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Microwave-assisted Solid-phase(SPPS)and Solution-phase (SPS) Synthesis of Biological Dipeptide ((*β*-alanine-*L*-histidine)

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Abstract

peptides have shown Promising effect as pharmaceutics with the potential to treat a wide variety of diseases. Peptides are mostly synthesized by biological technology or chemical methods. Solution phasepeptide synthesis (SPS) and solid phase peptide synthesis (SPPS) are two major chemical techniques for peptidesproduction.In this research, the synthesis of dipeptde(*β*-alanine-*L*-histidine)wasexaminedby the both SPS and SPPS methods. The solution phase synthesizeswasdonebyN-phthalic anhydride as a protective group andthe solid phase using standardtert-Butyloxycarbonyl (BOC) and Fluorenylmethyloxycarbonylchloride (Fmoc).Inthe both methods,themicrowave was assisted in different steps. The molecular structure of the dipeptide(*β*-alanine-*L*-histidine) wasdefined using different spectrometrymethods such as:UV-Vis, FT-IR, ^{1}HNMR and LC-Massanalysis.

Keywords:*Peptide, Microwave, Solution phase peptide synthesis, Solid phase peptide synthesis, Liquid chromatography–mass spectrometry.*

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Introduction

peptides are one of the best options for drug development due to their high specificity and low toxicity[1-7]. There are two major chemical techniques for peptides production: solution phase synthesis (SPS) and solid phase synthesis (SPPS). Classical SPS is based on the coupling of single amino acids in solution. The fragment condensation method has been used for the synthesis of long peptides. In this case, short fragments of the required peptide are first synthesized, then coupled together to form a long peptide. The prime advantage of SPS for peptide synthesis is that the intermediate products can be deprotected and purified to give the final desired peptide in high purity[8-10].The SPPS method is usually used for high production because of its simplified reaction and ordinary purification/isolation steps for the target products[11-14].In organic synthesis, when acompound hasseveral functionalgroups and reaction is accomplishedselectively at one functional group, other reactive functional groups must be protected. Therefore, adequate use of protective agents is very important to the synthetic strategy.

Protective groups like *N*-phthalic anhydride, tert-Butyloxycarbonyl (BOC) and Fluorenylmethyloxycarbonylchloride (Fmoc) areusedfor synthesis of peptides [15-21].In synthesisof peptides, the microwave method can be used as a green chemistry method. Microwave application is interesting, because of increasing the potential of synthetic method with low energy requirements and less waste, solvents and reaction times [22-27].

In this research, Synthesis of dipeptide(*β*-alanine-*L*-histidine)examinedby both solution and solid phase methods. The solution phase synthesizes of dipeptide(*β*-alanine-*L*-histidine)was doneby*N*-phthalic anhydride as a protective group. In this method, for loading first amino acid (*β*-alanine) to the phthalyl group, the microwave method (MM) and the reflux method (RM) were used and compared together. The morphology and the size of *N*-phthalyl-(*β*)-alanine were observed with a scanning electron microscope (SEM). The solid phase synthesis was done via the standardFluorenylmethyloxycarbonylchloride (Fmoc) andtert-Butyloxycarbonyl (BOC)as a protective groups.

The microwave was assisted for deprotection of Fmoc group from the first amin acid *(L*-histidine) and coupling of second amino acid (Boc-β-Alanine-OH) to the resin.The molecular structure of the dipeptide was defined using different spectrometry methods such as:UV-Vis, FT-IR, ^{1}H NMR and LC-Massanalysis.

Experimental

Material and methods

β-alanine,*L*-histidine hydrochloride monohydrate, phthalic anhydride, glacial acetic acid, benzene, sodium azide, petroleum ether, dioxane, oxalyl chloride, thionyl chloride ethanol, triethyl amine, phenyl hydrazine, diethyl ether and chloroform, methyl ethyl ketonewere obtained from Merck chemical Company.Trityl chloride resin (200~400 mesh), *N*-Fmoc-*N*-trityl-*L*-histidine was purchased (Bachem,Swiss). Boc-*β*-Alanine-OH (Aldrich, USA), coupling reagents: *O*-(Benzotriazol-1-yl)-*N, N, N′, N′*-tetramethyl-uroniumtetrafluoroborate (TBTU) (Fulka, USA). Scavengers: Anisole,phenol, solvents: trifluoroacetic acid (TFA), piperazine, *N*, *N*-di-isopropylethylamine (DIPEA), dichloro-methane (DCM), *N,N*-dimethyl formamide (DMF) and methanol (MeOH) were obtained from Merck chemical Company.

