcarbodiimide (DIC) (0.033 g, 1.76 mmol), and dimethylaminopyridine (20 mg, 0.3 mmol) in 5 ml DMF/CH_2Cl_2 (1 : 1) was added. The mixture was shaken at room temperature until the Kaiser test became 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-Thr-anchor polymer (0.22 mmol) was suspended in least amount of DMF. Thereafter, a solution of 25% piperidine/DMF was added and the mixture was shaken at room temperature for 45 min until the Kaiser test became 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 Fmoc-Lys to the peptide chain

To a solution of NH_2-Thr-anchor polymer (0.15 g, 0.22 mmol) in 2 ml DMF/CH_2Cl_2 (1 : 1), a solution of Fmoc-Lys (0.17 g, 1.76 mmol), HOBt (0.036 g, 1.76 mmol), and DIC (0.033 g, 8 mmol) in 4 ml DMF/CH_2Cl_2 (1 : 1) was added. The mixture was shaken at room temperature until the Kaiser test became 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-Thr-anchor resin was synthesized according to the above steps.

Synthesis of nonapeptide by modified solid-phase method with microwave technique (stepwise)

The nonapeptide (B_{22}–B_{30}) of insulin B chain was synthesized under the application of microwave energy using modified SPPS strategy described before. The coupling and deprotection reactions were carried out inside the domestic microwave oven at 2450 MHz using 10% of full power.

Synthesis of nonapeptide by modified solid-phase method using peptide synthesizer without microwave technique

In this step, the nonapeptide (B_{22}–B_{30}) of insulin B chain was synthesized using peptide synthesizer without microwave technique.

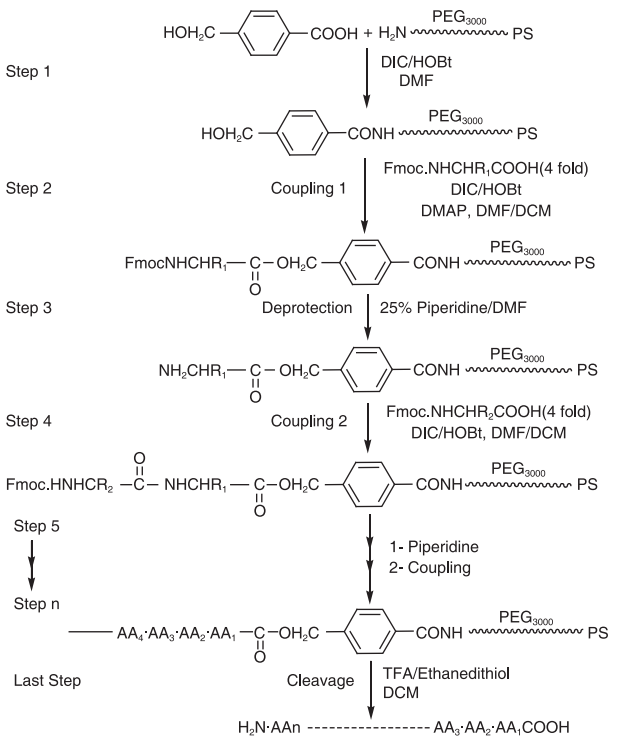
Coupling of the first Fmoc amino acid to the peptide chain using peptide synthesizer without microwave

To a solution of anchor polymer (0.15 g, 0.22 Meq), a solution of Fmoc amino acid (0.17 g, 1.76 mmol), HOBt (0.036 g, 1.76 mmol), and DIC (0.033 g, 1.76 mmol) in 2 ml DMF was added. This step was repeated for all amino acids to prepare activated amino acids. Thereafter, all steps that were carried out before without using microwave energy were repeated under nitrogen bubbling only.

Synthesis of nonapeptide by modified solid-phase method using peptide synthesizer with microwave technique

The nonapeptide (B_{22}–B_{30}) of insulin B chain was synthesized by coupling of the first Fmoc amino acid to the peptide chain using peptide synthesizer with microwave. Thereafter, all steps that were carried out before were repeated using microwave energy until the target peptide was obtained.

Scheme 1



Synthetic routes of nonapeptide (B_{22}–B_{30}) of human insulin B chain using modified SPPS.

