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Characterization and Purification of Viral Peptides Synthesized with Microwave Assisted Solid Phase Method

Zafer Ömer ÖZDEMİR* Murat TOPUZOĞULLARI Erdem KARABULUT Zeynep MUSTAFAEVA AKDESTE
Yildiz Technical University, Chemical and Metallurgical Faculty, Bioengineering Department, Davutpaşa Camp., Esenler, İstanbul 34220

*Corresponding Author
e-mail: ozdemirz@gmail.com

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Abstract:
Peptides and proteins play a central role in numerous biological and physiological processes in living organisms. This study was conducted for characterization and purification of three different viral capsid peptide sequences. These viral peptide sequences were synthesized with microwave assisted solid phase peptide synthesis in our research laboratory. Synthesized viral peptide sequences are NP 55-69, melanoma 155-163, and VP1 135-161. Viral capsid peptides are part of the viruses' outer shell of genetic materials. Viruses are recognized by immune system via capsid peptides. Depending on this property of capsid peptides, totally synthetic vaccine prototypes can be developed. LC-ESI-MS system was used to characterize the sequences and peptides were purified with preparative HPLC system.

Key words: LC-ESI-MS, RP-HPLC, solid phase peptide synthesis, synthetic vaccine

INTRODUCTION

Proteins and their smaller relatives' peptides are present in every living cell and possess a variety of biochemical activities. They appear as enzymes, hormones, antibiotics, and receptors. They compose a major portion of muscle, hair and skin. Consequently, scientists have been very interested in synthesizing them in the laboratory. This interest has developed into a major synthetic field known as "peptide synthesis" [1].

The general chemical requirements for peptide synthesis were to block the carboxyl group of one amino acid and the amino group of the second amino acid. Then, by activation of the free carboxyl group, the peptide bond could be formed, and selective removal of two protecting groups would lead to the free dipeptide [2]. After discovery of the solid phase peptide synthesis (SPPS) by Merrifield [3], research on peptide synthesis rapidly increased. In Merrifield's SPPS method, peptide grows on a polymeric solid particle, which is called resin. Resin is a polymeric material having functional groups for peptide binding. In contrary to solution phase peptide synthesis, SPPS is successful for synthesis of long peptide sequences [4].

Recently microwave energy is using in SPPS. Microwave irradiation is electromagnetic irradiation in the frequency range of 0,3 to 300 GHz. Microwave-enhanced chemistry is based on the efficient heating of materials by "microwave dielectric heating" effects. This phenomenon is dependent on the ability of a specific material (solvent or reagent) to absorb microwave energy and convert it into heat. The electric component of an electromagnetic field causes heating by two main mechanisms: dipolar polarization and ionic conduction [5, 6].

Mass spectrometry (MS) is the most powerful tool to characterize the synthesized peptides, which gives the molecular weight of molecule. The difference of theoretical and observed mass values of peptide molecule shows that the sequence is not accurate. LC-MS with electrospray ionization probe is widely used in peptide analysis. First LC-MS system was used in 1970's [7, 8]. Molecular weight of every size of molecules, like DNA molecule, can be observed and non covalent biomolecular interactions can also be examined with liquid chromatography electrospray mass spectrometry (LC-ESI-MS), since electrospray ionization (ESI) is a very 'soft' ionization technique [9].

LC-ESI-MS system has four main parts, which are High pressure liquid chromatography (HPLC) [10], ionizer, mass analyzer and detector. In ESI analysis, sample in a solution is ionized with sprayed to pressurized room by a capillary. 0,5-4,5 kV electric voltage is applied to this capillary. For ionization of the sample, some chemicals like trifluoroacetic acid (TFA), formic acid is added [11] to solution. Inert gas (N_2) is used for spray [12].

Like all chemical synthesis methods, by-products are formed during the SPPS of peptides, which must be removed for further biological studies. Reversed-phase HPLC is the most widely used method to purify crude peptide, since it has the capability of separating two polypeptides, which differ only by a single amino acid residue [13].

