ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

DEVELOPMENT OF SPIKE MHC-I T-CELL EPITOPE TARGETED PEPTIDES FOR VACCINE AND DIAGNOSTIC KIT AGAINST COVID-19

M.Sc. THESIS

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JUNE 2021

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

COVID-19 AŞISI VE TANI KİTİ İÇİN SPIKE MHC-I T-HÜCRE EPİTOP HEDEFLİ PEPTİTLERİN GELİŞTİRİLMESİ

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HAZİRAN 2021

Nihal Karakaş, a M.Sc. student of ITU Graduate School student ID 513191012, successfully defended the thesis entitled "DEVELOPMENT OF SPIKE MHC-I T-CELL EPITOPE TARGETED PEPTIDES FOR VACCINE AND DIAGNOSTIC KIT AGAINST COVID-19", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 15 June 2021
Date of Defense : 25 June 2021

FOREWORD

I would like to express my sincere gratitude to my advisor Asst. Prof Dr. Onur ALPTÜRK for his support, supervision, and providing all the necessary facilities during my MSc study. I would also like to thank him for his patience, helps, and everything that I learned from him.

I would like to sincerely thank my co-advisor Dr. Özgür YILMAZ for his support, guidance, and helps. I would also express my thanks to him for giving me a chance to work in his laboratory.

I would like to thank Mert DÖŞKAYA, Cemal ÜN, Hüseyin CAN, and Muhammet KARAKAVUK for their support and contribution to this project.

I would like to sincerely thank Sevgi GÜLYÜZ for her support, friendly approach, and invaluable scientific guidance. She always helped me in every field and found a solution to the problems that I encounter.

I would like to thank my all lab-mates, especially Sümeyra AYAN and Gizem BEYOĞLU for providing a fun, friendly, and peaceful working environment and sharing joyful moments during my studies. I am very appreciative of their sincere friendship, supports, and helps. It was a pleasure working with them.

I would like to thank The Scientific and Technological Research Council of Turkey (TÜBİTAK) for financially supporting me during my thesis study.

I would also like to thank Müge AKDEMİR and Seyedehnegar ARABİ for their valuable friendship, constructive suggestions, and helps.

I would like to express my special thanks to my dear family, my mother, my father, and my siblings for their endless support and patience during my education. I can feel how they are proud of me and I have felt their presence and support in every period of my life. I would also sincerely like to thank my best friends Ayşenur PAMUKÇU and Büşra KÖSE for their invaluable friendship and continuous support in every field of my life.

June 2021

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ABBREVIATIONS

2019-nCoV : 2019 novel coronavirus
Acn : Acetonitrile
Boc : t-butyloxycarbonyl
COVID-19 : Coronavirus Disease-2019
CT : Computed Tomography
DCC : Dicyclohexylcarbodiimide
DCM : Dichloromethane
DIC : Diisopropylcarbodiimide
DIPEA : *N*,*N*-Diisopropylethylamine
DMF : *N*,*N*-Dimethylformamide
DNA : Deoxyribonucleic acid
DTT : Dithiothreitol
EDC : Ethyl-3-(3-dimethylaminopropyl) carbodiimide
FDA : The Food and Drug Administration
Fmoc : 9-fluorenylmethyloxycarbonyl group
HBTU : O-(Benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate
hCoV-19 : human Coronavirus 2019
HOAt : 1-hydroxy-7-aza-benzotriazole
HOBt : triazoles 1-hydroxy-benzotriazole
HPLC : High-Performance Liquid Chromatography
KCN : Potassium cyanide
kDa : kilodalton
LC-HR/MS : Liquid Chromatography-High Resolution Mass Spectroscopy
mAU : Milli-absorbance unit
MBHA : 4-Methylbenzhydrylamine resin
MERS : The Middle East Respiratory Syndrome
MHC : Major Histocompatibility Complex
PCR : Polymerase Chain Reaction
PDA : Photodiode Array
PTFE : Polytetrafluoroethylene
RNA : Ribonucleic Acid
RP-HPLC : Reverse Phase High-Performance Liquid Chromatography
rRT-PCR : Real-Time Reverse Transcription Polymerase Chain Reaction
RT-PCR : Reverse Transcription Polymerase Chain Reaction
SARS : Severe Acute Respiratory Syndrome
SARS-CoV-2 : Severe Acute Respiratory Syndrome Coronavirus 2
SPPS : Solid-Phase Peptide Synthesis
TFA : Trifluoroacetic acid
TIPS : Triisopropylsilane
WHO : World Health Organization

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SUMMARY

A novel coronavirus disease caused by SARS-CoV-2 emerged as an outbreak at the end of 2019 in Wuhan, China. Then, the disease was declared a pandemic by spreading to the world. Due to the absence of a specific diagnosis, treatment, and protection methods for this novel coronavirus disease, it became a global threat. Therefore, it is obligatory to develop effective methods to detect, treat and prevent this disease. There are some available detection and treatment methods. However, there has been no protection method for COVID-19 yet.

The available detection methods are not adequate for SARS-CoV-2. The coronavirus has been currently detecting by the RT-PCR method, also chest CT can be employed for the patients. Also, there are some available drugs and vaccines which are used to diminish and relieve the symptoms of the COVID-19. However, these current methods are not specific to this disease and they are not able to cure and provide protection for this disease.

Besides diagnosis and treatment, protection and prevention are also very essential in any disease. There are some supportive treatments, however, there is no protection for coronavirus disease. The development of a protection method for coronavirus has huge importance for prevention from this disease and controlling the spreading of the disease, and also ending the pandemic.

There are some ongoing and promising studies to develop detection and protection methods for COVID-19. Some diagnostic tests and vaccine studies are approved and authorized in emergency use. However, they must be improved, also their efficacy and specificity must be increased. In addition to this, new vaccines and diagnostic kits that have better properties must be developed.

Consequently, it is obligatory to develop new and effective methods for the detection of, and protection from COVID-19 because of the ongoing pandemic. Based on the promising properties of peptides such properties as high specificity, selectivity, biocompatibility, non-toxicity, and ease to synthesize, it was decided to develop peptide-based diagnosis and vaccine for COVID-19 in this thesis.

In this context, the peptide sequences which are specific to coronavirus were designed by the in-silico studies. In this thesis, the designed peptides were synthesized by the Fmoc-mediated SPPS method and then the peptides were purified and characterized. For the purification of the peptides, the semi-preparative RP-HPLC method was used. Then, analytical RP-HPLC and LC-HR/MS were employed for the characterization of the peptides.

Keywords: Coronavirus, SARS-CoV-2, COVID-19, diagnostic kit, vaccine, peptide, Solid-phase peptide synthesis.

COVID-19 AŞISI VE TANI KİTİ İÇİN SPIKE MHC-I T-HÜCRE EPİTOP HEDEFLİ PEPTİTLERİN GELİŞTİRİLMESİ

ÖZET

2019 yılının aralık ayında Çin'in Wuhan kentinde SARS-CoV-2 virüsünün neden olduğu yeni bir korona virüs hastalığı ortaya çıktı. İlk başta sıradan bir soğuk algınlığı gibi baş gösteren bu hastalığın zamanla yapılan çalışmalarla yeni bir hastalık türü olduğu belirtildi. Ortaya çıkan bu yeni tip korona virüsün sebep olduğu hastalık ise COVID-19 hastalığı (Coronavirus Disease-19) olarak tanımlandı. Bulaşıcı olması sebebiyle bu hastalık tüm dünyaya yayılarak 2020 yılının mart ayında Dünya Sağlık Örgütü tarafından pandemi ilan edildi.

Bu yeni korona virüs hastalığı için spesifik bir teşhis, tedavi ve korunma yönteminin bulunmaması nedeniyle hastalık, küresel bir tehdit haline geldi ve milyonlarca kişinin hastalanmasına ve ölümüne yol açtı. Bu nedenle salgının daha fazla yayılmasını önlemek ve ölümlerin önüne geçebilmek adına bu hastalığı tespit etmek, tedavi etmek ve önlemek için etkili yöntemlerin geliştirmesi zorunludur. Şu an halihazırda kullanılan bazı tespit ve tedavi yöntemleri vardır. Fakat, COVID-19 hastalığından korunmak için henüz etkili bir yöntem bulunmamaktadır.

Şu an halihazırda kullanılan tespit ve tedavi yöntemleri SARS-CoV-2 virüsünün tanısı ve hastalığının tedavisi için yeterli değildir. Korona virüs hastalığını tespit edebilmek adına ilk aşamada RT-PCR (Gerçek zamanlı polimeraz zincir reaksiyonu) yöntemi kullanılmaya başlandı. Virüse spesifik bir yöntem olduğu için yüksek doğruluk oranına sahip bu yöntem, dünya çapında uygulanmaya başlandı ve halihazırda olan bir yöntem olduğu için hastalığın tespit aşamasında en çok başvurulan yöntem oldu. Semptomlarının pek çok hastalıkla ortak olması nedeniyle, hastalıktan tamamen emin olabilmek ve doğruluğunu ispatlayabilmek için RT-PCR testinden sonra hastalardan göğüs tomografisi de istenebilmektedir.

Kullanılan bu tespit yöntemlerinin yanı sıra maalesef bu hastalığa özgü bir tedavi yöntemi bulunmamaktadır. Diğer hastalıklarla ortak semptomlara sahip olması sebebiyle COVID-19 hastalığına yakalanmış kişilerde semptomları azaltabilmek ve hafifletmek için kullanılan bazı ilaçlar vardır. Ancak bu yöntemler hastalığı tedavi etmede çok başarılı değildir. Bu yöntemler sadece baş ağrısı, kas ağrısı, ateş, halsizlik, öksürük, boğaz ağrısı gibi yaygın semptomların azaltılması ve hastayı rahatlatmak amaçlı kullanılır. Etkili bir tedavi olmamasının yanı sıra yeni ortaya çıkan bir hastalık olduğu için maalesef hastalığı önlemeye yönelik de bir yöntem bulunmamaktadır.

Herhangi bir hastalıkta teşhis ve tedavinin önemi olduğu kadar o hastalıktan korunmak ya da hastalığa yakalanmadan hastalığı önleyebilmek de çok büyük öneme sahiptir. Korona virüs hastalığı için bazı destekleyici ve semptomlarını azaltmaya yönelik tedavi yöntemleri bulunmakta ama korona virüs hastalığından korunmak ve bu hastalığa karşı bağışıklık kazanabilmek adına etkili bir yöntem bulunmamaktadır. Korona virüsten ve bu virüsün sebep olduğu hastalıktan korunmak için yeni yöntemler geliştirilmesi, hastalığa yakalanmayı önlemek ve hastalığın yayılmasını kontrol altına

almak ayrıca da toplumsal bağışıklık sağlayarak pandemiyi de sona erdirebilmek adına büyük önem taşımaktadır.

COVID-19 salgın hastalığının ilk ortaya çıktığı andan itibaren pek çok bilim insanı bu virüsle ve hastalıkla ilgili çalışmalara başlamıştır. Şu an bu hastalığı teşhis edebilmek adına tanı kiti gibi çeşitli yöntemler geliştirilmiş ve geliştirilmeye de devam etmektedir. Bunun yanında pek çok ülkeden pek çok bilim insanı pandemiyi sona erdirmek için aşı geliştirebilmek adına gece gündüz çalışmaktadır. Bu çalışmaların sonucu olarak acil kullanım için bazı tanı kitleri ve aşı çalışmalarına Dünya Sağlık Örgütü tarafından onay ve yetki verilmiş ve kullanılmaya başlanmıştır. Şu an hali hazırda ticari olarak da üretilen çok fazla sayıda ve markada tanı kiti vardır. Ama maalesef bazı tanı kitlerinin doğruluk oranı çok düşüktür. Yanlış negatif ya da yanlış pozitif sonuç verebilmektedir. Tanı kitlerinin yanı sıra onaylanmış aşılar da bulunmaktadır ve acil kullanım için yetki verilmiş ve dünyada pek çok ülkede insanlar aşılanmaya başlanmıştır. Ancak bu geliştirilen mevcut yöntemler daha da iyileştirilmeli, bu yöntemlerin etkinlik ve özgüllükleri daha da artırılmalıdır. Buna ek olarak, daha iyi özelliklere sahip ve daha etkili yeni aşılar ve tanı kitleri geliştirilmelidir.

Sonuç olarak, devam eden pandemi nedeniyle COVID-19 hastalığını en yüksek doğruluk değeriyle hata payı olmadan tespit edebilmek için ve bu hastalığa karşı koruma sağlayacak yeni ve etkili yöntemlerin geliştirilmesi zorunludur. Bu tez kapsamında da COVID-19 hastalığını tespit etmek için yanal akışlı tanı kiti ve bu hastalığa karşı koruma sağlayabilecek aşıların geliştirilmesi amaçlanmıştır. Bu bağlamda ana bileşen olarak peptitlerin kullanılmasına karar verilmiştir. Peptitlerin yüksek özgüllüğe sahip olmaları, seçici olmaları, biyouyumlu olmaları, toksik olmamaları ve kolay sentezlenebilir olmaları gibi umut vaat eden özelliklerine dayanarak, bu tezde COVID-19 için peptit bazlı tanı kiti ve aşı geliştirilmesine karar verilmiştir.

Bu bağlamda, korona virüse özgü peptit dizileri in-siliko çalışmalarla tasarlanmıştır. Bu tez kapsamında, tasarlanan peptit sekansları Fmoc aracılı katı fazlı peptit sentezi yöntemi ile sentezlenmiştir. Daha sonra sentezlenen peptitler yarı preparatif ters faz yüksek performanslı sıvı kromatografisi kullanılarak saflaştırılmıştır. Saflaştırılan peptitler analitik ters faz yüksek performanslı sıvı kromatografisi ve yüksek çözünürlüklü sıvı kromatografisi kütle spektroskopisi kullanılarak karakterize edilmiştir.

Tez kapsamında RQIAPGQTGK, CYFPLQSYGF, PFAMQMAYRF, TEILPVSMTK, KWPWYIWLGF, LQYGSFCTQL ve EQYIKWPWYI sekanslı peptitler sentezlenmiş ve karakterizasyon sonuçlarına göre sırasıyla %98, %94, %98, %91, %91, %90 ve %85 olacak şekilde yüksek saflıklarda başarılı bir şekilde sentezlenebilmiş ve saflaştırılmıştır.

İn-siliko çalışmalarla belirlenen bu peptitler sentezlendikten sonra tanı kiti ve aşı geliştirilmesi sırasında kullanılacaktır. Bu bağlamda bundan sonraki çalışmalarda ELISA yöntemi kullanılarak peptitlerin bağlanma afiniteleri belirlenecektir. Yüksek afiniteye sahip peptitler belirlenerek bir sonraki aşamaya geçilecek ve klinik çalışmalara başlanacaktır.

COVID-19 hastalığını tespit etmeye yönelik geliştirilecek olan yanal akışlı tanı kitinin, yüksek spesifisite ve sensitiviteye sahip olması için peptit bazlı olarak tasarlanmasına karar verilmiştir. Peptitin tanı kiti üzerindeki tanıma bölgesine immobilize edilmesinin

ardından üzerine damlatıldığı kan veya vücut sıvısı örneğiyle COVID-19 hastalığı spesifik olarak tespit edilebilecektir.

COVID-19 hastalığına karşı aşı geliştirilmesi için ise sentezlenen peptitlerin afinite çalışmalarından sonra en yüksek afiniteye sahip peptitlerin belirlenerek aşının ana bileşenini oluşturması amaçlanmıştır. Bu aşamadan sonra klinik çalışmalara geçilerek sırasıyla aşı geliştirme fazları takip edilecektir.

Bu çalışmalar sonucunda yüksek spesifisite ve sensitiviteye sahip yanal akışlı tanı kitleri ve hastalığa karşı uzun soluklu koruma sağlayabilecek aşılar geliştirilebilecektir.

Anahtar kelimeler: Korona virüsü, SARS-CoV-2, COVID-19, tanı kiti, aşı, peptit, katı-fazlı peptit sentezi.

1. INTRODUCTION

1.1. Coronavirus Disease

The coronavirus disease that is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was emerged in 2019 and named COVID-19. The disease became a global threat by spreading to the world. Thus, it was declared an outbreak in January 2020 and then as a pandemic in March 2020 by World Health Organization [1]. It has simply known as coronavirus, then it was named as 2019 novel coronavirus (2019-nCoV), also called human coronavirus 2019 (HCoV-19 or hCoV-19) [2]. According to today's data, more than 100 million cases and more than 2,5 million deaths have been approved by the WHO for the COVID-19 as of the date of March 2021.