Results and discussion

Modified solid-phase method of nonapeptide (B_{22}–B_{30}) synthesis of human insulin B chain without microwave technique

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 method. The following nonapeptide (B_{22}–B_{30}) of human insulin B chain is the synthetic model.

H-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-anchor resin.

In the present work, the protocol used for synthesis of the model peptide chain by the slow batch-modified SPPS (Scheme 1) includes the use of Fmoc group for protection of amino group. The Fmoc group can be cleaved with a base such as piperidine, and therefore can be used in conjunction with acid-labile side chain-protecting groups such as tBu compared with solely acid-labile Boc-group; the Fmoc group thus furnishes decisive improvements. Its UV absorption also opens up the possibility of spectroscopically monitoring the coupling and deprotection reactions during the peptide synthesis process.

The Fmoc group was deprotected using 25% piperidine/DMF. The electron-withdrawing fluorene ring system of the Fmoc group renders the lone hydrogen on the β carbon very acidic, and therefore susceptible to removal by weak bases. After β-hydrogen extraction, the Fmoc group proceeds through a carbanion intermediate to form dibenzofulvene. The coupling reactions were carried out using 1-hydroxybenzotriazol (HOBt) ester of Fmoc amino acids introduced by DIC, which forms soluble urea [21].

The interest in HOBt esters of Fmoc amino acids is based on well-established properties of these esters, such as suppression of racemization during coupling and rapid coupling of either *in situ* or the preformed esters. HOBt esters of Fmoc amino acids are easily formed with carbodiimides (e.g. DIC). Cleavage of the peptide linker was carried out using (10 ml) a mixture of TFA/ethanedithiol (4/1) for each gram of resin. 1,2-Ethanedithiol has been shown to be most

efficient as scavenger for the t-butyl trifluoroacetate compared with anisol/phenol and thiophenol, which are normally used for this purpose. All side chain-protecting groups were cleaved simultaneously. The time required for maximum coupling and deprotection reactions is represented in Table 1.

Modified solid-phase method of nonapeptide (B_{22}–B_{30}) synthesis of human insulin B chain with microwave technique

We describe here the application of microwave technology to enhance coupling and deprotection efficiency in solid-phase peptide synthesis. Here, the nonapeptide (B_{22}–B_{30}) of insulin B chain was synthesized using the Fmoc-SPPS strategy. The reaction was carried out inside the microwave; HOBt/DIC was utilized as activating reagent [22,23]. In addition, four-fold excess of Fmoc amino acid was used instead of eight-fold commonly used in conventional SPPS. All resin, washing solvents, cleavage mixture, and activated Fmoc amino acid were manually inserted into the reaction vessel. Complete coupling and deprotection reactions were followed up by the Kaiser test [24]. The time required for complete coupling and deprotection reactions in the synthesis of nonapeptide (B_{22}–B_{30}) of insulin is represented in Table 1. Peptide chain was identified by correct amino acid analysis as shown in Table 2.

The above results indicated clearly that the rate of both coupling and deprotection reactions is markedly enhanced using microwave energy method II. There is a marked decrease in the time required for complete coupling (2–9 min compared with 600–1200 min) in the modified SPPS method I. In addition, the deprotection reactions as shown in Table 1 required 0.5–1.5 min to be completed compared with 45 min required for Fmoc deprotection without microwave energy.

Modified solid-phase method of nonapeptide (B_{22}–B_{30}) synthesis of human insulin B chain using peptide synthesizer with microwave technique

The nonapeptide (B_{22}–B_{30}) of insulin B chain was synthesized under the application of microwave peptide synthesizer using modified SPPS strategy described before. The coupling and deprotection reactions were carried out inside the domestic microwave oven at 2450 MHz using 10% of full power. UV–visible spectrophotometer was used for measuring the coupling capacity of Fmoc amino acid and also for follow-up of the deprotection reaction according to the characteristic absorbance band in UV region of the Fmoc group. The time required for maximum coupling and deprotection reactions is represented in Table 2.