In this study, peptide sequences defined from foot-and-mouth disease, influenza and melanoma viruses were synthesized with microwave assisted SPPS method. These peptides were analyzed using LC-ESI-MS, and crude peptides were purified with reversed-phase HPLC system.

MATERIALS AND METHODS

All chemicals used in this study were obtained from commercial sources. Wang resins [14], amino acids and coupling reagents purchased from NovaBiochem. The other chemicals purchased from Sigma-Aldrich. These peptides were synthesized by microwave assisted SPPS method (Liberty, CEM) in dimethylformamide (DMF) media, using 2-(1H-Benzotriazole-1-yl)–1,1,3,3– tetramethyluronium hexafluorophosphate / 1-Hydroxybenzotriazole (HBTU/HOBt) as activator and N,N–Diisopropylethylamine / N-Methyl–2-pyrrolidinone (DIEA/NMP) as activator bases.

Preloaded Wang resin was used in the synthesis of peptides. Microwave energy used for the deprotection step was 55 W to maximum 45 °C for 2 min and it was 25 W to maximum 75 °C for 3 min for the coupling step. Peptides were cleaved from resin using cleavage cocktail prepared with TFA/Thioanisole/EDT/Water 90/5/2.5/2.5 (v/v). Cold (-20°C) ether was used for precipitation. After the centrifugation, precipitated peptides were dried in vacuum. Synthesized viral peptides were VP1 135-161, defined from foot- and-mouth disease virus's (FMDV) capsid protein sequences [15], (nonstructural protein) NP 55-69, defined from influenza virus [16] and Melanoma 155-163, defined from melanoma virus [17]. Tryptophan amino acid was also added to the N-terminus of the sequences in order to analyze them with fluorescence spectrometry for further research. Synthesized peptide sequences are:

NP 55-69: WRLIQNSLTIERMVLSA, Melanoma 155-163: QLSLLMWIT, VP1 135-161: SKYSTTGERTRGDLGALAARVATQLPA.

LC-MS system (Shimadzu LC-MS 2010 EV) with electro spray ionization (ESI) probe was used for characterization of synthetic peptides. HPLC column was Teknokroma Tracer Exel 120 ODS-A 5 µm in the dimensions of 20 cm length and 2.1 mm inlet diameter. Elution was gradient from eluent A (water, 0.1% TFA) to eluent B (acetonitrile, 0.1% TFA) and at a flow rate of 2.0 ml/min. When ideal gradient was defined, HPLC purification were performed with these parameters. Crude peptides purified with preparative-HPLC system (Shimadzu). Elution is the same as Liquid chromatography-mass spectrometry (LC-MS) system. Shim-pack PRC-ODS HPLC column (20 mm x 25 cm) was used for purification. Ultra pure water from Millipore MilliQ Gradient system was used.

RESULTS

Fig. 1 shows the total ion chromatogram and mass spectrum of peak I obtained from LC-ESI-MS analysis of VP1 135-161 sequence after synthesis. In ESI method, a series of multiply charged ions of the molecule are formed. The molecular weight of molecule is calculated using m/z values of these ions. In figure 1B, there are m/z values of 559.30, 698.70, 931.35 and 1396.35, which are the molecular ions of $[M+5H^+]^{5+}$, $[M+4H^+]^{4+}$, $[M+3H^+]^{3+}$ and $[M+2H^+]^{2+}$, respectively. Molecular weight of the peptide calculated from these ions is 2791.09, which has the theoretical value of 2791.125. It is clear that this peak belongs to VP1 135-161 sequence. Other peaks have the molecular weights of 2634.56, 2478.04 and 2773.45. 2634.56 is the molecular weight of VP1 135-161 sequence, which has an arginine deletion. Mass difference between 2791.09 and 2478.04 shows two arginine deletions in the sequence. Arginine deletion can be prevented or decreased by increasing arginine concentration and microwave energy in coupling steps. 2773.45 is the molecular weight of dehydrated form of VP1 135-161 sequence.