Sample characterization

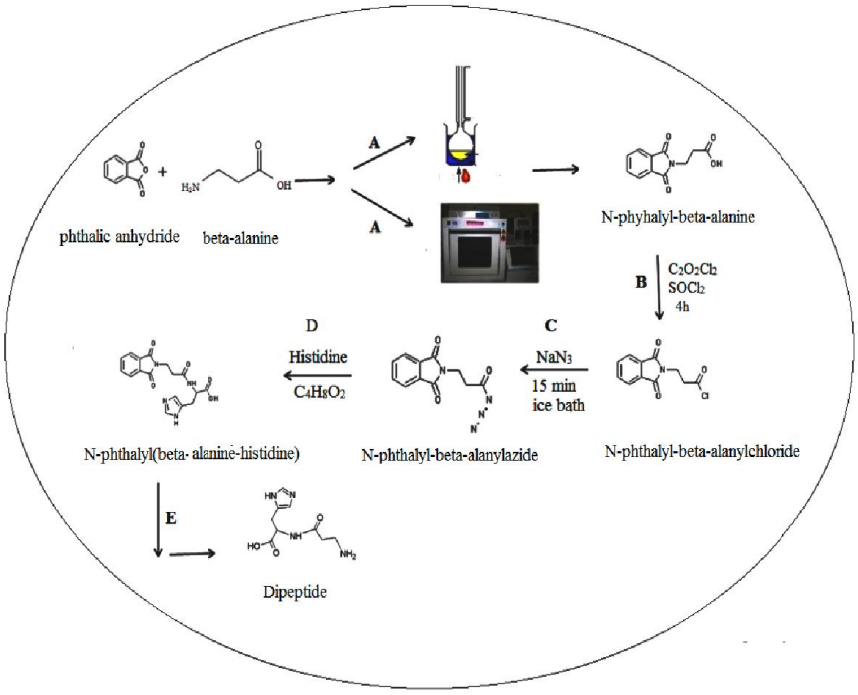
PG Instruments T80 Double Beam UV-Visibleiblespectrophotometer was used for UV-Visible measurements. The infrared spectra were recorded on a Shimadzu FT-IR-8400S spectrophotometer in the range of 400–4000 cm^{-1}. Proton NMR spectra were recorded on a Bruker DRX250 (300 MHz) spectrometer in $CDCl_3$ and water. Laboratory microwave oven, MicroSynth, Milestone (Company Italy) was employed. The morphology and size were observed with a scanning electron microscopy (SEM) Tescan-Vega II. Mass-Spectra was recorded on LC–MS Triple Quad 6410 Agilent Technologies use series 1200 HPLC system (Tokoyo,Japan) column: C-18, 250 9 4.6 mm, 5 µm, mobile phase: A: H_2O, B: methanol, flow rate: 1 ml/min, 20 µL, total run time: 40 min.

Solution phase synthesis of dipeptide(β-alanine-L-histidine)

In this method, the preparationof dipeptide (*β*-alanine-*L*-histidine) was investigated in 5 steps(Scheme1).

Synthesis of N-phthalyl-(β)-alanine

The preparation of *N*-phthalyl-(*β*)-alanine (Scheme**1-A**) was done by reflux (RM) and microwave methods (MM). The molecular structures of the compound were definite by UV-Vis, FT-IR, and ^1H NMR spectroscopy. The morphology of*N*-phthalyl-(*β*)-alaninewereinvestigated by SEM images.



Scheme 1. Solution phase synthesis of (*β*-alanine-*L*-histidine).

Synthesis of N-phthaloyl-(β)-alanine via reflux method (RM)

Phthalicanhydride(5.38 g) and (*β*)-alanine (3.23 g) were heated under reflux in glacial acetic acid (32.5 ml)for 3 h (bath temperature =118 °C). The acetic acid was evaporated under vacuum. Distilled water (7 ml)was added to the residue and the mixture was refluxed for 1 hour. After cooling, the resulting mixture was extracted with ether-water (1:4). The precipitated solid was filtered and dried under vacuum to give white crystals of the *N*-phthaloyl-*(β)*-alanine.(Yield = 80.6 %, m.p. =150 °C)[28].