In December 2019, the coronavirus emerged as a pneumonia outbreak in Wuhan, China. Then, the outbreak was reported as a novel strain of coronavirus and named 2019-nCoV by the World Health Organization (WHO), later renamed as SARS-CoV-2 by the International Committee on Taxonomy of Viruses [3].

Coronavirus is an RNA-related virus that causes various diseases in different species, especially in mammals [4]. This virus can cause mild or lethal infectious in the respiratory tract in humans. It is emerged as a common cold as mild infectious in humans, while SARS, MERS, and COVID-19 can emerge as lethal diseases [5]. Coronaviruses have risk factors that can cause the death of the infected.

Coronaviruses belong to the Coronaviridae family. The coronavirus is used as a common name for any member who belongs to the Orthocoronavirinae subfamily. They can be categorized into four genera; Alpha-coronavirus, Beta-coronavirus, Delta-coronavirus, and Gamma-coronavirus. 45 different species have been recognized officially by the year 2020 [6].

The coronavirus infection in animals was firstly emerged in the chickens and reported in the 1920s. Then, the virus was seen in different animals. The virus could firstly be isolated in 1933 and cultivated in 1937. The infection caused by a coronavirus in

humans emerged and then started to studies about human coronavirus in the 1960s. Thereafter, human coronaviruses have been identified as HCoV NL63 and SARS-CoV in 2003, HCoV HKU1 in 2004, MERS-CoV in 2013, and finally as SARS-CoV-2 in 2019 [7].

Coronaviruses are large, spherical, and enveloped viruses that have a positive sense and single-stranded RNA genome. The coronaviruses are one of the largest RNA viruses. Their genome size is approximately 30 kilobases [8, 9]. The diameter of the virus ranges from 50 to 200 nm and it has an average of 120 nm in diameter [10]. The average molecular weight is calculated at approximately 40000 kDa.

Coronavirus has an envelope that is composed of the anchored envelope (E), membrane (M), and spike (S) proteins in a lipid bilayer structure [11]. The coronavirus structure is shown in Figure 1.1.

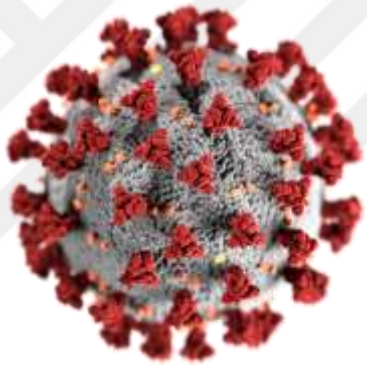

Figure 1.1 : The structure of the coronavirus. Red structures represent the spike proteins (S), Grey shows the lipid bilayer envelope, Yellow represents the envelope proteins (E) and orange structures represent the membrane proteins (M) [5].

The M and E proteins are the structural proteins that define the structure, shape, and size of the virus, while the S protein interacts with the host cell [12]. The M and E proteins are composed of 218 to 263 amino acid residues and 76 to 109 amino acid residues, respectively. The spike proteins that are the surface proteins have an essential role because they are responsible for the interaction with the host cells. A coronavirus has 74 spikes on its surface. Each spike consists of an S protein trimer. The S protein has two subunits as S1 and S2. S1 is responsible for the binding of receptor and membrane fusion between the virus and the host cell. The S2 subunit creates an anchor. Then, the virus can fuse in the host cell [13, 14].

The infection starts with the attachment of the spike protein of the virus to the receptor of the host cell. The host cell surface is cleaved by the protease of the host cell, then activated by the host cell receptor that is attached to the spike protein. As a result of the cleavage and the activation, the virus can fuse the host cell. After the entrance, the genome of the coronavirus acts as messenger RNA and the ribosome of the host cell starts to translate the genome. The positive-sense genome of the virus is replicated. This replicated genomic RNA is the progeny virus genome anymore. Progeny viruses are released from the host cell and infect the other host cells [14].

Coronaviruses generally target epithelial cells. The coronavirus causes infection on the epithelial cells of the digestive tract in animals, while the human coronavirus can cause the infection on the epithelial cells of the respiratory tract. The newly emerged SARS coronavirus infects the epithelial cells of the lungs of humans [14, 15].

The coronavirus can transmit through airborne as droplets or aerosols from an infected person to another person by coughing, sneezing, speaking, or breathing. For this reason, the people who carry the virus must isolate themselves, wear a face mask and pay attention to personal hygiene to prevent the spreading of the virus. It is also possible to spread by fomite or any contaminated surface [15, 16].

The symptoms can come out between 2 or 14 days after exposure to infection of the virus [17]. There are various symptoms of the COVID-19 ranging from, none (asymptomatic) to mild symptoms or severe and lethal [18]. The common symptoms can be defined as headache, cough, fever, sore throat, muscle pain, fatigue, nasal congestion, loss of smell and taste, breathing difficulties, and respiratory failure [19].

1.2. Diagnosis, treatment, and prevention methods for the coronavirus disease

There is no specific diagnosis and treatment method for coronavirus disease. Some methods can be used for these purposes however they are not adequate and effective. Besides, there is no prevention and protection method for coronavirus disease. For this reason, the disease has still been spreading and threatening the world. Because of the ongoing pandemic, it is obligatory to develop rapid, reliable, and effective methods to diagnose, treat and prevent coronavirus disease [20]. There are some ongoing and approved studies about the diagnosis and vaccine for Coronavirus. However, these vaccine and diagnostic kit candidates must be improved and their efficacy must be

increased. Besides, new vaccines and diagnostic kits that have better properties must be developed.

To detect the presence of coronavirus, the real-time reverse transcription-polymerase chain reaction (rRT-PCR) method has been using [21]. This test can detect the RNA fragment, but cannot detect the infectious virus. This test is applied by using respiratory samples taken by the nasal swab. The results are obtained at least in a few hours or at most in two days [22]. In addition to the PCR test, some blood tests can be employed to detect the virus. However, blood tests require taking the blood samples two weeks apart, hence the results cannot be obtained immediately. Besides, chest radiography or chest computed tomography (CT) can also be useful for the imaging and diagnosis of coronavirus disease. However, this method is not highly recommended because this method is not easy to handle and practical. Also, it can be employed for the advancing period of the disease [15]. Because of the adequateness of these diagnosis techniques, new testing methods have been developing for rapid and reliable diagnosis of this disease.

There is no specific treatment for coronavirus disease 2019 (COVID-19) yet. There some supportive treatments are available to relieve and diminish the symptoms. However, they are not specific and adequate to cure this disease, and also their effectiveness is very low and limited. Most cases of coronavirus disease have mild symptoms. Hence, it can be applied to supportive treatment such as some available medications to relieve mild symptoms like fever, cough, headache, and other body aches [16]. Also, it is highly recommended to resting, proper diet, vitamins, minerals, and personal hygiene. Patients with more severe symptoms need intensive care in the hospital. These people have generally breathing difficulties and respiratory failures. Thus, it is required to non-invasive ventilation to support their breathing. Furthermore, an intensive care unit can be needed for mechanical ventilation for serious patients [23]. These kinds of treatments have been applied however their benefits are still investigating and under consideration. There are some studies to develop more effective and specific methods for the treatment of coronavirus disease.

Besides diagnosis and treatment, protection and prevention are also very essential in any disease. There are some supportive treatments, however, there is no protection for coronavirus disease. The treatment methods can be applied in case of infection.

However, protection from coronavirus is more important to prevent and control the spreading of the disease and also to end the pandemic.

There are various ongoing researches on the protection and prevention methods being studied. The most effective method to prevent or protect against any disease is vaccination. Several vaccines have been developed in clinical trials by using different techniques against the SARS-CoV-2. Against the human coronaviruses, the vaccination is targeted to the protein structures on the virus [24]. Also, they aim to block the entrance in the host cell, polymerases, proteases, or replication [25]. Most vaccine candidates are focused on and target the spike proteins on the coronavirus as the primary antigen for COVID-19.

1.3. Diagnostic Kit

The diagnosis is a process to determine the causes of any problem or situation. In the medical aspect, it is defined as the identification or analysis of any disease or disorder by examination of the signs and symptoms. It is also a decision reached as a result of such an examination.

A rapid and reliable diagnosis is very essential for the decision about the treatment of the disease. It can be very difficult to detect any disease. Because the symptoms may not appear at the beginning of the disease. Also, a symptom can be a sign of many diseases or disorders and some symptoms are common for several diseases. Hence, it can cause failure in the diagnosis. Therefore, the diagnosis technique must be accurate and specific to that disease [26]. There are some methods to detect any disease or disorder. The diagnostic kits are the most rapid and practical ones.

The diagnostic kits supply a quick and reliable response. They are very practical and easy to perform. The results can be obtained within minutes or a few hours. The diagnostic kits are very suitable for the point-of-care test which allows detecting the antibody, antigen, or any biological molecule directly. Therefore, they are very suitable and useful for preliminary studies to detect the disease. There are different types of diagnostic kits such as lateral flow test, vertical flow test or dipstick test, and so on. The most known and employed diagnostic kit is the lateral flow test [27]. The basic illustration of the lateral flow test is as shown in Figure 1.2.

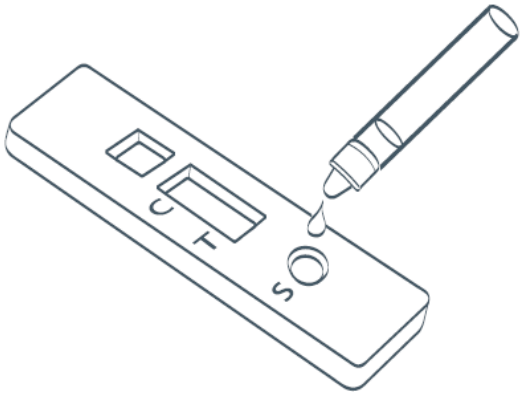

Figure 1.2 : The basic illustration of the lateral flow test.

While the RT-PCR is the most accurate method between the diagnostic test, the rapid diagnostic test is the most employed method to detect the COVID-19. RT-PCR has higher sensitivity, reliability, and specificity according to the rapid diagnostic tests. However, the rapid diagnostic test is preferred because of the adequate properties and rapid response. If the sensitivity, accuracy, and specificity are relatively low, the test results can be confirmed by further tests such as PCR [27].

1.3.1. Serodiagnostic assay

The medical diagnosis is mostly carried out serologically. Serology is defined as a scientific study is performed by serum or other body fluids. The serologic essay is a kind of diagnostic method is carried out by the identification of antibodies and antigens in the serum [28]. Such antibodies are produced in the body as a result of an infection. The presence of antibodies in the blood sample indicates any disease, pathogen, or infection. Therefore, the serological test can be employed to diagnose any infection, disease, disorder, or autoimmune illness. Also, this test can be used to determine the blood type or in forensic investigations [29]. The procedure to perform a serological test is very simple. The blood sample is taken from the patient. The sample is dropped on the test mechanism. Then, the result is obtained within minutes [30]. The serologic test is an immunochromatographic assay that allows the visual results. Thus, the results can be seen with the naked eye.

1.3.2. Diagnostic kit for coronavirus disease

At the beginning of the outbreak, there was no specific diagnosis method to detect the coronavirus. After the definition of the coronavirus disease, the countries focused on the diagnosis of the coronavirus. Then, the studies have been started to develop a more

specific, rapid, and proper method to diagnose coronavirus disease. Now, there are some available tests like nucleic acid tests, antigen tests, and serology tests for the coronavirus disease [26, 27].

These tests can detect the SARS-CoV-2 virus which causes coronavirus disease. Also, they can detect antibodies, antigens, or nucleic acids. These tests are very rapid that result within minutes. They are so easy to perform and require minimal training.

For the diagnosis of the COVID-19, the most useful and frequently employed application is rapid antigen testing. Mostly, lateral flow types are used and approved by global governments. They have lots of advantages and benefits. They are quick and easy to perform with minimal training, give results in 5-30 minutes, and offer a cost advantage. The rapid antigen test is very useful in the mass testing or population screening for the COVID-19. These tests can determine the current or past presence of the coronavirus infection. The virus can be detected directly or the antibodies which are produced as a response against the infection can be detected by these tests [31]. The commonly used lateral flow-rapid antigen test for the coronavirus disease is as shown in Figure 1.3.

Figure 1.3 : The lateral flow-rapid antigen test for the coronavirus disease.

1.4. Vaccine

A vaccine is defined as a biological solution that is used to simulate and mimic any disease to develop immunity in the body against that disease. The vaccine helps to immune system to develop protection for the disease.

The vaccine stimulates the disease. The immune system recognizes the vaccine components as foreign and threat to the body and then starts to produce antibodies and immune response. Then, if the immune system encounters that agent again in the future, recognize and remember that agent and the immune response is produced immediately and any microorganism related to that agent is destroyed [32].

The vaccines are composed of killed or inactivated disease-causing microorganisms, derived products from them, biological agents synthetically resemble, or some chemicals like toxins. While most vaccines are produced by inactivated, dead, weakened, or attenuated micro-organisms, synthetic vaccines are composed of synthetic materials such as peptides, antigens, or carbohydrates [33].

The vaccines can be used for both the prevention or treatment of a disease. So, the vaccine may be applied before any disease or after the occurrence of a disease. There are two kinds of vaccines as prophylactic and therapeutic. A prophylactic vaccine is used to prevent to have or decreases the effects of any disease or infection in the future. A therapeutic vaccine is employed to fight and cure any occurred disease in the body [34]. According to the consequences of scientific studies, vaccines are the most effective and highly safe method to prevent any disease or fight against any infectious disease. However, there are some limitations to the vaccines [35].

The vaccination can fail sometimes, because of some reasons both vaccine-related or host-related. The vaccine-related failures can be an inadequate vaccine, vaccine attenuation, or vaccination method. The host-related failures are generally because of the immune system of the host. The immune system of the host may not produce an immune response against the vaccine. Besides, sometimes, even if the immune system of host response and produce antibody, it might not be effective or adequate. However, even weak or inadequate immune response can help to fight the infection thus, it lowers the mortality rate and morbidity or supplies faster recovery according to without vaccination [35].

1.4.1. Vaccine types

The vaccines can be categorized according to the biological contents of vaccines. The types of vaccines can be classified as inactivated, attenuated, subunit, conjugate, and RNA vaccines [36]. Some experimental vaccines are being actively studied. The experimental vaccines include dendritic cell vaccines, DNA vaccine, T-cell receptor

peptide vaccines, and the vaccines that are created by using recombinant vector and plasmids [37, 38].

Inactivated vaccines include virulent or microorganisms that are inactivated or destroyed by heat, radiation, or chemicals. Attenuated vaccines are produced by live but attenuated and weakened microorganisms. The attenuated organisms become less dangerous for the body. Thus, the body can develop immunity against that microorganism. In the subunit vaccines, a fragment is used rather than the whole agent to develop an immune response. To produce a conjugate vaccine, it is used some particular bacteria which have a polysaccharide surface that is not immunogenic. A desired biological material is linked to this surface, then the immune system recognizes the polysaccharide and does not respond against that material [36]. The RNA vaccine is included a packaged RNA in a vector-like lipid. It is a very novel type of vaccine [39]. The vaccine studies are focused on the RNA vaccine against COVID-19 because of the RNA-related structure of the coronavirus. There are several RNA vaccines to fight the COVID-19 pandemic and some of them received the authorization.

1.4.2. Vaccine development

There are five main stages for vaccine development; preclinical stage, Phase-I, Phase-II, Phase-III, and Phase-IV. In the preclinical stage, the vaccine type, dose range and formulation, and components of the vaccine are determined. The preliminary studies are carried out in this stage. The vaccine candidates are produced and tested for safety, activity, toxicity, immunogenicity, and adverse effects. It is very essential to determine the immune response against the vaccine candidate. After completing the preliminary studies, it moves through to Phase-I [40].

In the Phase-I stage, the vaccine candidates are introduced to healthy people. The vaccine candidates are tested as a placebo or an adjuvant-containing cocktail to understand the effect of these materials on healthy people and the immune response against these candidates. After the application of the vaccine or the placebo, the data on immune response and health outcomes are collected and recorded. The observed side effects, adverse effects, and the results on the people are noted. According to the result of these studies, it is decided whether to continue with the Phase-II stage. In this stage, vaccine escalation studies are also carried out to minimize adverse effects. For the escalation studies, the vaccine is applied to healthy people by increasing dosage or

frequency. The escalation studies start with two or three groups with 10 healthy volunteers. The same dosage that is the lowest amount to produce the immune response is given to each group. Then, the different dosage is applied to another group to collect data about the dosage and the adverse effects of the vaccine. According to the results, it is defined that the dosage of the vaccine and whether a booster is needed [40].