The by-products were removed and peptide was purified with reversed phase HPLC (RP-HPLC) system. UV Chromatogram obtained from purification step is shown in Fig.2A. The fraction shown as 12 in the chromatogram is VP1 135-161 sequence. Fig. 2B is the mass spectrum of this peak. After collection of this peak, peptide was lyophilized.

Fig. 3A is the total ion chromatogram of NP 55-69 sequence, which has the theoretical molecular weight of 2030.43. The peak eluting at the retention time 17.4 minutes has the molecular weight of 2030.52 calculated using the molecular ions of 1015.95 and 677.60 (Fig 3B). This peak belongs to NP 55-69 sequence.

Other peaks have the molecular weights of 1375.25 and 1916.78. Mass difference between 1916.78 and 2030.52 is 131, which shows the deletion of methionine. Molecular weight of 1375.25 can be related to the sequence which has the deletions of two arginine molecules, two (iso)leucine molecules and valine. Since isoleucin and leucin have the same molecular weights, it cannot be identified which amino acid is missing [18].

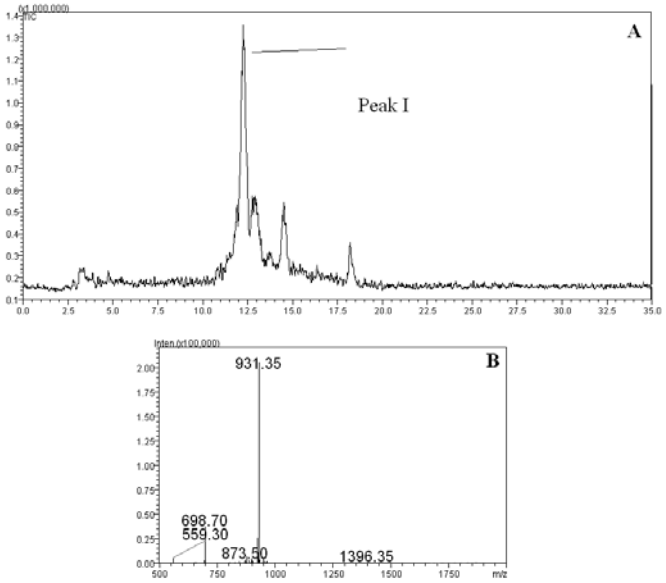

Figure 1. A) Total ion chromatogram of crude VP1 135-161. B) Mass spectrum obtained from total ion chromatogram of VP1 135-161 between the retention times of 12.1-12.5 minutes.

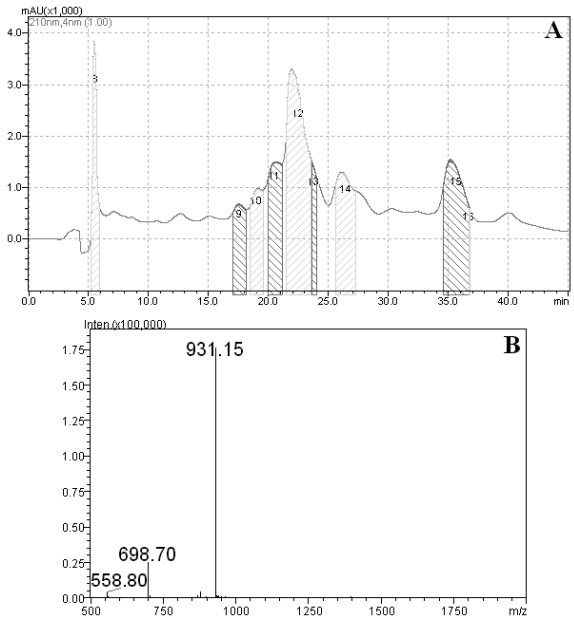

Figure 2. A) UV chromatogram of preparative RP-HPLC of VP1 135-161. B) Mass spectrum of fraction 12 shown in Fig. 2B.