Table 1 The time in minutes required for complete coupling and deprotection reactions in the synthesis of nonapeptide (B_{22}–B_{30}) of insulin

Activated amino acids	Modified SPPS without microwave (I)		Modified SPPS with microwave (II)	
	Coupling	Deprotection	Coupling	Deprotection
Thr	1200	45	—	—
Lys	960	45	6	0.5
Pro	1200	45	8	1.0
Thr	1080	45	9	1.5
Tyr	960	45	6	1.0
Phe	960	45	5	1.0
Phe	960	45	5	1.0
Gly	600	45	2	0.5
Arg	1320	45	9	—

Table 2 The time required in minutes for complete coupling and deprotection reactions in the synthesis of nonapeptide (B_{22}–B_{30}) of insulin

Activated amino acids	Modified SPPS using peptide synthesizer with microwave (III)		Modified SPPS using peptide synthesizer without microwave (V)	
	Coupling	Deprotection	Coupling	Deprotection
Thr	—	—	—	—
Lys	13	1.5	70	9
Pro	17	2.0	80	11
Thr	12	2.5	75	13
Tyr	11	2.0	65	10
Phe	10	1.5	60	8
Phe	10	1.5	60	8
Gly	7	1.0	45	6
Arg	18	—	85	—

Modified solid-phase method of nonapeptide (B_{22}–B_{30}) synthesis of human insulin B chain using peptide synthesizer without microwave technique

In this part, we used peptide synthesizer only without using the microwave energy for comparing between the results of peptide synthesizer with and without microwave. The nonapeptide (B_{22}–B_{30}) of insulin B chain was synthesized with the application of peptide synthesizer without microwave energy using the modified SPPS strategy. The coupling and deprotection reactions were performed outside the microwave oven using peptide synthesizer unit and UV–visible spectrophotometer, which was used for measuring the coupling capacity of Fmoc amino acid and for follow-up of the deprotection reaction by characteristic absorbance band of the Fmoc group. The time required for maximum coupling and deprotection reactions is represented in Table 2.

The above results indicated clearly that the rate of both coupling and deprotection reactions is enhanced using peptide synthesizer with the microwave method III. Although coupling reactions were carried out using only four-fold excess of amino acids in case of using peptide synthesizer with microwave method III instead of eight-fold in case of using peptide

synthesizer without microwave method IV, there is a decrease in the time required for complete coupling (7–18 min) compared with (45–85 min) that using peptide synthesizer without microwave method V. In addition, deprotection reactions as shown above required 1–2.5 min to be completed compared with 6–13 min required for Fmoc deprotection using peptide synthesizer without microwave method IV.

Here, the nonapeptide (B_{22}–B_{30}) of insulin B chain that was synthesized using four different methods was purified and identified by amino acid analysis and is illustrated in Table 3.

Table 3 indicates that, for most of the amino acids, the theoretical and experimental results were equivalent.

The crude peptide chain after polymer cleavage was obtained by centrifugation, dissolved in distilled water, and subjected to HPLC. The crude peptide that was synthesized using modified SPPS with and without microwave energy was analyzed by HPLC and by positive-mode electrospray ionization mass spectroscopy ($^{+}$P ESI–MS). Figure 3 shows the ESI–MS of the crude peptide synthesized using modified SPPS without microwave energy. The target peptide is represented by the peak at *m/z* of 1298.66. The spectrum indicates the presence of impurities, immature, branched peptide, and remaining particles of the polymeric support.

Figure 4 shows the ESI–MS of the crude peptide synthesized using modified SPPS with microwave energy. The target peptide is represented by the peak at *m/z* of 1298.67. ESI–MS of the crude peptide indicates the presence of single major product (single peak) having the mass as calculated.

Infrared spectroscopy

The infrared spectra of the nonapeptide (B_{22}–B_{30}) of insulin B chain, which was prepared by two different methods, modified SPPS (slow batch) and modified SPPS with microwave peptide synthesizer, are shown in Figure 5.