The phase-II stage is continued based on the results that are obtained from Phase-I on the healthy volunteers. The phase-II trials are carried out with more volunteers to define the effect of the vaccine on a different range of people [40].

The Phase-III studies are very similar to Phase-II. It is continued with defining and monitoring the effects of the vaccine on the larger scale of people as adverse effects, immunogenicity, and toxicity. After obtaining good results such as safety, effectiveness, and quality, the vaccine must be approved before production. The Food and Drug Administration (FDA) is responsible for the examination and approval of a vaccine [40].

After the approval of the vaccine, the long-term effects are monitored and the data on the vaccine usage and the adverse effects are collected continuously in the last stage that is the Phase-IV. As a final stage, the vaccine is licensed and it is started to the general production and marketing [40].

Vaccine development is a very difficult and complicated process. All parameters are very essential and they must be considered in every stage. The vaccine development trials can take months or years to reach the achievement because the reaction of the vaccine and creating an immune response take time.

1.4.3. Vaccine for coronavirus disease

At the beginning of the outbreak, there was no therapeutic or protective vaccine against SARS in humans, although there were some approved vaccines against several diseases caused by coronaviruses in some animals [41]. Before SARS-CoV-2, some vaccines have been studied and tested against viruses in the Coronaviridae family which causes human diseases like the Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) [42]. However, there had been no specific vaccine against coronavirus disease in humans. The novel COVID-19 vaccine studies aim to develop a vaccine to prevent Coronavirus disease or provide immunity against the COVID-19 [43].

Because of the ongoing pandemic, it is obligatory and urgent to develop an effective and specific vaccine for COVID-19. Before the COVID-19, there had never been produced a vaccine in less than one year for an infection, and studies for vaccine development normally take several years [44]. However, vaccine development studies have been accelerated and some vaccine candidates that have the most efficiency are authorized for emergency use for COVID-19.

As of February 2021, there are more than 60 vaccine candidates in clinical research which demonstrate high efficacy against COVID-19 infections. The trials were terminated for some candidates and ten vaccines are authorized, including the **Pfizer–BioNTech** vaccine and the **Moderna** vaccine as RNA vaccine; **CoronaVac** from Sinovac, **BBIBP-CorV** and **WIBP** from Sinopharm, and **BBV152** from Bharat Biotech as an inactivated vaccine; **Ad5-nCoV** from CanSino Biologics, **Oxford–AstraZeneca** vaccine and **Sputnik V** from the Gamaleya Research Institute as viral vector vaccine; **EpiVacCorona** from the Vector Institute as a peptide vaccine. Pfizer-BioNTech COVID-19 Vaccine and Moderna COVID-19 Vaccine are authorized vaccines for emergency used. The vaccination around the world has been started by these vaccines [45].

1.5. Peptide-Based Diagnostic Kit and Vaccine

Peptide-based diagnosis has been developed and commercially available for decades. Peptides are employed as a diagnostic candidate because of the benefits such as easy synthesis, accessibility, and providing accurate and rapid detection. Besides, peptides provide sensitive, specific and point-of-care detection [46].

Peptides play an essential role in the diagnosis of a disease. Diseases can be detected easily and specifically by using peptide which is the part of any pathogen or infectious cause of a disease. Thus, diseases can be detected easily [47].

Based on these benefits, peptides can be used to develop a diagnostic method to detect coronavirus disease. Specific peptide sequences can be defined by the genetic material of the coronavirus, then they are used as diagnosis assay candidates to detect the COVID-19. In this context, there are some literature studies to develop a peptide-based diagnosis method to detect the novel coronavirus.

Zimina et al. developed a peptide-based biosensor for diagnosis of the coronavirus disease. Zimina and her team developed two peptide sequences. Then, a biosensor was created by covalently binding these peptides on the biochip platform of the biosensor [48].

A peptide-based magnetic chemiluminescence enzyme immunoassay was developed by Cai et al. for serological diagnosis of COVID-19. Twenty peptide sequences were defined and synthesized in this study. By using these peptides and serum samples, a peptide-based immunoassay was developed [49].

The peptide-based vaccine is a type of vaccine that is composed of the peptide. These vaccines serve as an alternative to classical and conventional vaccines. For the peptide vaccines, the peptide which has approximately 20-30 amino acid residues is synthesized as a vaccine candidate. Peptide vaccines decrease the side effects because of biocompatibility and non-toxicity. Besides, these vaccines have no heterogeneous multicomponent preparation as the other types of vaccines. A peptide can trigger the immune system due to its highly immunogenic properties [50]. Peptide vaccines have many advantages according to the other types of vaccines. They are safe and very easy to synthesis at a low cost; they have specificity and selectivity and increased stability. In addition to these properties, peptide-based vaccines show high activity.

The peptide sequence is designed based on the genetic material of the infectious, disease or pathogen. Hence, the peptide vaccine contains the specific amino acids of the antigen or antibody epitopes related to the disease and infectious or pathogenic agent. Thus, peptide vaccines can be designed and used for any disease, they have no target limitation [51].

A peptide-based vaccine can be a proper and good option for the prevention of COVID-19. There are some literature studies to develop a peptide-based vaccine for coronavirus disease, which are given as follows.

Kalita et al. designed a multi-peptide subunit-based epitope vaccine against COVID-19. The designed vaccine was composed of 33 highly antigenic epitopes found on the coronavirus proteins [52].

Bhattacharya et al. designed an epitope-based peptide vaccine. They defined 13 MHC-I and 3 MHC-II epitopes on the spike protein of SARS-COV-2, that can be vaccine candidate for COVID-19 [53].

A T-cell epitope-based peptide vaccine by using the envelope proteins was designed for COVID-19 by Abdelmageed et al. They defined ten peptides as a vaccine candidate binding to MHC-I and MHC-II [54].

1.6. General Information About Peptides

A peptide is a continuous, linear, and short-chain polymer that is composed of amino acids. Amino acids are basic units and biological molecules which play a very essential role for the body. They serve as a building block of the proteins. There are 20 main amino acids in the body. All amino acids have unique properties, characteristics, and structures [55, 56].

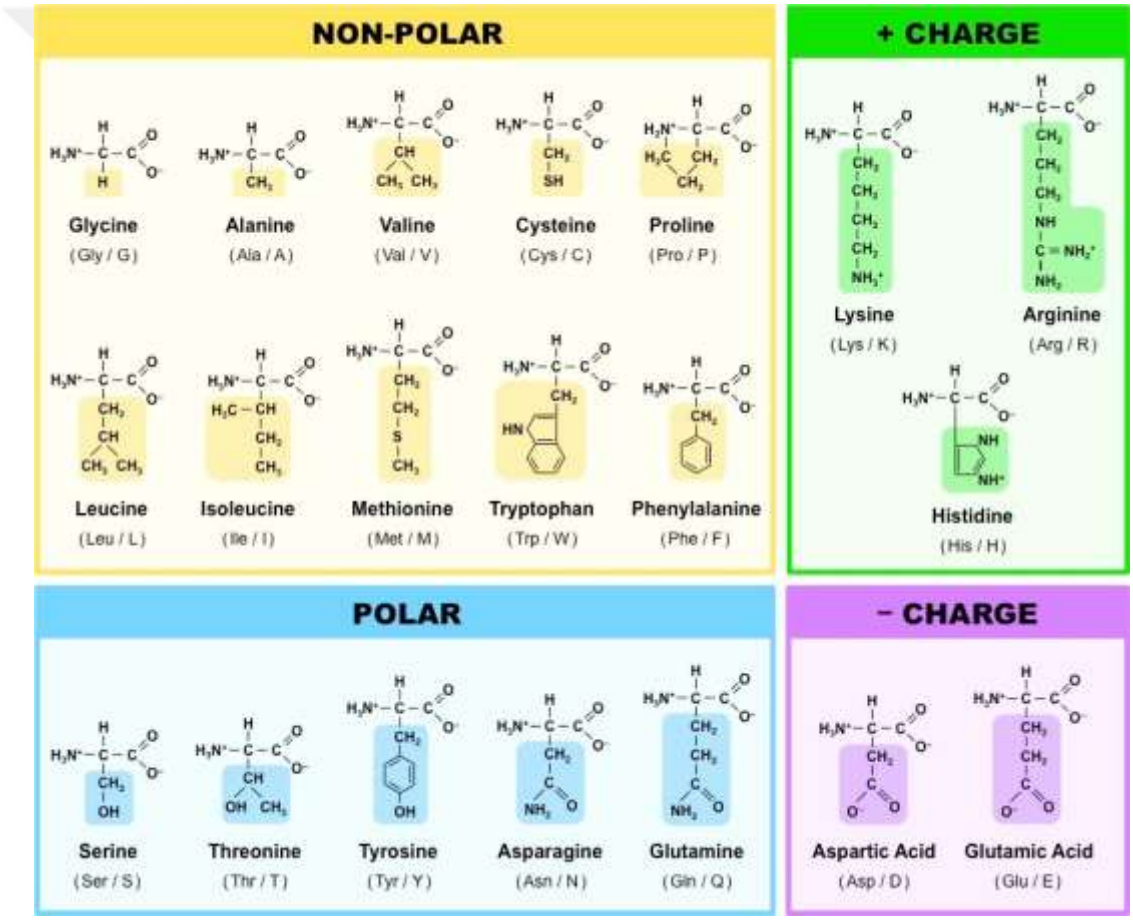

Figure 1.4 : Chemical structures and properties of the proteinogenic amino acids.

Besides, the side chain of each twenty amino acids is different from each other. The side chain refers to the radical group of the amino acid. Figure 1.4. shows the structures, properties, and side chains of the proteinogenic amino acids.

The peptides then the proteins are formed by getting these 20 amino acids together in a different order and residue number. Peptides are linear sequences that are shorter than proteins and longer than oligopeptides. Peptides are sequences that include about twenty amino acid residues. The sequence that is composed of fewer than fifteen amino acids is called oligopeptide. The oligopeptide structure with two amino acids is called a dipeptide which is the simplest peptide. If a sequence is composed of up to almost fifty amino acids, is called a polypeptide as shown in Figure 1.5. Then, polypeptides are organized in a more complex structure to create protein [55, 56].

Proteins are large biological molecules composed of one or more than one polypeptide sequence. The biological activity of the protein is determined by the properties of amino acids that participated in the structure of that protein. They have lots of key and biological functions in the organisms like catalysis of the metabolic reactions, transportation between the cells, replication, giving shape to the cells, and so on. They control almost all processes in the living cells. Proteins are the building blocks of the cells [57].

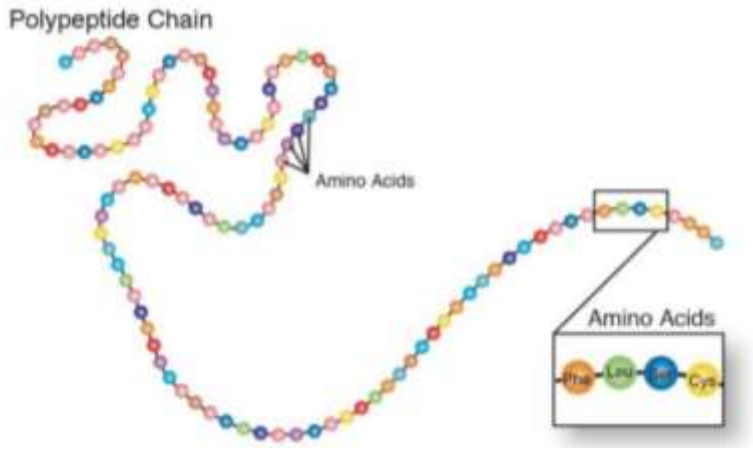

Figure 1.5 : The structure of the polypeptide chain.

Each amino acid has a basic chemical structure as shown in Figure 1.6. There is a central C atom in the structure of amino acid. Furthermore; a hydrogen atom, a carboxyl group (COOH), an amino group (NH_2), and a functional R group specific for each amino acid are found around the C atom. All amino acid is composed of carbon (C), oxygen (O), hydrogen (H), and nitrogen (N) atoms, however, the other elements can be found on the side chain of the amino acid.

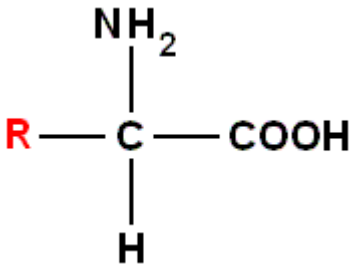

Figure 1.6 : The basic chemical structure of amino acids.

Amino acids are linked by the peptide bond. This bond is a kind of covalent bond and is also called an amide bond. This bond links carbon and nitrogen atom of two adjacent alpha-amino acids. The peptide bond is synthesized due to the reaction of the amino group of one amino acid with the carboxyl group of the other amino acid. The formation of the peptide bond is a kind of de dehydration and condensation reaction. This reaction consumes energy that is derived from ATP and one molecule of water (H_2O) is released so is named a dehydration process [58].

1.7. Peptide synthesis

The carboxylic acid of one amino acid residue and amine of another amino acid residue come together side by side. The Carboxyl group loses one oxygen and one hydrogen atom while the amino group loses one hydrogen atom. A molecule of water (H_2O) is produced as a result of this reaction [58]. Thus, the two amino acids are got together by peptide bond (-CO-NH-) and these bounded amino acids are called a dipeptide. The reaction is shown in Figure 1.7.

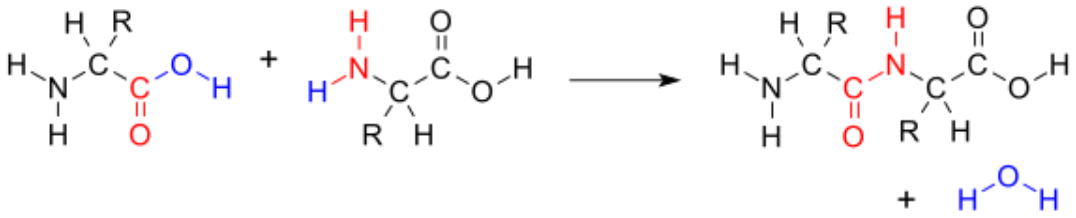

Figure 1.7 : The formation of the peptide bond.

Except for the cyclic peptides, almost all peptide sequences have two end groups as an amino group (NH_2) and a carboxyl group (COOH). The end with the amino group is called as N-terminal, the other end with the carboxyl group is the C-terminal.

The proteins have a very complex structure and hence it is very difficult to chemically synthesis a protein in lab conditions. Therefore, a particular peptide sequence that is located in the protein sequence, is easier to synthesis. Namely, the peptides can be easily synthesized chemically in lab conditions. There are some developed methods for the synthesis of a peptide. Peptides can be synthesized by using the classical solution-phase method and solid-phase peptide synthesis (SPPS) method. The classical solution-phase technique is more useful for large-scale production for industrial purposes. Hence, this technique can be replaced with the solid-phase method which is easier to perform [59].

1.7.1. Solid-phase peptide synthesis

Solid-phase synthesis is a frequently employed method in chemistry. This method allows step by step synthesis of molecules by covalently bonding on a solid phase material in a particular order in a reaction vessel, with a selective protecting group strategy. This method is utilized for the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), peptide, and some other molecules which require synthesis in a certain sequence [60]. This technique was pioneered by Robert Bruce Merrifield in the 1950s. Subsequently, his work won him the Nobel Prize in Chemistry in 1984 [61].

In the SPPS method, the rapid assembly of the peptide sequence is carried out by covalently linking an amino-protected amino acid in a particular order on a solid support. The covalent bond that is mostly an amide bond or ester bond, is built between the carbonyl group of the amino acid and the solid support. The resin is a mostly used material as solid support in peptide synthesis [62]. After the formation of the amide bond between the resin and the first amino acid, the protection group on the amino group of the amino acid is removed and the deprotected amino group reacts with the carbonyl group of the amino protected next amino acid. These steps are repeated and the synthesis cycle is continued till obtaining the desired peptide sequence. After the completion of all reactions, the obtained peptide is removed from the solid support by the cleavage [63]. Peptides are biologically synthesized N to C direction in the body. However, the chemical synthesis of peptides starts with the C-terminus and progressed to the N-terminus although in the opposite direction of the synthesis in the cells.

There are three main components in the SPPS method; solid support, coupling reagent, and the protection group. The solid support is generally polymeric material consists of

small resin beads [64]. These beads are functionalized with some reactive groups like hydroxyl or amine groups. The first amino acid attached to the solid support covalently and by the bonding of the amino acids, the peptide sequence grows on the solid support. Due to the covalent attachment of the peptide to the support, the side products, excess chemicals, and reagents can be easily removed by washing during the synthesis [65].