Synthesis of N-phthaloyl-(β)-alanine via microwave method (MM)

A mixture of phthalic anhydride (1.48g) and (*β*)-alanine (0.89 g)were added in a microwave vessel. The microwave was programmed to give a maximum internal temperature of 130 °C and setting the power range in 380 W for 10 minutes. Then, the reaction mixture was cooled in room temperature and tested by TLC method and again put in microwave and heated for 5 minutes and tested with TLC. The reaction mixture was extracted with water-chloroform (1:3), dried with (Na_2SO_4), filtered and the solvent removed (Yield = 85.6 %, m.p=146 °C)[29].

Synthesis of N-phthalyl-β-alanylchloride

For stirred solution of *N*-phthaloyl-(*β*)-alanine (1.12 g) in (20 ml)mixture of cyclohexane-benzene (1:1), thionyl chloride (0.9ml) was added drop wise. After that freshly distilled DMF(0.01ml) was added. The mixture was shaken at room temperature for 4 h. The solvents were evaporated under vacuum. The residue was washed with dry cyclohexane and then evaporated in vacuum to dryness. A yellow crystalline solid was obtained. (Scheme**1-B**) (Yield = 87 %, m.p. = 92°C)[28].

Synthesis of N-phthalyl-β-alanylazide

This step accomplished by the method of Kroll and Hoberman[29]. Which (0.5g)phthalyl-*β*-alanylchloride was dissolved in(5ml)of cold acetone, and the solution mixed with (0.07g)of sodiumazide that dissolved in (2ml)of water. The reaction mixture was shaken for 10 minutes in an ice-bath. White oil separated initially,which solidified after about 30 minutes.The residue was obtained by filtration and dried in vacuum oven(Scheme**1-C**) (Yield = 85 %).

Synthesis of N-phthaly1 dipeptide (β-alanine-L-histidine)

Histidine hydrochloride monohydrate (0.26g) was dissolved in (2.5ml)of sodium hydroxide. The phthalyl-*β*-alanylazide(0.3g) was dissolved in (6ml) of dioxane and the resulting solution poured into the histidine solution. The mixture was stayed in the refrigerator overnight. The reaction mixture was neutralized with (4.5 ml) sulfuric acid. The resulting mixture was filtered and concentrated under vacuum. The residue was washed with (5ml) methanol for three times and again put in the refrigerator. A white crystalline solid was obtained (Scheme**1-D**) (Yield =86%, m.p. = 230°C)[30].

Synthesis of dipeptide (β-alanine-L-histidine)

In the last step of the synthesis, thepeptide was cleaved from the*N*-phthaloyl groups by the method of Shuman and Boissonas[31].The phthalyl dipeptide (0.08 g) was dissolved in ethyl alcohol (1ml), triethylamine(0.04ml) and phenyl hydrazine (0.06 ml). The mixture was refluxed for three hours. At the end of this time, the yellow solution was appeared,thatcooled and acidified with glacial acetic acid (0.05 ml) and the mixture poured into methyl ethyl ketone (3.5ml). Then, theobtained precipitate dissolved in water and recrystallized by addition of ethyl alcohol. The white residue is dipeptide (Scheme **1-E**) (Yield = 85%, m.p. =258 °C).

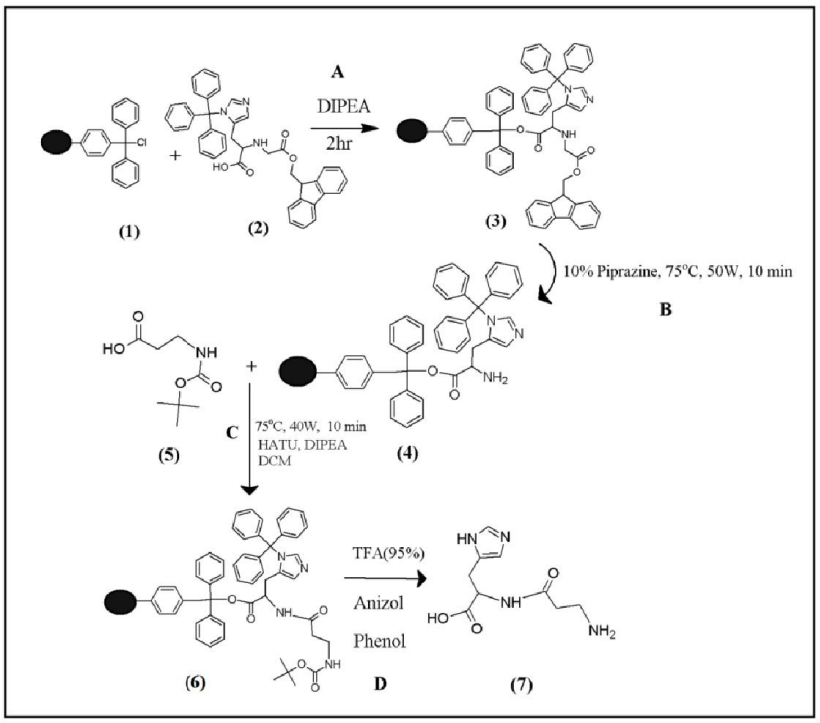
Solid-phasesynthesis of dipeptide (β-alanine-L-histidine)