Table 3 The amino acid analysis of nonapeptide (B_{22}–B_{30})

Methods number	Amino acids							
	Thr	Lys	Pro	Thr	Tyr	Phe	Gly	Arg
I								
Calculated	1	1	1	1	1	2	1	1
Found	1	0.96	0.94	0.81	0.44	1.8	1	0.86
II								
Calculated	1	1	1	1	1	2	1	1
Found	1	1	0.97	0.94	0.49	1.97	1.1	0.98
III								
Calculated	1	1	1	1	1	2	1	1
Found	1	1	0.96	0.93	0.48	1.98	1	0.99
IV								
Calculated	1	1	1	1	1	2	1	1
Found	1	0.97	0.95	0.82	0.42	1.85	1	0.89

The infrared spectra of nonapeptide (B_{22}–B_{30}) of insulin B chain, which were synthesized by two different methods, SPPS with microwave peptide synthesizer and modified SPPS slow batch, 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.

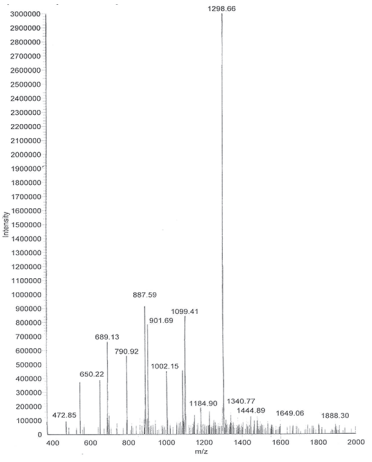
Finally, we can interpret that these results provide a bird's eye view of the benefits of microwave energy with SPPS in the enhancement of coupling and deprotection reactions in peptide synthesis. In addition, the application of microwave energy with SPPS provides an efficient tool for peptide synthesis, as microwave energy can effectively disrupt intermolecular aggregation and prevent β-sheet formation. Microwave technique proved to be the better approach used for this purpose in the present work.

In addition, from the advantages of the microwave technique, the side reactions that result in racemization, cyclization, or premature peptide formation are reduced. Microwave energy produces peptides with yields and purity better than the conventional method. It can accelerate the rate of coupling and deprotection reactions, reducing the reaction time from hours to minutes.

Conclusion

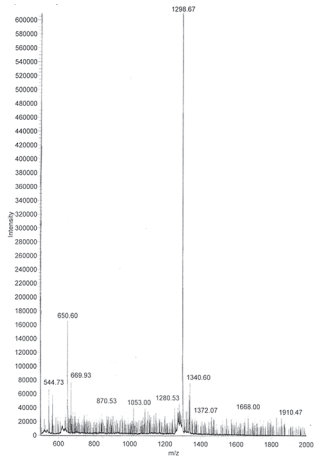
The microwave application with SPPS shows many benefits such as following:

Figure 3



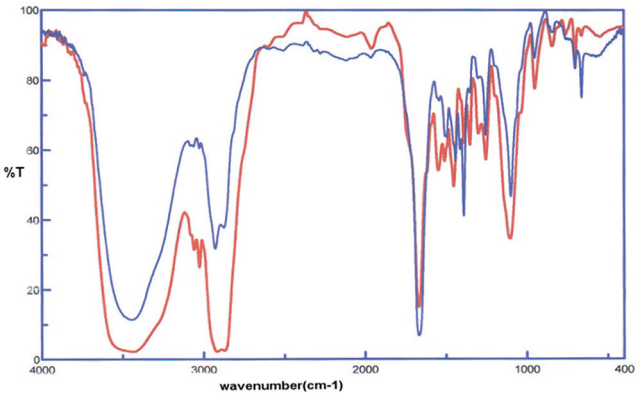
Electrospray ionization mass spectroscopy data for the crude nonapeptide (B_{22}–B_{30}) of insulin B chain synthesized using modified SPPS without microwave energy.

Figure 4



Electrospray ionization mass spectroscopy data for the crude nonapeptide (B_{22}–B_{30}) of insulin B chain synthesized using modified SPPS with microwave energy.

Figure 5



The infrared spectra of the target peptide using modified SPPS slow batch, blue color, and modified SPPS using microwave peptide synthesizer, red color.

(1) Enhancement of coupling and deprotection reactions.
(2) Effectively disrupts intermolecular aggregation and prevents β-sheet formation.
(3) Reducing side reactions that result in racemization and cyclization.
(4) Better yields and purity.
(5) Accelerates the rate of coupling and deprotection reactions.

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Conflicts of interest

There are no conflicts of interest.

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