The solid support is selected according to the physical and chemical properties of the material. The support must be physically stable and insoluble to allow the quick filtration of the excess chemical and liquids during the synthesis. Proper support is inert to chemicals and reagents participated during the process. However, it must be swellable in the solvents to link the first amino acid and to allow the passing through of the chemicals [62, 65].

The peptide bond forms slowly between and carboxylic acid and amine therefore it mostly requires an activator. The activators which are used for this purpose are called coupling reagents. There is a wide range of commercially available coupling reagents which have different effects each on the particular couplings. The most famous and frequently used coupling reagents are carbodiimides, aminium/uronium, and phosphonium salt. Besides these reagents, there are some commercially available reagents like propanephosphonic acid anhydride.

Carbodiimides are a type of reagents used in amide bond formation. The most employed carbodiimides are dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). These reagents are used with some additives such as 1-hydroxy-7-aza-benzotriazole (HOAt) and the triazoles 1-hydroxy-benzotriazole (HOBt) [62].

There are aminium/uronium and phosphonium salt as the coupling reagents that leave out the carbodiimides and they include HOAt and HOBt. The aminium/uronium reagents are HATU (HOAt), HBTU/TBTU (HOBt), and HCTU (6-ClHOBt). Besides, the phosphonium reagents are PyAOP (HOAt) and PyBOP (HOBt) [66]. In addition to these reagents, propanephosphonic acid anhydride has become commercially available since the 2000s. It is a very useful reagent that may be used for the formation of the amide bond.

The N-terminus of the amino acids must be protected to prevent the undesired side reactions and the functional group on the side chains must also be protected by an appropriate protection group depending on the protection strategy and the structure of the side chain. The undesired side reactions cause failure in the synthesis of the peptide in the desired sequence and also, side reactions decrease the yield. Therefore, protection is very essential for peptide synthesis. The most used protection groups in the peptide synthesis are 9-fluorenylmethyloxycarbonyl group (Fmoc) which is base-labile and t-butyloxycarbonyl (Boc) which is acid-labile. While the Fmoc and Boc protection groups can be removed easily during the synthesis, the protection on the functional groups must be stable during the synthesis. These functional groups are removed in the final deprotection step [62].

In the Fmoc/tBu mediated SPPS, it is used the base-labile Fmoc protection on the N-terminal of the amino acid with side-chain protection and acid-labile resin linkage. The peptide is cleaved from the resin by the TFA in the final step. The Boc/Bzl mediated approach uses the TFA-labile Boc protection on the N-terminal and the side chain of the amino acid. This protection group is removed at the same time as the cleavage of the peptide from the resin [62]. The cleavage mechanisms for the Boc and Fmoc group protection are shown in Figure 1.8. and 1.9.

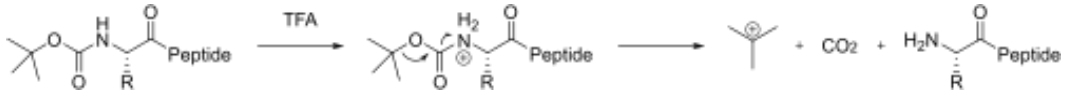

Figure 1.8 : Cleavage of the Boc group protection.

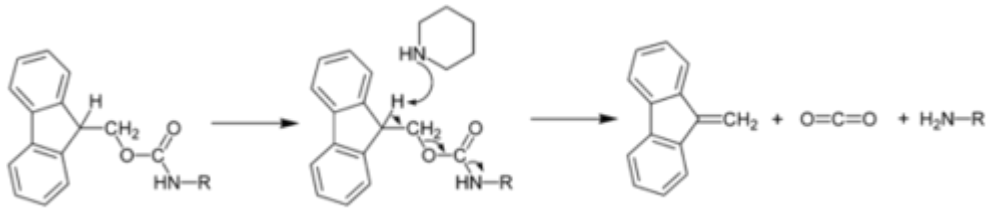

Figure 1.9 : Cleavage of the Fmoc group protection.

Besides, there are some other protection groups like Benzyloxy-carbonyl protecting group, allyloxycarbonyl (alloc) protecting group, and different types of thiol protecting groups. These protection groups can be rarely employed in the case of necessity.

1.7.2. Fmoc-mediated SPPS

In the Fmoc-mediated SPPS method, the peptide assembly on a solid support. Resin is the most employed solid support for peptide synthesis. As a first step, the resin is swollen in a proper chemical solvent and the protection group of the resin is removed. Then, the activated first amino acid is bound to the C-terminus of the solid support at the -COOH group end. After the coupling of the amino acid, the Fmoc protection group on the N-terminal of amino acid is removed by piperidine treatment. These steps are repeated until the desired sequence is obtained. After the elongation is completed, the peptide is cleaved and removed from the solid support. The schematic illustration of Fmoc-mediated solid-phase peptide synthesis is as shown in Figure 1.10. [67].

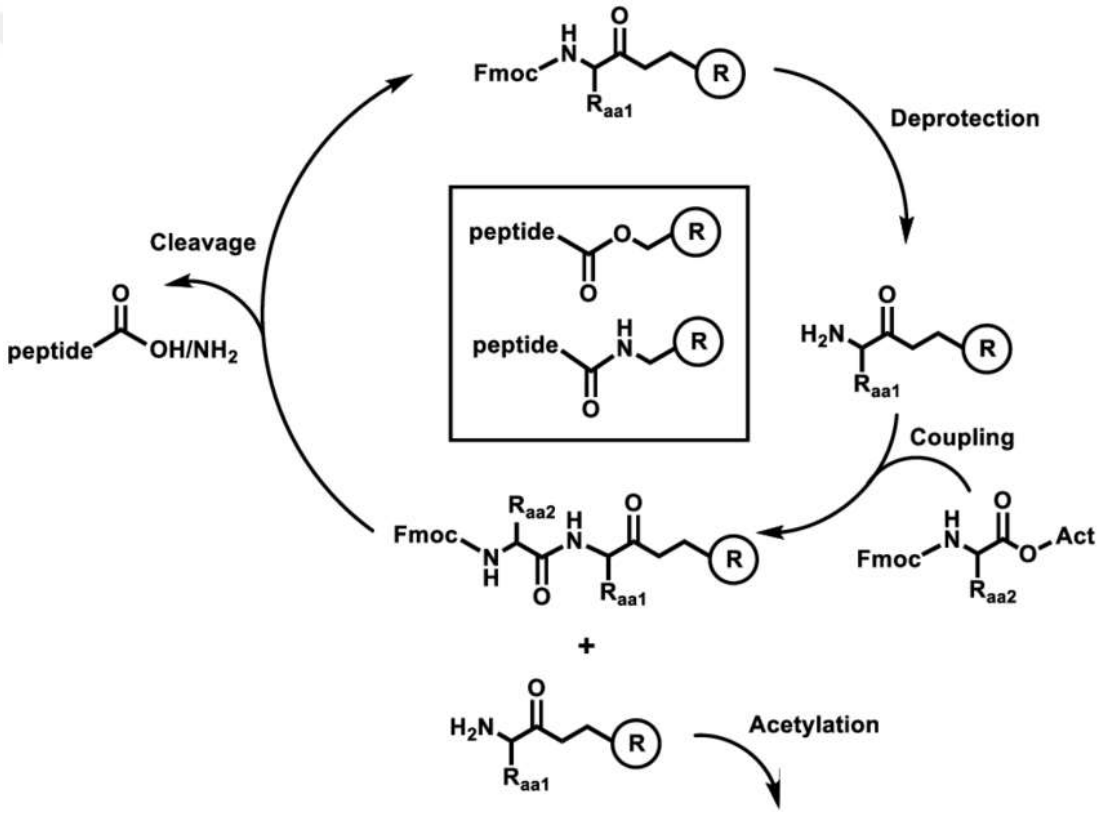

Figure 1.10 : Schematic illustration of the Fmoc-mediated Solis-Phase Peptide Synthesis.

2. PURPOSE OF THE THESIS

A novel coronavirus disease emerged as an outbreak at the end of 2019. Then, the disease was declared a pandemic by spreading to the world. Because of the absence of a specific diagnosis, treatment, and prevention method for this novel coronavirus disease, it became a global threat. Therefore, it is obligatory to develop effective detection and prevention methods for this disease. Because of the high specificity and selectivity properties of peptides, it was decided to develop a peptide-based diagnosis and vaccine for COVID-19 in this thesis.

In the context of the purpose of this thesis, the full genome of SARS-CoV-2 was analyzed to define the antigenic proteins and epitopes. Then, the most probable peptide sequences that can be used to develop a vaccine or diagnostic assay were discovered by in silico studies. According to the preliminary studies, the docking analysis was carried out. The probable antigenic epitopes were docked with their MHC-I alleles. As a result of these studies, the peptide sequences were designed. These design studies were carried out by Prof. Dr. Cemal Ün, Assoc. Prof. Mert Döşkaya, Assoc. Prof. Hüseyin Can, and their teams at Ege University. The sequences are given below;

- RQIAPGQTGK
- CYFPLQSYGF
- PFAMQMAYRF
- TEILPVSMTK
- KWPWYIWLGF
- LQYGSFCTQL
- EQYIKWPWYI

In this context, the purpose of my thesis is to synthesize these peptides by Fmoc-mediated SPPS, followed by their purification and characterization. Then, these peptides will be utilized as vaccine and diagnostic assay candidates. The structures of these peptides are given below in Figure 2.1. to 2.7.

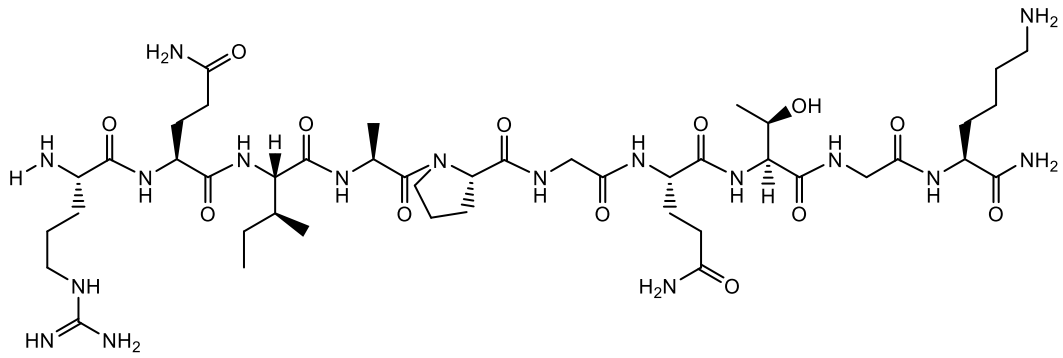

Figure 2.1 : The chemical structure of RQIAPGQTGK.

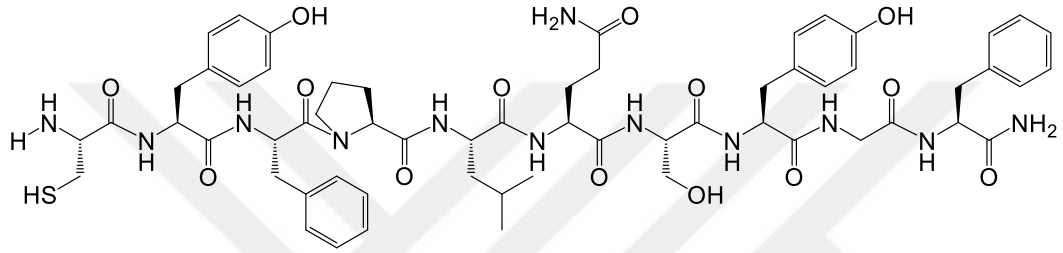

Figure 2.2 : The chemical structure of CYFPLQSYGF.

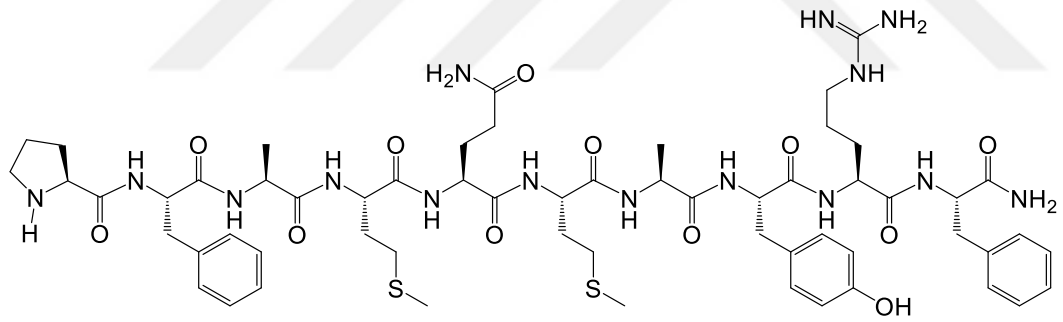

Figure 2.3 : The chemical structure of PFAMQMAYRF.

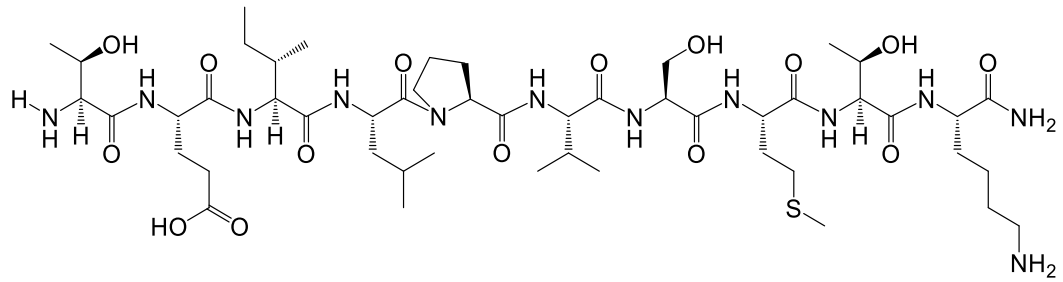

Figure 2.4 : The chemical structure of TEILPVSMTK.

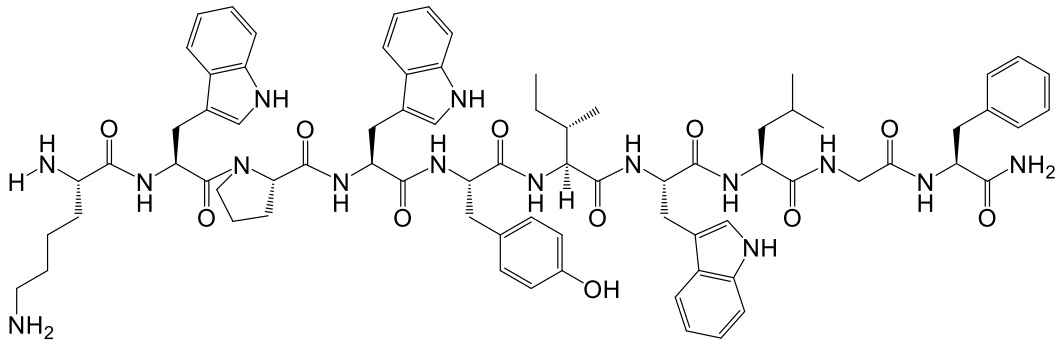

Figure 2.5 : The chemical structure of KWPWYIWLGF.

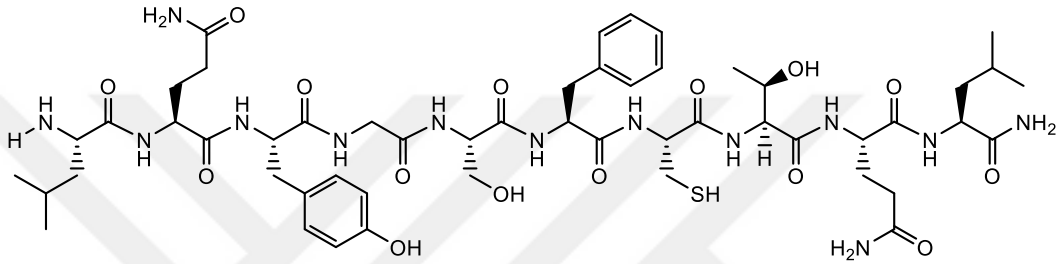

Figure 2.6 : The chemical structure of LQYGSFCTQL.