The dipeptide (his-*β*-alanine**)**was manually synthesized on solid phase using standard Fmoc and Boc Strategy[20, 32-34].Briefly, the peptide sequence(Scheme **2**)his-*β*-alanine was assembled on trityl chloride resin(2 g) (Scheme**2-1**)and swelled in dichloromethane(1h). *N*-Fmoc-*N*-trityl-*L*-histidine (2.5 g, 2 equivalents) (Scheme**2-2**), and *N*, *N*-isopropylethylamine (DIPEA) (1 ml), DCM (2.5 ml), DMF (5 ml) were added to the reaction vessel. The mixture was shaken for 2h**(**Scheme**2-A)**. After 2 h resin was filtered and washed entirely under nitrogen atmosphere with DMF (5 ml × 3), DCM (5 ml × 3), respectively.

After, first amino acid is loaded to the resin(Scheme**2-3**) the unreacted sites must be end-capped with methanol (MeOH) to ensure that subsequent reactions do not react at those unloaded sites. In cappingprocess mixture of DCM, MeOH, and DIPEA in a (80:15:5) ratio wereadded to the resin and shacked for 15 minutes.Then resin washed under nitrogen atmosphere with DMF (5 ml × 3), DCM (5 ml × 3) respectively.The microwave was assisted forremoval of the Fmoc group by addition of 10% piperazine;at 75 °Cand setting the power range at50 Watt for 10 minutes**(**Scheme**2-B)**.

The monitoring of the completion of the Fmoc cleavage(Scheme**2-4**), performed with color detector (acetaldehyde/chloranil) for detection of free terminal amino groups.This detector will transformedfrom white to green if an amino group is present.Forloading second amino acid**(**Scheme**2-5)**, again microwave was assisted.Boc-*β*-alanine-OH (0.75 g, 2 equivalents) was treated with coupling reagents [HATU (1.2 g, 2 equivalents) in DIPEA (1 ml), DCM (10 ml)] to form a solution, which was added to the resin and occurred at 75 °C at 40 Wfor 10 minutesin microwave[35](Scheme**2-C**).

The reaction was terminated by performing the chloranil test, colorless beads positive for coupling the second amino acid.After coupling resin was filtered and washed with DMF (5 ml × 3), DCM (5 ml × 3) and MeOH (5 ml × 3) respectively,and dried under vacuum.In the last step of the synthesis(Scheme**2-D**), dipeptide(Scheme**2-6**) was cleaved from the resin with a mixture of trifluoroacetic acid (TFA) (1 ml), anizol (0.3 ml), phenol (0.3 g) and was shaken at room temperature for 2 h. At the end of 2 h,theresin was filtered and washed with DMF (5 ml × 3), DCM (5 ml × 3). The filtrate was evaporated under reduced pressure and the resulting mixture precipitated by adding diethylether(Scheme**2-7**). (Yield = 90 %, m.p. =258 °C).



Scheme 2. Steps of solid-phase synthesis of (*β*-alanine-*L*-histidine).

Results and discussion

Characterization of prepared dipeptide

Synthesis of dipeptide(*β*-alanine-*L*-histidine) examined by both solution and solid phase methods. The synthesis of dipeptide (histidine-*β*-alanine)was structurally confirmed by UV-Visible, FT-IR,^{1}H NMR and LC-Mass techniques. In the first step of SPS method, the preparation of *N*-phthalyl-(*β*)-alanine was investigated by the reflux method (RM) and microwave method (MM). In reflux method, the reaction was completed after 5h, whereas in microwave method, only within a fewminutes. Therefore, relative to reflux method, the microwave method, significantly reduced synthesis time and amounts of reagents.