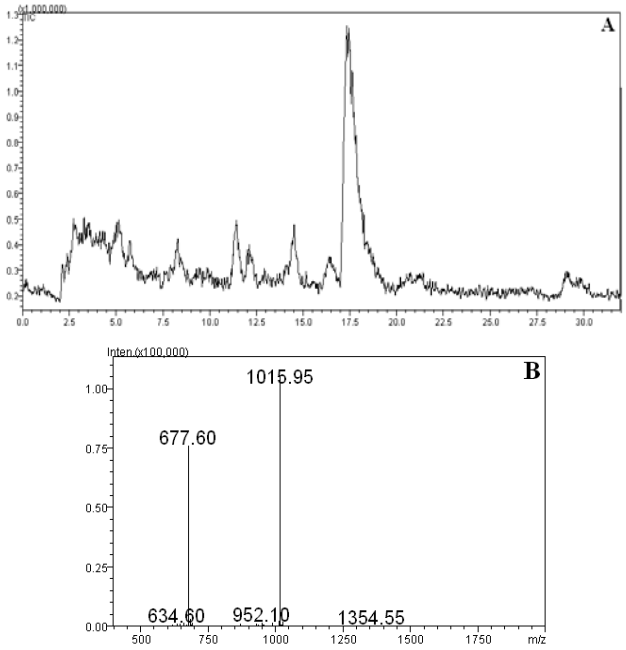

Figure 3. A) Total ion chromatogram of crude NP 55-69. B) Mass spectrum obtained from total ion chromatogram of NP 55-69 between the retention times of 16.9-19.3 minutes.

Figure 4A shows the total ion chromatogram of melanoma 155-163 sequence. Mass spectrum of peak I is shown in Fig. 4B. Theoretical molecular weight of the sequence is 1290.59 and observed molecular weight of peak I is 1290.15. It is seen from the figure that peptide molecule has only mono-charged ion. Molecular weight was calculated using only this value.

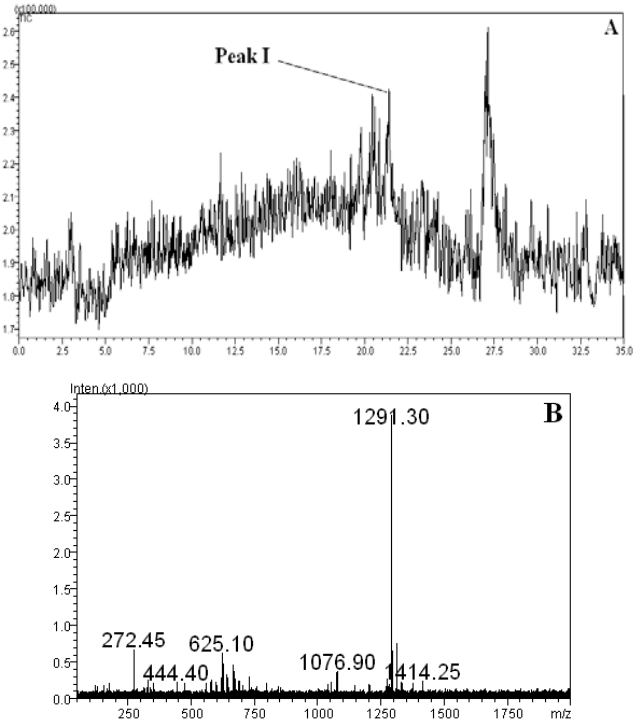

Figure 4. A) Total ion chromatogram of crude melanoma 155-163. B) Mass spectrum of peak I obtained from total ion chromatogram.

DISCUSSION

As it is seen from the results, long peptide sequences (longer than 15 amino acid residues) are synthesized effectively with microwave assisted SPPS method. Aggregation is one of the main problems especially for synthesizing long peptide sequences. Using microwave irradiation prohibits the aggregation of elongated peptide chains. Viral peptides which are synthesized, characterized in this research will be used in bioconjugation with proteins and water-soluble polymers by our research group. Generally, vaccines may consist of live or killed viruses [19]. Viral capside peptide is the viruse's outer shell protein part which recognized by element of immune system [20]. Depending on this property and preparation of their bioconjugates with biodegradable polymers, developing a totally synthetic vaccine prototype is possible [21, 22].

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