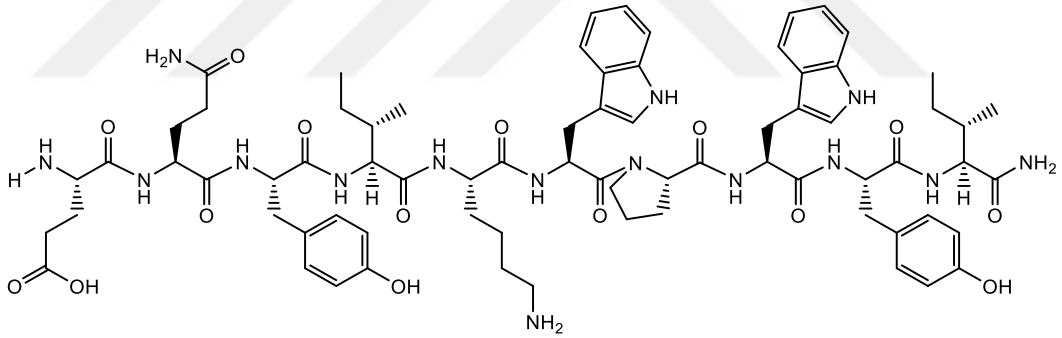

Figure 2.7 : The chemical structure of EQYIKWPWYI.

3. EXPERIMENTAL PART

3.1. Materials and Methods

N,*N*-Dimethylformamide, and isopropyl alcohol were purchased from Isolab. Dichloromethane, piperidine, dithiothreitol, triisopropylsilane, acetonitrile, *N*-butanol, and phenol were purchased from Sigma Aldrich. Pyridine and trifluoroacetic acid were purchased from Merck. Acetic Anhydride was purchased from Acros Organics. Potassium cyanide was purchased from Ega Chemie. *N*,*N*-Diisopropylethylamine was purchased from Alfa Aesar. N^{α}-Fmoc- N^{δ}-trityl-L-glutamine, Fmoc-glycine, Fmoc-O-tert-butyl-L-threonine, Fmoc-S-trityl-L-cysteine, Fmoc-L-methionine, Fmoc-L-valine, N^{α}-Fmoc-N^{in}-Boc-L-tryptophan, N^{α}-Fmoc-N^{ω}-(2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl)-L-arginine, Fmoc-L-isoleucine, Fmoc-L-leucine, N^{α}-Fmoc-N^{γ}-trityl-L-asparagine, Fmoc-L-phenylalanine, Fmoc-L-proline, Fmoc-L-alanine, Fmoc-L-aspartic acid β-tert-butyl ester, Fmoc-L-glutamic acid γ-tert-butyl ester hydrate, N^{α}-Fmoc-N^{im}-trityl-L-histidine, Fmoc-O-tert-butyl-L-tyrosine, Fmoc-O-tert-butyl-L-serine, N^{α}-Fmoc-N^{ε}-Boc-L-lysine, rink amide MBHA resin, O-(Benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate and ninhydrin were purchased from the Chem-Impex International.

The peptides were purified and their purities were assessed by Shimadzu brand Reverse Phase High-Performance Liquid Chromatography. The mass of the peptides was determined by using Thermo Fisher Scientific Q Exactive Orbitrap LC-HR/MS Mass Spectrometry. The purified peptides were dried by using Martin Christ brand Freeze Dryer (Lyophiliser), Millipore brand pure water device was used for supplying pure water to be used in the studies. Isolab brand vortex and Bandelin brand Ultrasonic Bath were employed for agitation of the samples.

3.2. Synthesis of Peptides

3.2.1. Swelling of the rink amide MBHA resin

50 mg of (0.022 mmol, 0.44 meq/g) rink amide MBHA resin was weighed. The weighed resin was applied to the column and 10 ml of DMF was added to the resin. The resin was left to swell in DMF for 30 minutes. Then, DMF was drained by the vacuum system.

3.2.2. Cleavage of Fmoc groups on MBHA resin and amino acids

To remove the Fmoc protection group from resin and amino acids, pre-swollen MBHA resin and coupled amino acid were treated with piperidine solution (20% Piperidine in DMF) two times. First, 855 µl of piperidine solution was added and waited for 10 minutes. After 10 minutes, the solution was drained and 855 µl of piperidine solution was added again and waited for 10 minutes. Then, the solution was drained by the vacuum system and the column was washed with 5 ml of DMF 10 times.

3.2.3. Coupling of the amino acids

The amino acid cocktail was prepared with amino acid (0.121 mmol, 5.5 eq.), HBTU (0.11 mmol, 5 eq.), DMF, and DIPEA (42,2 µl, 10 eq.). The required amounts of amino acids and reagents for each amino acid cocktail are given in Tables 1 and 2. The amounts are given in Table 3.1. and 3.2., are for one amino acid cocktail.

Table 3.1 : The amounts of reagents in coupling reactions.

Reagent	MW (g/mol)	Equivalent	Amount
HBTU	379,25	5	45 mg
DIPEA	129,25	10	42,2 ml

Each amino acid cocktail was prepared with 1,5 ml of DMF. Also, these values are for synthesis with 50 mg (0.022 mmol, 0.44 meq/g) of resin.

Table 3.2 : The amounts of amino acids in a coupling reaction.

Amino acid	MW (g/mol)	mmol	Equivalent	Amount (mg)
A (Ala)	311.3	0.121	5.5	37.67
R (Arg)	648.77	0.121	5.5	78.50
N (Asn)	596.77	0.121	5.5	72.21
D (Asp)	411.5	0.121	5.5	49.79

Table 3.2 (continued) : The amounts of amino acids in a coupling reaction.

Amino acid	MW (g/mol)	mmol	Equivalent	Amount (mg)
C (Cys)	585.7	0.121	5.5	70.87
Q (Gln)	610.7	0.121	5.5	73.89
E (Glu)	443.49	0.121	5.5	53.66
G (Gly)	297.3	0.121	5.5	35.97
H (His)	619.3	0.121	5.5	74.94
I (Ile)	353.4	0.121	5.5	42.76
L (Leu)	353.4	0.121	5.5	42.76
K (Lys)	468.6	0.121	5.5	56.70
M (Met)	371.5	0.121	5.5	44.95
F (Phe)	387.4	0.121	5.5	46.88
P (Pro)	337.4	0.121	5.5	40.83
S (Ser)	383.4	0.121	5.5	46.39
T (Thr)	397.5	0.121	5.5	48.10
W (Trp)	526.6	0.121	5.5	63.72
Y (Tyr)	459.5	0.121	5.5	55.60
V (Val)	339.4	0.121	5.5	41.07

To prepare the amino acid cocktail, 0,121 mmol of amino acid and 45 mg (0.11 mmol, 5 eq.) of HBTU were weighed and dissolved in 1,5 ml of DMF. Then, 42,2 µl of DIPEA was added to the cocktail and mixed by vortex, and the cocktail was poured into the column. The first amino acid was reacted overnight to ensure the binding of the amino acid to resin properly. The other amino acids were reacted with the resin for 1 hour.

3.2.4. Kaiser test (Ninhydrin assay)

After the coupling of the amino acids, the Kaiser test can be applied to access the extent of coupling. This test is employed especially after the coupling of the first amino acid and also after the coupling of the amino acids which have a bulky structure such as R, Y, W, F, P, and V. According to the result of the Kaiser test, the synthesis is continued. If the Kaiser test is positive, the synthesis is carried on with the next amino acid. However, if the test result is negative, the same amino acid is re-coupled. These reagents are prepared as follows;

- Reagent A: 16.5 mg of KCN is dissolved in 25 ml of distilled water. 1 ml of this solution is taken and diluted with 49 ml of pyridine.
- Reagent B: 1 g of ninhydrin is dissolved in 20 ml of n-butanol.
- Reagent C: 40 g of phenol is dissolved in 20 ml of n-butanol.

The Kaiser test is performed as follows;

2-3 drops are taken from each reagent and mixed in two different test tubes. After the coupling step of amino acid, 10-15 beads of resin are taken and the beads are put into one of the test tubes. This tube which includes resin is labeled as 'sample' and the other tube is labeled as 'reference'. Then, these two tubes are heated at 110 ºC for 5 minutes. After 5 minutes, the color change of the tube that includes the resin is compared with the reference tube. If there is no change in color, this indicates the coupling is completed. If the color turns dark blue, it means the coupling is not succeeded.

During the synthesis of the peptides, after the coupling of the first amino acids and after the coupling of the R, Y, W, and F, the Kaiser test was applied. In addition to these, after the coupling of the amino acids that come after V and P, the Kaiser test was employed. The test results were generally positive thus the synthesis was progressed. However, the results of some Kaiser tests were obtained as negative. In such cases, the same amino acids were re-coupled and the synthesizes were completed.

3.2.5. Capping

After coupling of the amino acids, the capping procedure is applied to cap the unreacted and free amine groups on the resin. The capping solution was prepared as in Table 3.3. for 0.022 mmol resin. Then, the prepared capping solution was added to the column and after 10 min, the capping solution was drained by the vacuum system. Then, the resin was washed with 5 ml of DMF 3 times.

Table 3.3 : The amounts of chemicals to prepare capping solution.

Chemical	For 0.1 mmol resin	For 0.022 mmol resin
Acetic Anhydride	200 µl	44 µl
Pyridine	200 µl	44 µl
DMF	2 ml	440 µl

3.2.6. Cleavage of the peptide

After the removal of the Fmoc protection group on the last amino acid, the piperidine solution was drained by the vacuum system. The resin was washed with 5 ml of DMF 2 times then washed with 5 ml of DCM 10 times. DCM is used to remove the DMF in this step. The cleavage cocktail was prepared with 1617 µl of TFA, 16,5 µl of TIPS, 16,5 µl of dH2O (98% TFA, %1 TIPS, and %1 dH2O) to cleave the peptide from the resin. 825 µl of the cocktail was added to the column and waited for 1 hour and the solution was collected in the flask by the vacuum system. The cleavage was carried out in 2 hours (1-hour x 2 times). After the cleavage protocol, the peptide was collected in the volumetric flask. The volatile materials were removed under reduced pressure.

If the sequence contains cysteine, the cleavage cocktail is prepared with DTT. Cysteine has a functional Thiol group on its side chain. The oxidation of these thiol groups may build a disulfide bridge. The cleavage cocktail for the sequence that contains cysteine was prepared with 100 mg of DTT, 50 µl of dH2O, 50 µl of TIPS, and 1,9 ml of TFA. 1 ml of the prepared cocktail was added to the column and waited for 1 hour. Then, the cocktail was drained and 1 ml of the cocktail was added again. Then, the volatile materials were removed under reduced pressure. The obtained crude peptides were purified by HPLC and then characterized by LC-HR/MS.

3.3. Purifications of Peptides

The physical properties of peptides are very essential in the purification step. Because the peptides are dissolved and then loaded to the HPLC device for purification.

As seen in Figures 3.1. to 3.7., most of the peptides that are synthesized within the scope of this thesis, are highly hydrophobic. Five of these synthesized peptides CYFPLQSYGF, PFAMQMAYRF, KWPWYIWLGF, LQYGSFCTQL, and EQYIKWPWYI are hydrophobic. The two of them are hydrophilic; RQIAPGQTGK and TEILPVSMTK. The peptides are dissolved in an acetonitrile/water mixture with a dropwise addition of TFA. Afterward, the samples are agitated in the ultrasonic bath to ensure full solubility of peptides.

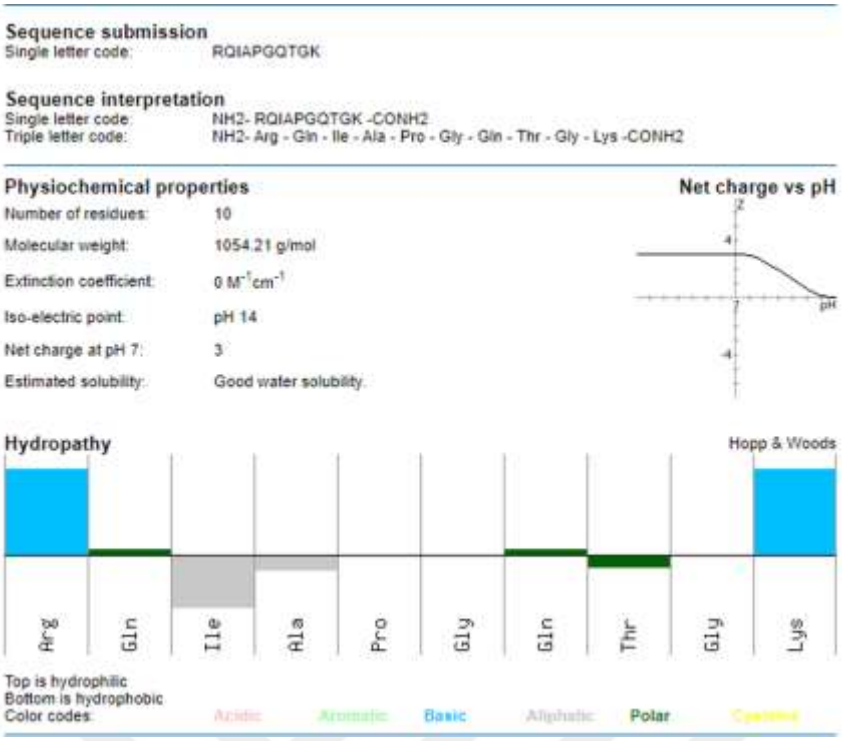

Figure 3.1 : The estimated physical properties of RQIAPGQTGK.

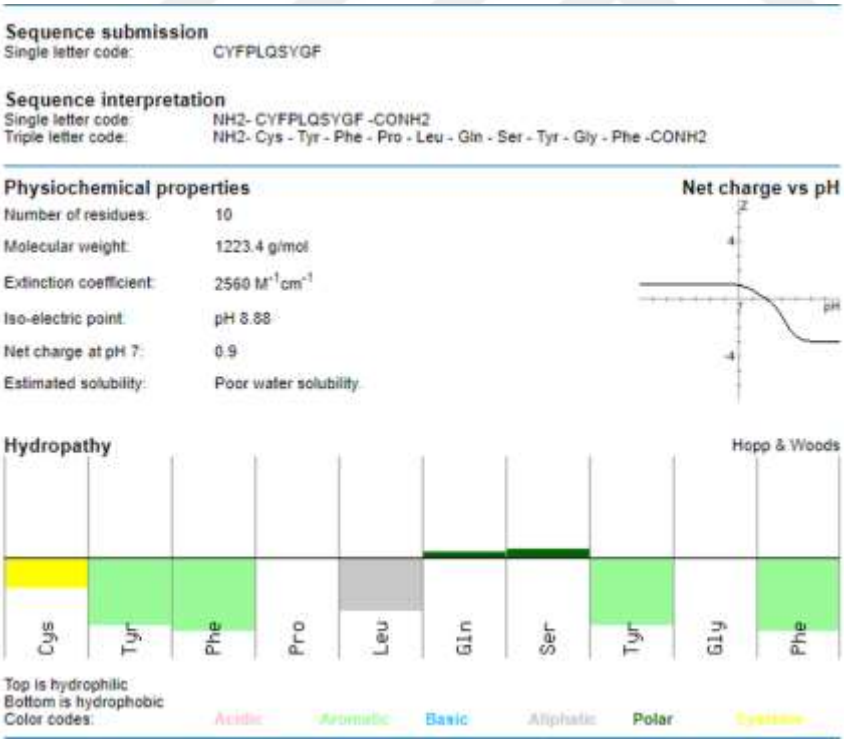

Figure 3.2 : The estimated physical properties of CYFPLQSYGF.

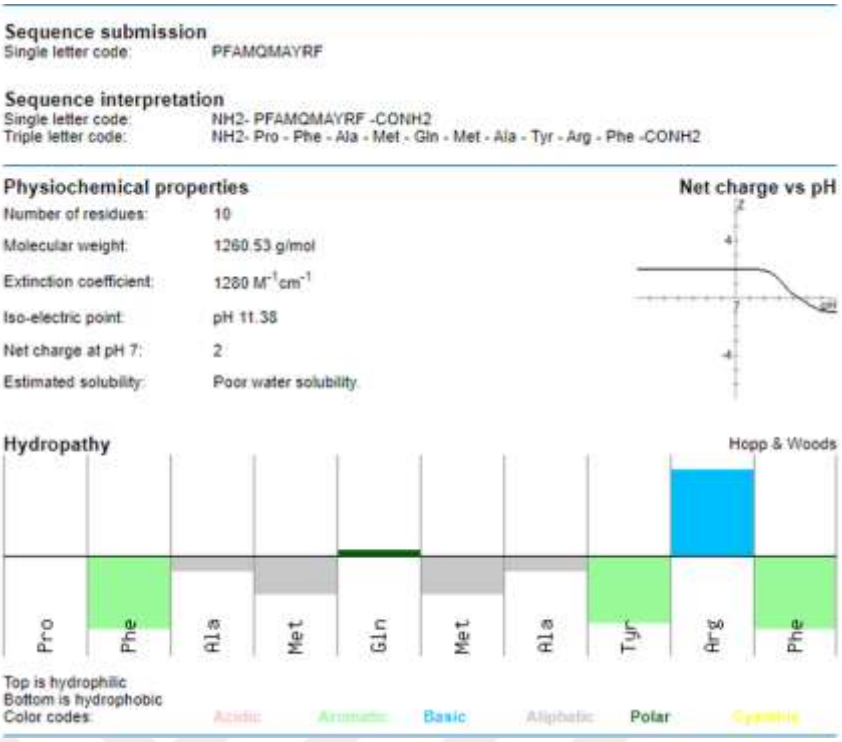

Figure 3.3 : The estimated physical properties of PFAMQMAYRF.