The The UV-Vis absorbance spectra of *N*-phthaloyl-(*β*)-alanine by RM and MM methods was prepared in chloroform at 25 °C. The results show that the maximum peak of *N*-phthaloyl-(*β*)-alanine was appeared at 246 and 244 nm for reflux and microwave methods, respectively. The observed slight difference can be due to the difference in particle size of *N*-phthaloyl-(*β*)-alanine according to the SEM images are shown in Figure 1 (**A-C**) and (**D-F**) respectively. In reflux method, the time of reaction might be sufficient to form the separate phases, which has led to the bigger particle size with wider distribution, but in the microwave method, the size of the particles with smaller and narrow distribution were obtained[36].FT-

IR Spectrum of *N*-phthalyl-(*β*)-alanineby reflux (RM) and microwave (MW) methods are completely correspound together.

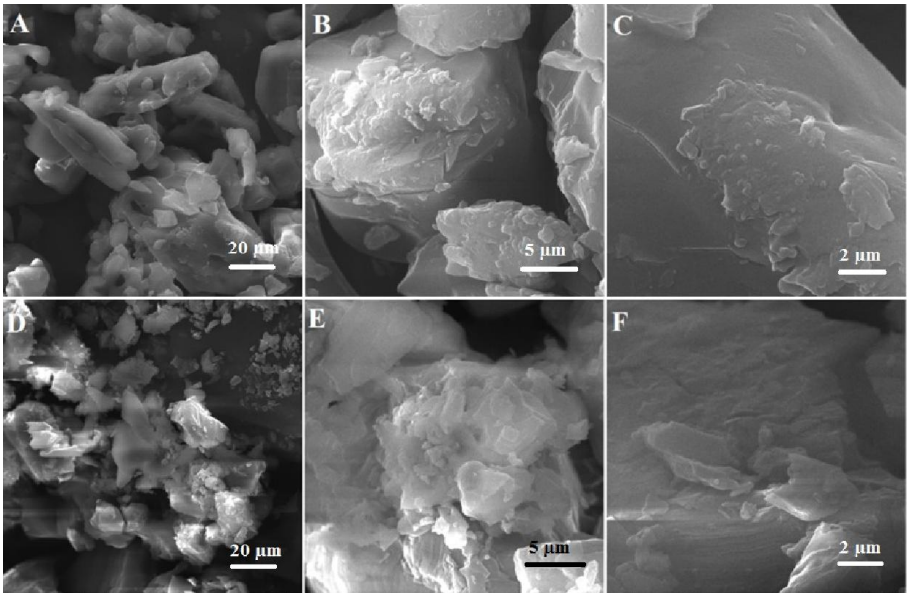


Figure 1.SEM images of*N*-phthalyl-(*β*)-alanine prepared by (A-C) reflux method and (D-F) microwave method.

The data of proton NMR spectra of *N*-phthalyl-(*β*)-alanine is:^{1}H NMR(300 MHz, $CDCl_3$), δ= 7.74 (2H, C_6H_4), 4.010 (1H, CO_2H), 2.27 (1H, CH).

In continue, other synthesized steps of SPS method were studied using FT-IR (KBr) or^{1}H NMR(300 MHz)spectra include:

N-phthalyl-β-alanyl chloride:

FT-IR (KBr) ν_{max} (cm^{-1}): 1801 (Cl-C=O), 1710(N-C=O), 1421 (CH=). ^{1}H NMR(300 MHz, $CDCl_3$):δ= 7.74 (2H, C_6H_4), 4.03(2H, CH_2N), 3.363(2H, CH_2CO).

N-phthalyl-β-alanylazide:

FT-IR (KBr) ν_{max}(cm^{-1}): 2920, 2923 (N_3-C=O), 1716 (N-C=O), 1569 (CH=).

N-phthalyl (β-alanine-L-histidine):

FT- IR (KBr) ν_{max}(cm^{-1}): 2858, 3091 (OH), 1631 (N-C=O), 1566 (HO-C=O).