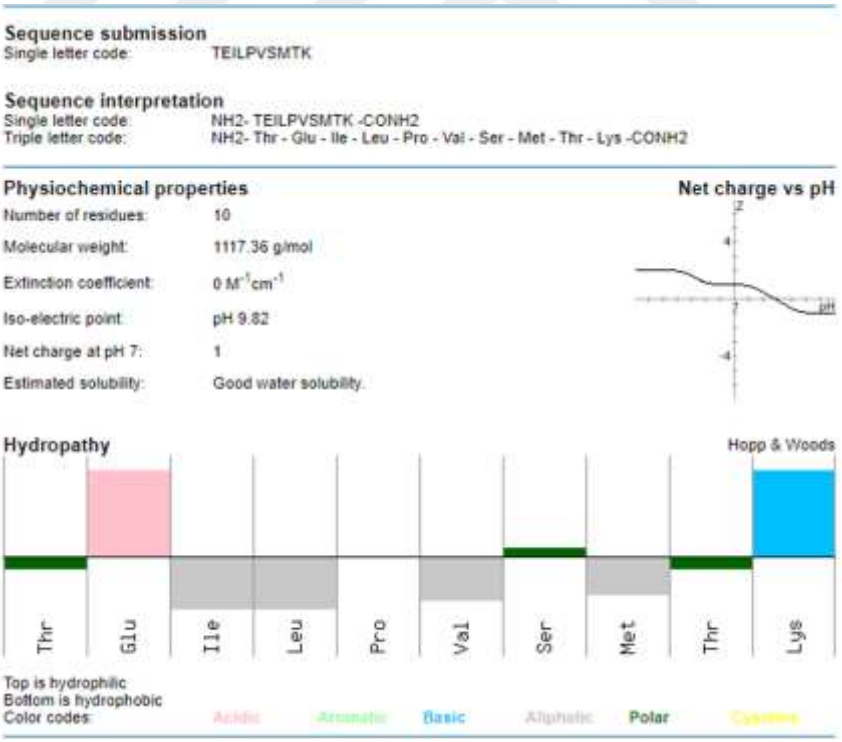

Figure 3.4 : The estimated physical properties of TEILPVSMTK.

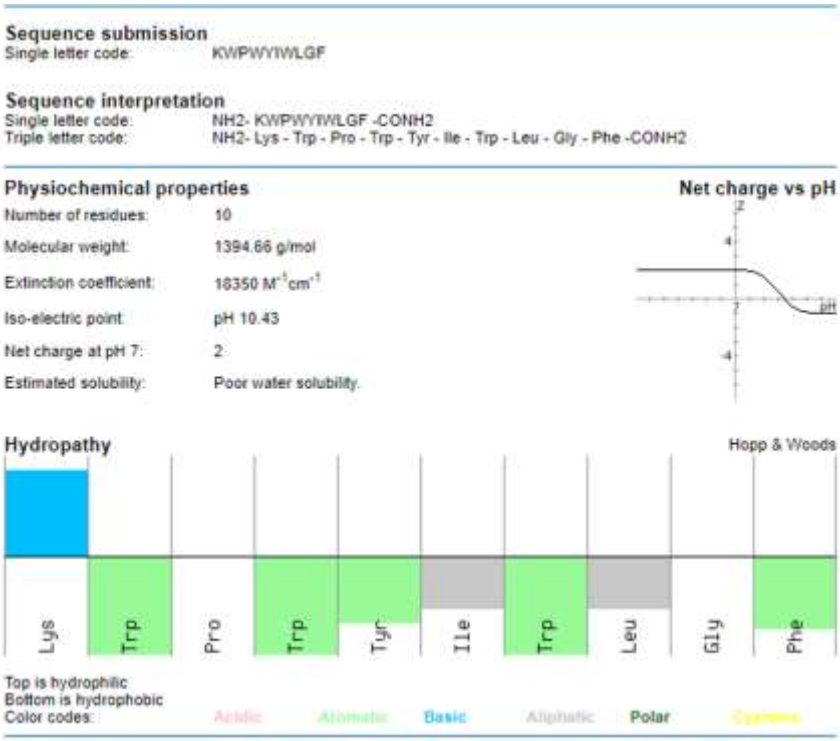

Figure 3.5 : The estimated physical properties of KWPWYIWLGF.

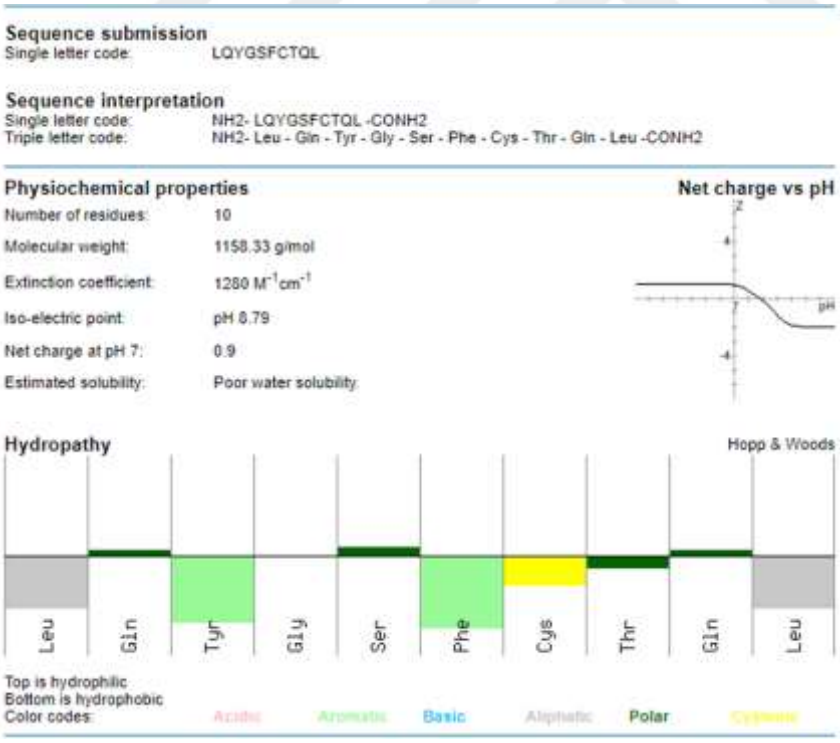

Figure 3.6 : The estimated physical properties of LQYGSFCTQL.

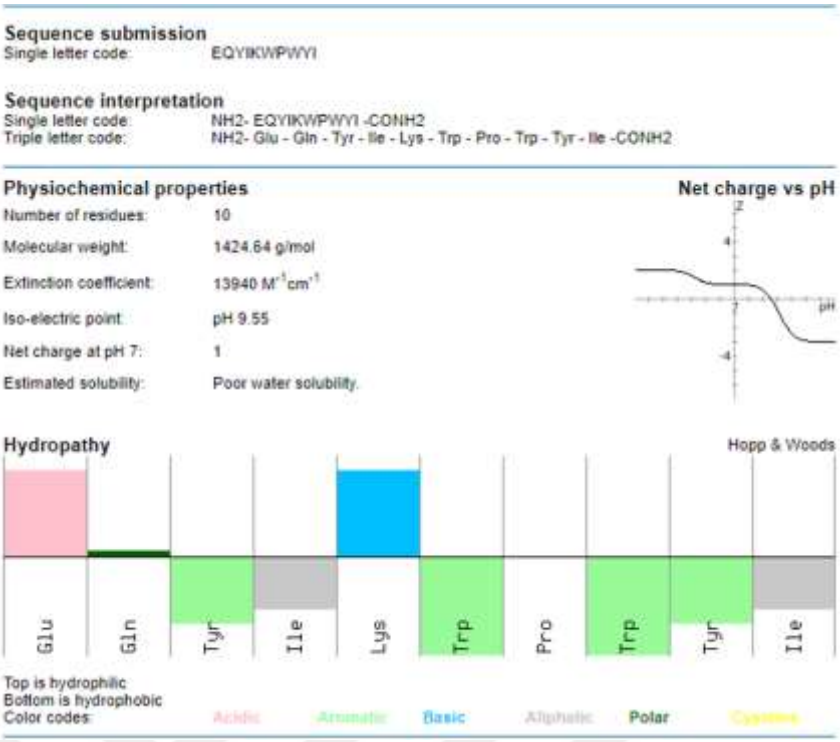

Figure 3.7 : The estimated physical properties of EQYIKWPWYI.

The homogeneously obtained peptide solutions were transferred to the HPLC vials by filtering with a filter with 0.45 µm of pore size. Nylon filter was used for hydrophilic peptides, while the polytetrafluoroethylene (PTFE) filter was used for hydrophobic peptides. The prepared vials that include the peptide solution were loaded to the HPLC and the peptides were purified by the semi-preparative HPLC method under the conditions that are specified below;

- Flow rate: 5.0 ml/min
- Mobile Phase A: Distilled water (0.1% TFA)
- Mobile Phase B: Acetonitrile (0.085% TFA)
- Column: Thermo Scientific Hypersil GOLD C18 column (250 mm x 10.0 mm, 12µm)
- Detector: PDA Detector, 214 nm
- Injection Volume: 1800 µl
- Column Temperature: 25 ºC
- Duration Time: 55 minutes
- Solvent gradient: 5% to 100% (Phase B)

After purification of the peptides, the samples were dried by using Lyophiliser for 48 hours. Figure 23 shows the dried peptide by Lyophiliser. After completely drying the peptides, the peptide samples were weighed and the obtained amounts of peptides were recorded. Then, the synthesized peptides were characterized.

3.4. Characterization of Peptides

3.4.1. Characterization of the peptides by analytical RP-HPLC

The purity of the peptides is determined with analytical RP-HPLC. The HPLC was run with the method that is given below and the obtained percent purity results of peptides are given in Tables 4.1 to 4.7.

- Flow rate: 1.0 ml/min
- Mobile Phase A: Distilled water (0.1% TFA)
- Mobile Phase B: Acetonitrile (0.085% TFA)
- Column: GL Sciences Inertsil ODS-3 C18 Analytical Column (150 mm x 4.6 mm, 5µm)
- Detector: PDA Detector, 214 nm
- Injection Volume: 30 µl
- Column Temperature: 25 ºC
- Duration Time: 35 minutes
- Solvent gradient: 5% to 100% (Phase B)

3.4.2. Characterization of the peptides by LC-HR/MS

The analysis of the molecular weight indicates whether the peptide has been properly synthesized. Mass Spectrometry is used to analyze the molecular weights of the samples. In this project, the characterization of the molecular weight of the synthesized and purified peptides was performed by the Thermo Scientific Q Exactive Orbitrap LC-HR/MS device, in the Bezmialem Vakıf University. For characterization of the peptides, a very small amount (approximately 0,05 mg) of the dried peptide was taken and analyzed. The spectrums and the calculations based on the spectrums were specified in the next section.

4. RESULTS AND DISCUSSION

4.1. Results of Analytical RP-HPLC

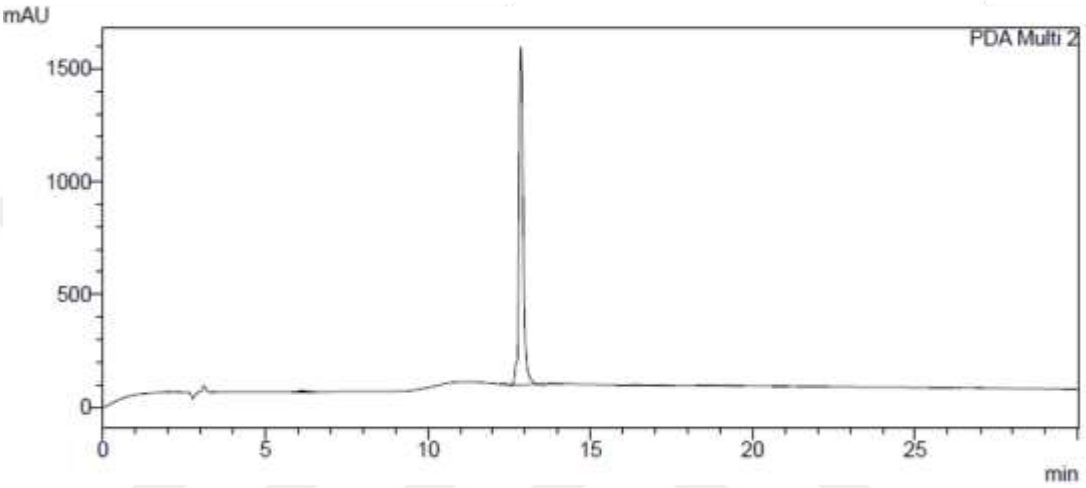

Figure 4.1 : The chromatogram of the analytical RP-HPLC analysis of RQIAPGQTGK.

Table 4.1 : The percent purity of RQIAPGQTGK.

Peak #	Peak Start	Peak End	Retention Time	Area	Height	Area %
1	5.835	6.517	6.131	71134	5266	0.5038
2	12.245	12.555	12.328	25635	2569	0.1816
3	**12.576**	**13.493**	**12.853**	**13859210**	**1494256**	**98.1618**
4	13.493	14.165	13.774	43500	4745	0.3081
5	16.096	17.557	16.337	58683	2313	0.4156
6	18.251	18.453	18.371	5127	644	0.0363
7	18.453	19.691	18.649	40052	1331	0.2837
8	20.608	20.853	20.734	3337	455	0.0236
9	20.853	21.109	20.948	12066	1963	0.0855

According to the HPLC chromatogram of the RQIAPGQTGK, as shown in Figure 4.1.; the areas of the peaks were calculated as a percentage value. The percentage of values was recorded in Table 4.1. The percentage of areas shows the percent purity of

the peptides. Accordingly, the peptide was obtained with a high purity of 98% as shown in Table 4.1.

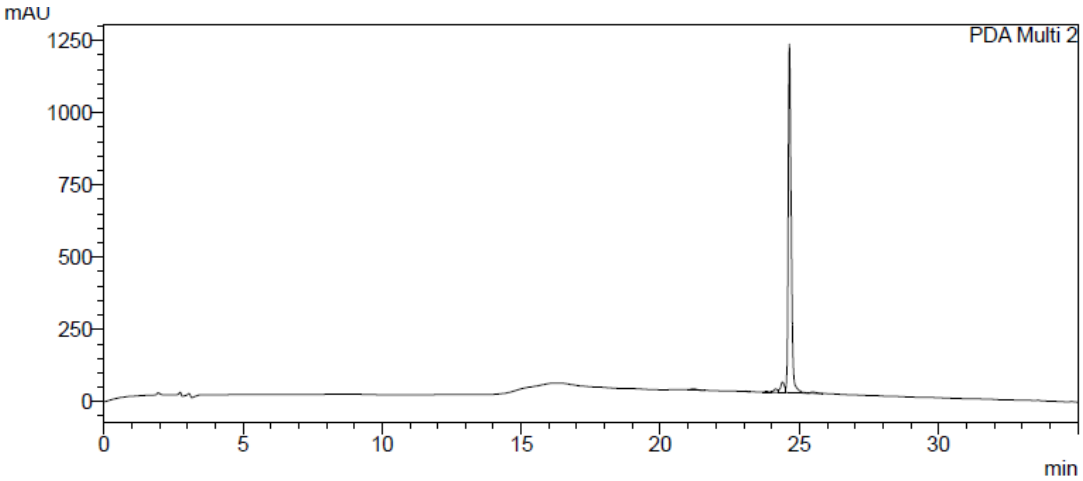

Figure 4.2 : The chromatogram of the analytical RP-HPLC analysis of CYFPLQSYGF.

Table 4.2 : The percent purity of CYFPLQSYGF.

Peak #	Peak Start	Peak End	Retention Time	Area	Height	Area %
1	21.013	21.611	21.197	56788	4107	0.5838
2	23.008	23.221	23.065	3129	521	0.0322
3	23.701	23.968	23.809	25582	3016	0.2630
4	23.968	24.235	24.143	114688	11713	1.1791
5	24.235	24.501	24.389	356468	36707	3.6647
6	**24.501**	**25.323**	**24.637**	**9106484**	**1206163**	**93.6207**
7	25.323	25.845	25.468	63858	4955	0.6565

After the purification of the CYFPLQSYGF, the chromatogram was obtained as given in Figure 4.2. According to the chromatogram, the areas of the peaks were calculated and recorded in Table 4.2. Figure 4.2. and Table 4.2. show that the purity of this peptide is determined to be ~94%.

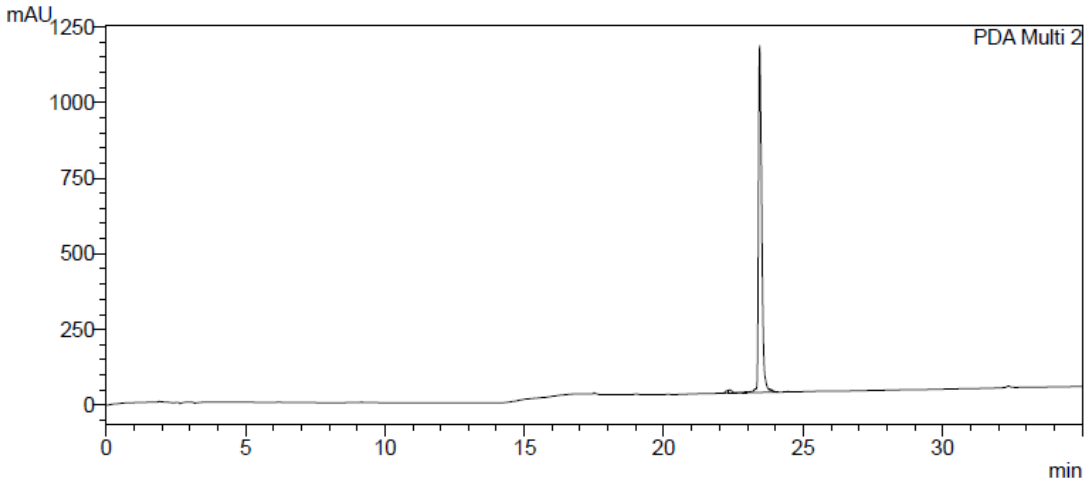

Figure 4.3 : The chromatogram of the analytical RP-HPLC analysis of PFAMQMAYRF.

Table 4.3 : The percent purity of PFAMQMAYRF.

Peak #	Peak Start	Peak End	Retention Time	Area	Height	Area %
1	22.123	22.315	22.266	47458	7939	0.4921
2	22.315	22.613	22.374	79105	9737	0.8202
3	22.613	22.805	22.741	6814	753	0.0707
4	22.805	22.997	22.918	19166	2478	0.1987
5	**22.997**	**24.085**	**23.438**	**9472967**	**1145674**	**98.2241**
6	23.787	23.947	23.821	11366	2146	0.1178
7	24.299	24.651	24.444	7358	845	0.0763

According to Figure 4.3 and Table 4.3. the results show that the PFAMQMAYRF was obtained as highly pure. According to the calculation, the peptide has a purity of 98%.

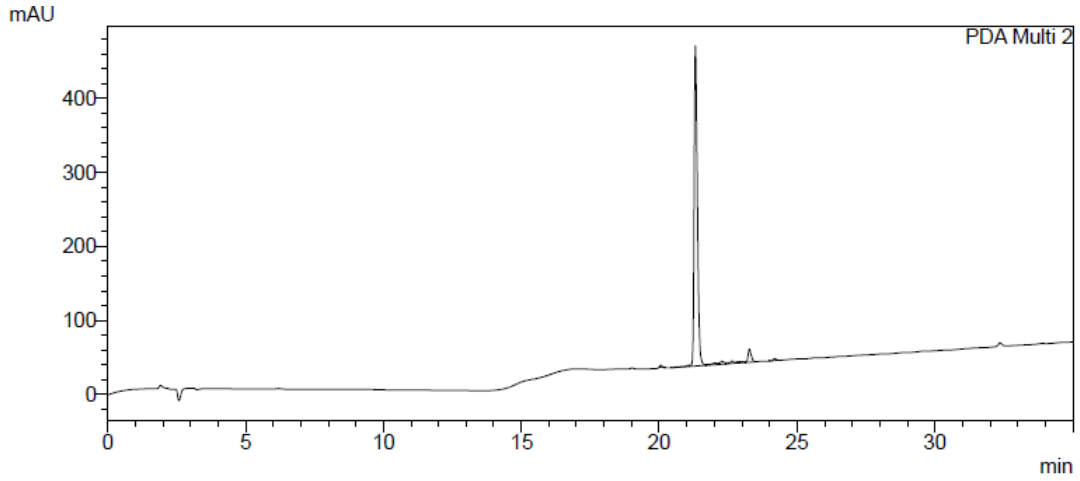

Figure 4.4 : The chromatogram of the analytical RP-HPLC analysis of TEILPVSMTK.

Table 4.4 : The percent purity of TEILPVSMTK.

Peak #	Peak Start	Peak End	Retention Time	Area	Height	Area %
1	20.011	20.203	20.054	12290	2281	0.3202
2	20.416	20.715	20.619	3232	345	0.0842
3	20.715	21.109	21.067	15924	1375	0.4148
4	**21.109**	**21.696**	**21.310**	**3509115**	**432363**	**91.4115**
5	21.696	22.144	22.017	34081	2055	0.8878
6	22.144	22.432	22.269	40318	3890	1.0503
7	22.432	22.539	22.496	7454	1201	0.1942
8	22.539	22.763	22.641	27410	3256	0.7140
9	22.763	22.923	22.844	13222	1702	0.3444
10	22.923	23.125	23.010	12602	1429	0.3283
11	23.125	23.531	23.267	142600	17784	3.7147
12	23.979	24.373	24.182	20562	2674	0.5356

According to the result of the analytical HPCL, the chromatogram of TEILPVSMTK was obtained as in Figure 4.4. As shown in Table 4.4., the purity of this peptide is 91%.

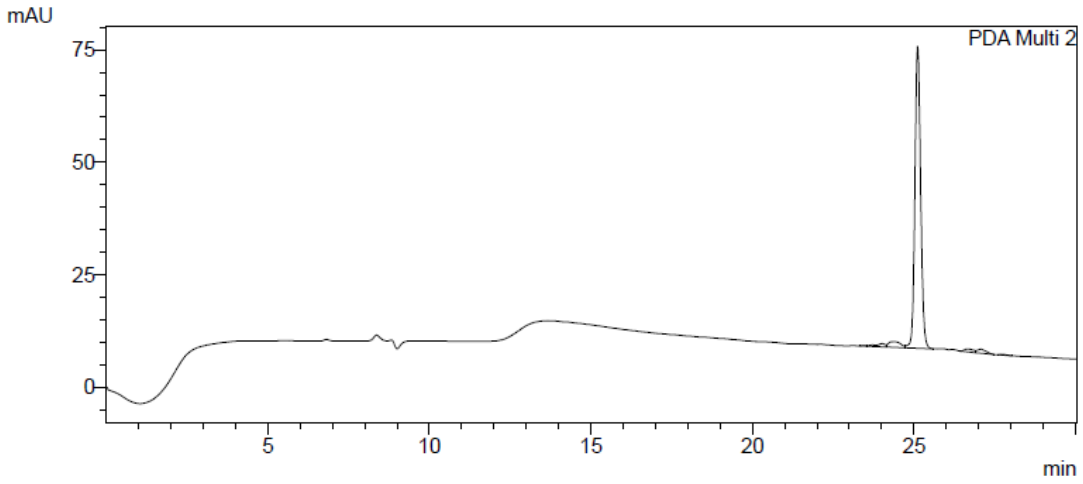

Figure 4.5 : The chromatogram of the analytical RP-HPLC analysis of KWPWYIWLGF.

Table 4.5 : The percent purity of KWPWYIWLGF.

Peak #	Peak Start	Peak End	Retention Time	Area	Height	Area %
1	23.306	23.530	23.413	1115	94	0.1221
2	23.530	23.818	23.697	4187	295	0.4583
3	23.818	24.138	24.004	10405	746	1.1389
4	24.138	24.703	24.357	32043	1218	3.5072

Table 4.5 (continued) : The percent purity of KWPWYIWLGF.

Peak #	Peak Start	Peak End	Retention Time	Area	Height	Area %
5	**24.703**	**25.599**	**25.108**	**835027**	**67063**	**91.3977**
6	26.442	26.911	26.661	12773	622	1.3980
7	26.911	27.477	27.062	16115	877	1.7638
8	27.594	28.021	27.759	1955	177	0.2139

The chromatogram of purified peptide shows its purity is 91% which is highly good.

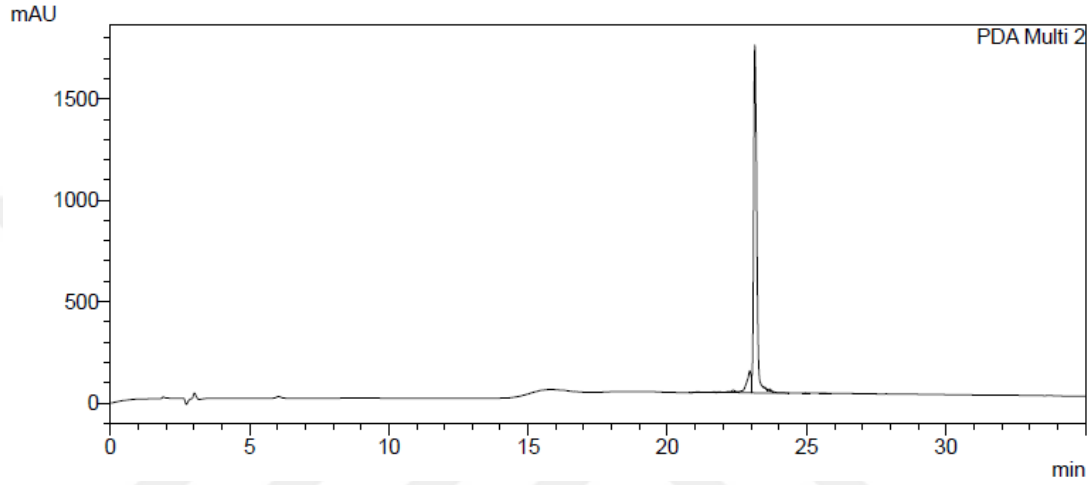

Figure 4.6 : The chromatogram of the analytical RP-HPLC analysis of LQYGSFCTQL.

Table 4.6 : The percent purity of LQYGSFCTQL.

Peak #	Peak Start	Peak End	Retention Time	Area	Height	Area %
1	20.800	21.344	21.104	30687	1843	0.2047
2	21.504	21.781	21.669	21438	2692	0.1430
3	21.781	22.069	21.855	27109	2761	0.1808
4	22.069	22.261	22.191	42942	5853	0.2864
5	22.261	22.549	22.356	127153	12046	0.8481
6	22.549	23.019	22.951	1192991	108695	7.9575
7	**23.019**	**24.363**	**23.138**	**13472313**	**1714650**	**89.8631**
8	23.456	23.595	23.487	19087	3803	0.1273
9	23.605	23.808	23.667	44559	8254	0.2972
10	24.843	25.120	24.945	4285	620	0.0286
11	25.451	25.856	25.597	9479	869	0.0632

According to the chromatogram shown in Figure 4.6., the peak areas were calculated and recorded in Table 4.6. As seen in Table 4.6., the peptide was obtained with a good purity which is ~90%.

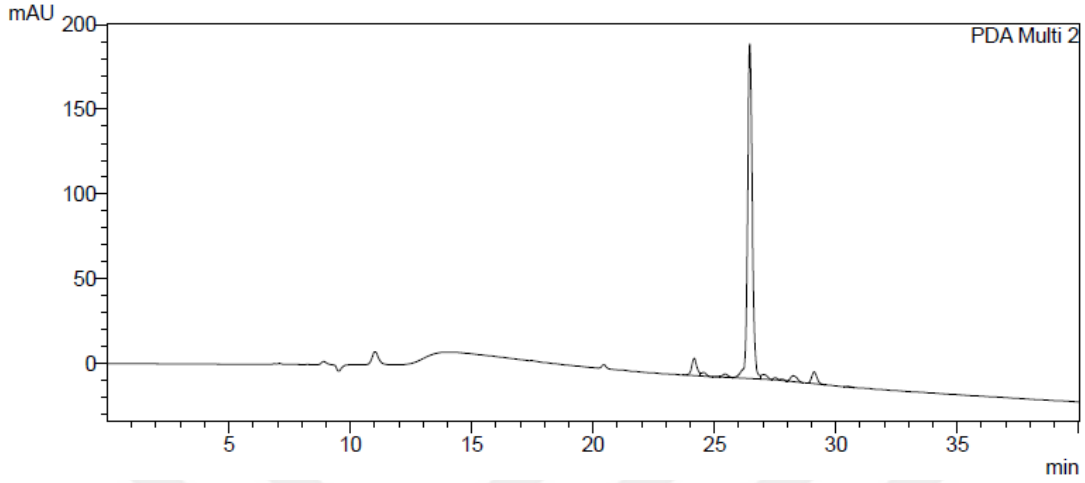

Figure 4.7 : The chromatogram of the analytical RP-HPLC analysis of EQYIKWPWYI.

Table 4.7 : The percent purity of EQYIKWPWYI.

Peak #	Peak Start	Peak End	Retention Time	Area	Height	Area %
1	23.722	24.437	24.162	154001	10205	4.9161
2	24.437	24.949	24.551	30701	2356	0.9801
3	24.949	25.226	25.153	4490	414	0.1433
4	25.226	25.759	25.434	29373	1866	0.9377
5	**25.759**	**26.879**	**26.454**	**2668203**	**197322**	**85.1751**
6	26.879	27.349	27.035	48312	2717	1.5422
7	27.359	27.658	27.499	13365	1257	0.4266
8	27.658	27.978	27.776	10016	910	0.3197
9	27.999	28.629	28.239	63866	3425	2.0387
10	28.831	29.567	29.104	105426	7059	3.3654
11	30.346	30.751	30.499	4855	390	0.1550

As a result of the analytical HPLC, the chromatogram given in Figure 4.7., was obtained. The peptide was obtained with a purity of 85%.

4.2. Results of LC-HR/MS

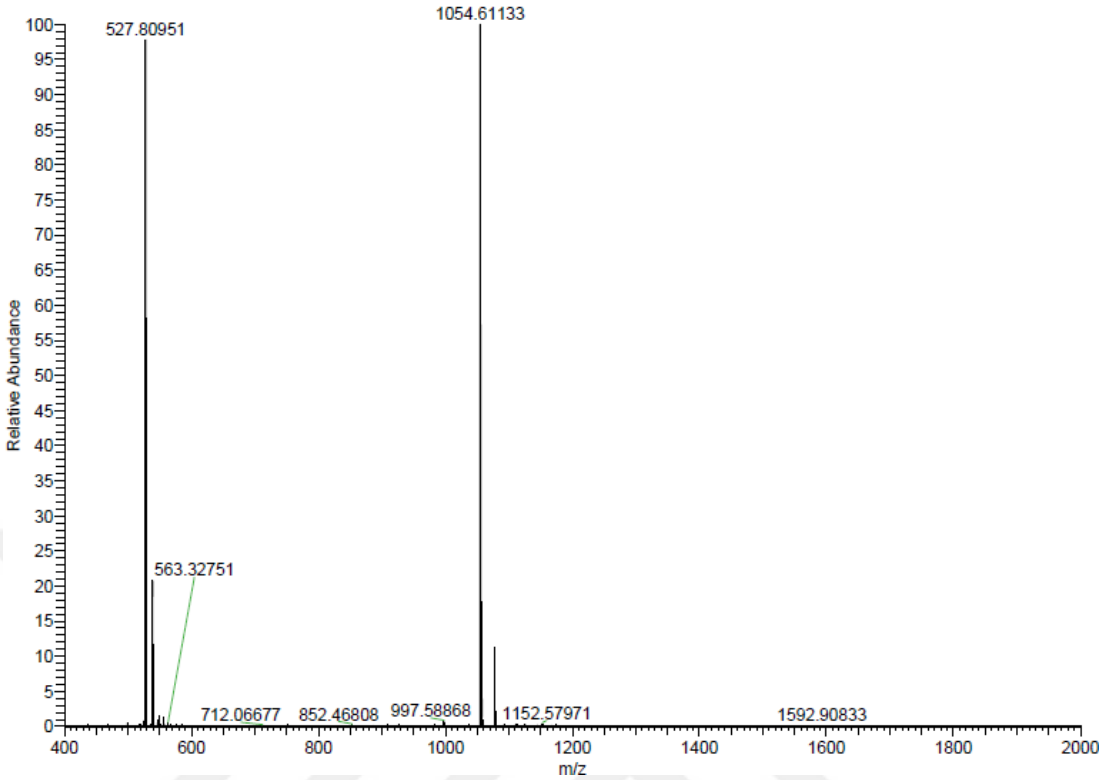

Figure 4.8 : The mass spectrum of RQIAPGQTGK.