Finally, the synthesis of a dipeptide (*β*-alanine-*L*-histidine) with both SPS and SPPS methods, approved by UV-Vis, FT-IR, ^{1}H NMR spectra, and LC-Mass analysis. The results ofthe both methods were corresponded together.The UV-Visible absorbance spectra of the *dipeptide*was

obtained in water at 25°C, the UV absorptions appeared at 214 and 268 nm which can be related to electronic transitions of$\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$respectively. The following spectral data for dipeptide was obtained, from theFT-IRspectra (KBr, cm^{-1}) with ν_{max}: 3238 (NH_2), 2613-3300(OH), 1643 (N-C=O), 1564 (-C=N), and from^{1}H NMRspectra (300 MHz, D_2O):δ=7.48 (imidazole ring);δ=4.69(2H, CH_2N),δ=4.24 (d, CO_2H).The LC–MS analysis revealed a single mass peak in $[M+H]^+$and $[M]^-$which corresponds to the calculated molecular weight ofdipeptide,$C_9H_{14}N_4O_3$,calculated:226.23, found: m/z $[M+H]^+$: 227.000 and m/z $[M]^-$: 224.800 (Figure 2).

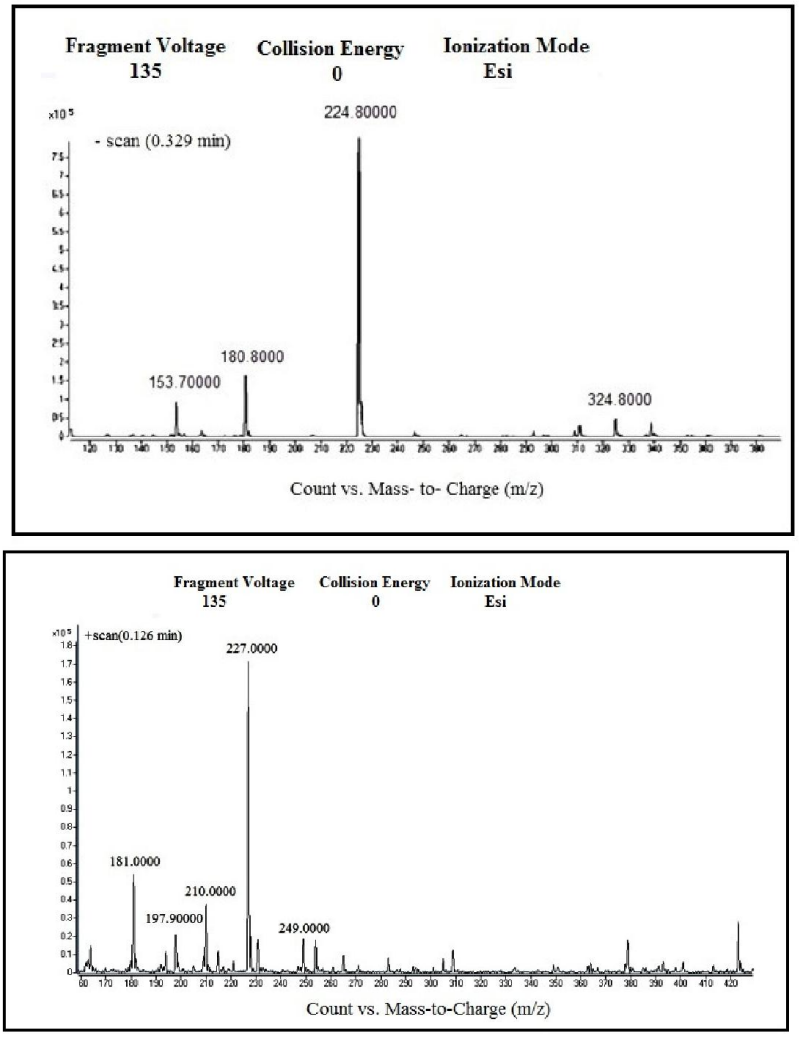


Figure 2. LC–MS chromatogram (+scan and –scan) of(*β*-alanine-*L*-histidine).

Conclusion

In this work, the synthesis of a dipeptide (his-*β*-Alanine) was donebysolution phasepeptide synthesis (SPS) and solid phase peptide synthesis (SPPS). In the both methods, microwave

was assisted in different steps thatincreasing the potential of synthetic method with low energy requirements and less waste, solvents and reaction timein high yield and purity. The advantages of SPPSvs. SPS is:Fast production of dipeptide byincreasing the amount of reactant, quick purification by filtration.Also, the disadvantages are:Expensive resin and limited scale-up.

Acknowledgements

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