Mw: Exact Mass= 1053.62 g/mol

$[M+H]^{+}$= 1054.61133 g/mol

In conclusion, the mass of the peptide was calculated as 1053.603505 g/mol from the spectrum in Figure 4.8. as the experimental mass of the peptide in the protonated state is 1054.61133 g/mol. This result indicates this peptide was synthesized successfully.

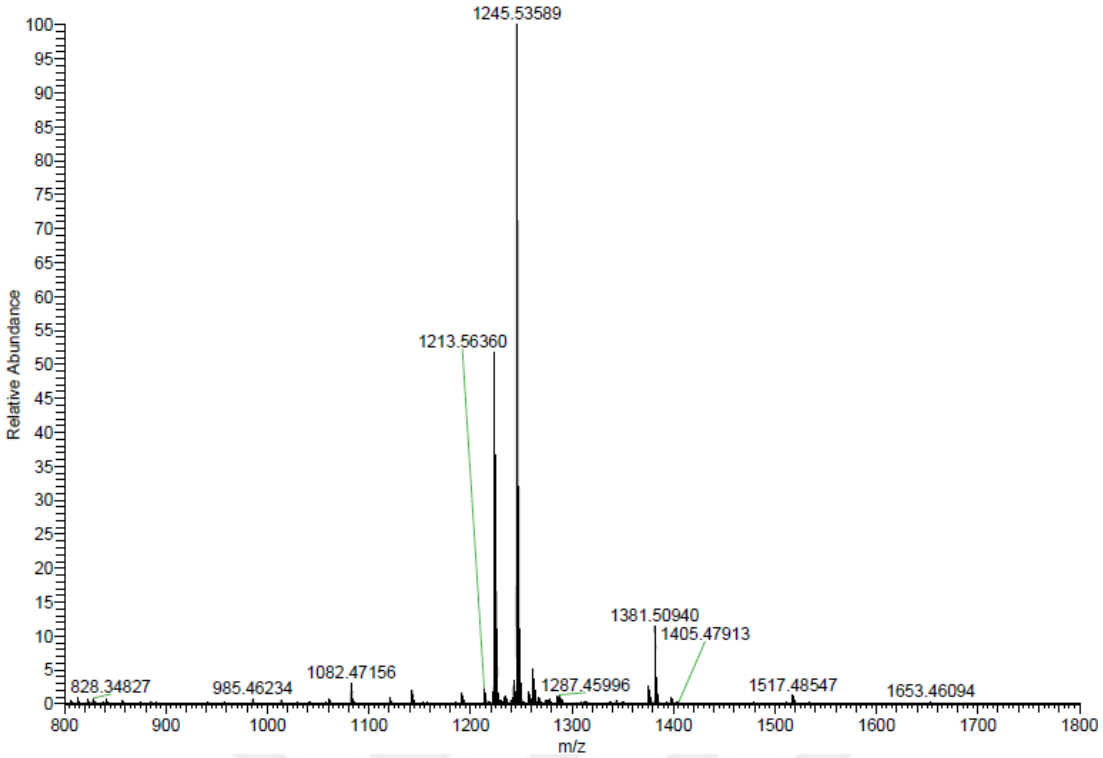

Figure 4.9 : The mass spectrum of CYFPLQSYGF.

Mw= Exact Mass: 1222.55 g/mol

[M+Na] += 1245.53589 g/mol

The experimental mass of the peptide, which is 1245.53589 g/mol in the protonated stage, was calculated as 1.222.54612 g/mol. This result shows that this peptide was synthesized successfully.

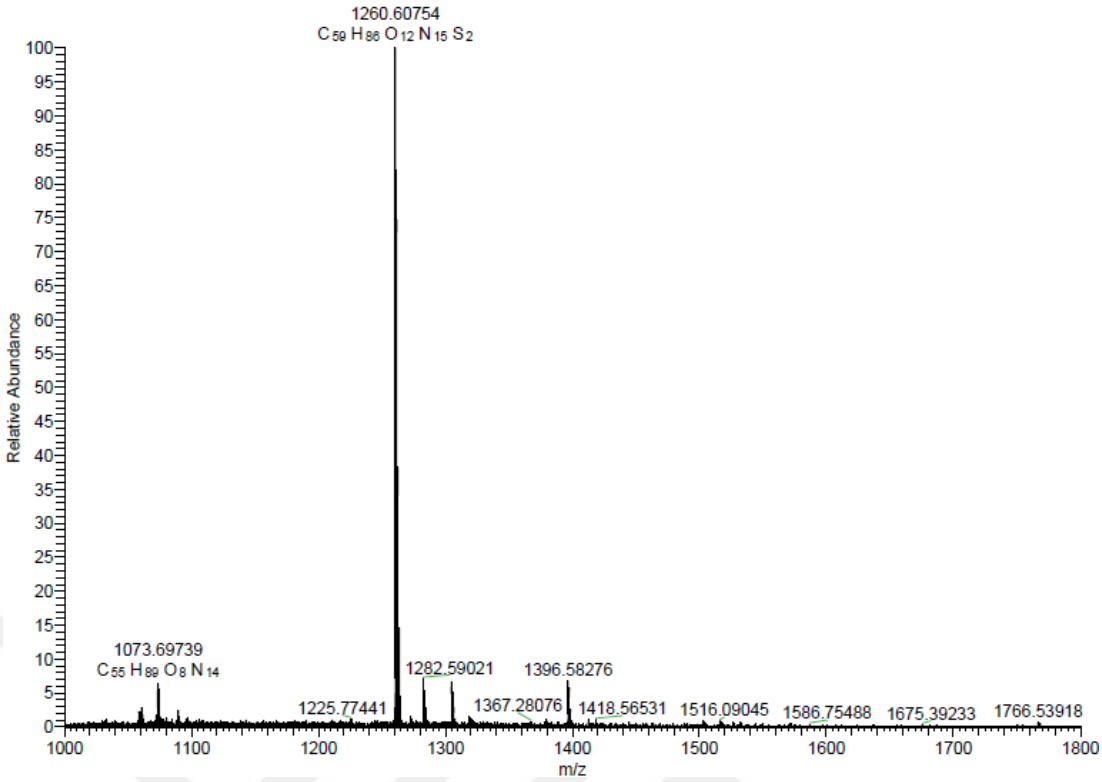

Figure 4.10 : The mass spectrum of PFAMQMAYRF.

Mw: Exact Mass= 1259.60 g/mol

[M+H] += 1260.60754 g/mol

The mass value of the peptide was obtained as 1260.60754 g/mol in the protonated stage from the spectrum in Figure 4.10. Then, the experimental value was calculated as 1259.599715 g/mol. These calculations indicate that this peptide was synthesized successfully.

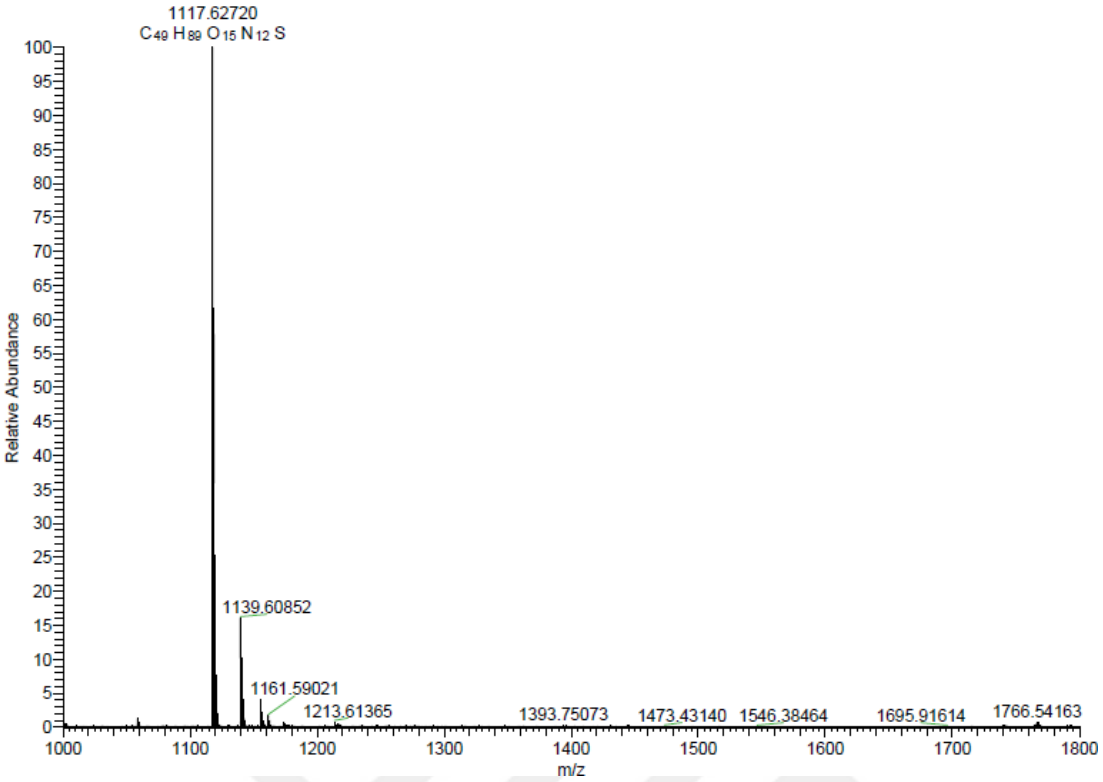

Figure 4.11 : The mass spectrum of TEILPVSMTK.

Mw= Exact Mass: 1116.64 g/mol

[M+H] += 1117.62720 g/mol

As a conclusion, the experimental mass of the peptide was calculated as 1116.619375 g/mol, from the protonated state of 1117.62720 g/mol obtaining from the spectrum in Figure 4.11. The result indicates that this peptide was successfully synthesized.

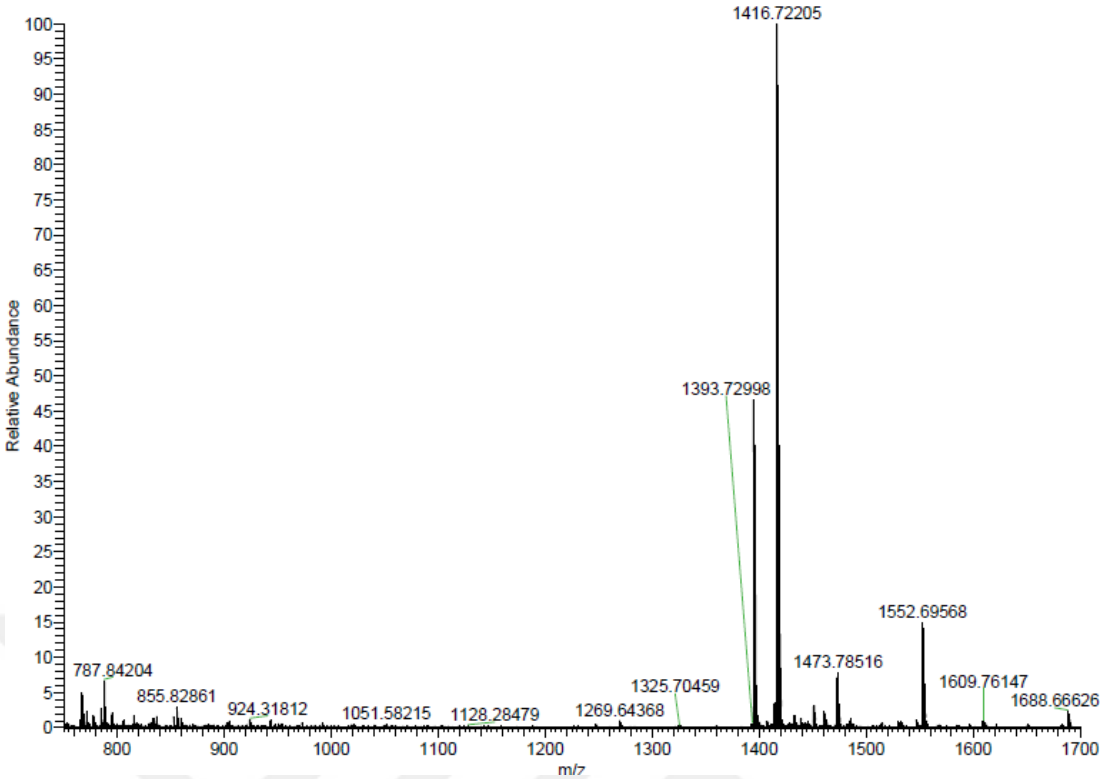

Figure 4.12 : The mass spectrum of KWPWYIWLGF.

Mw= Exact Mass: 1393.75 g/mol

[M+Na] += 1416.72205 g/mol

The mass value of the peptide was obtained as 1416.72205 g/mol in the protonated stage from the spectrum in Figure 4.12. Then, the experimental value was calculated as 1,393.73228 g/mol. The results indicate that this peptide was synthesized successfully.

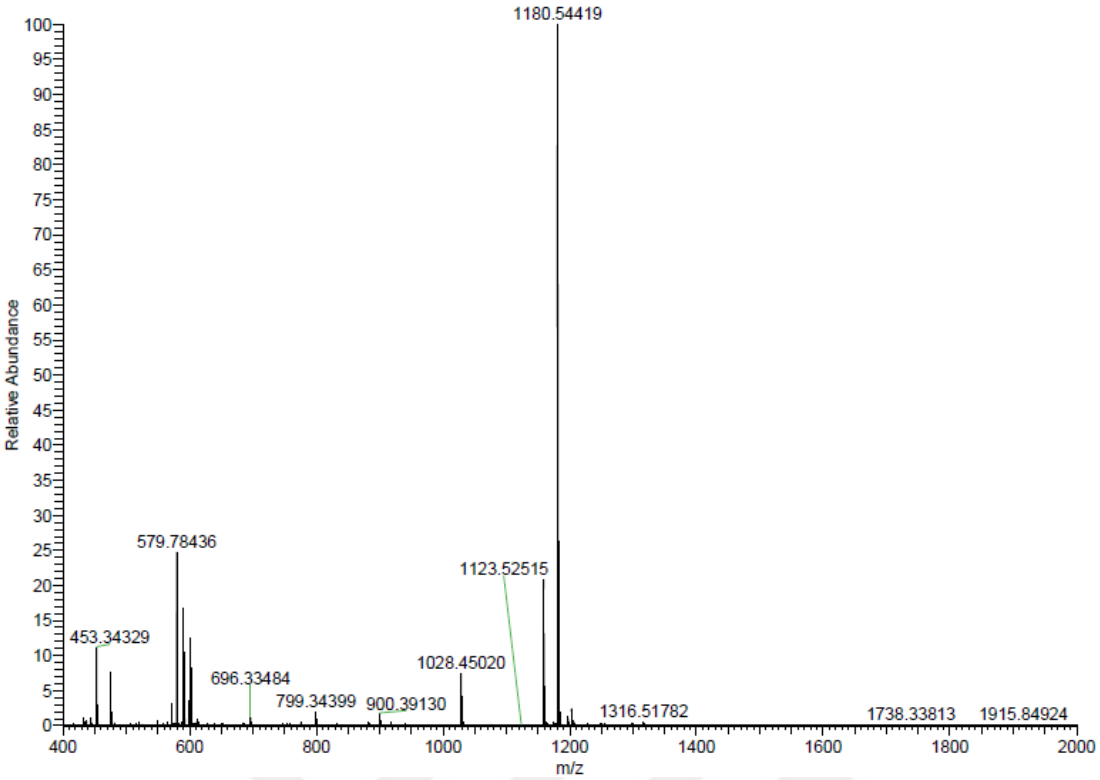

Figure 4.13 : The mass spectrum of LQYGSFCTQL.

Mw= Exact Mass: 1157.57 g/mol

[M+Na] += 1180.54419g/mol

The mass value in the protonated stage was obtained as 1180.54419 g/mol from the spectrum in Figure 4.13. then, the experimental mass of the peptide was calculated as 1,157.55442 g/mol. As a result, the calculations indicate that the peptide was synthesized successfully.

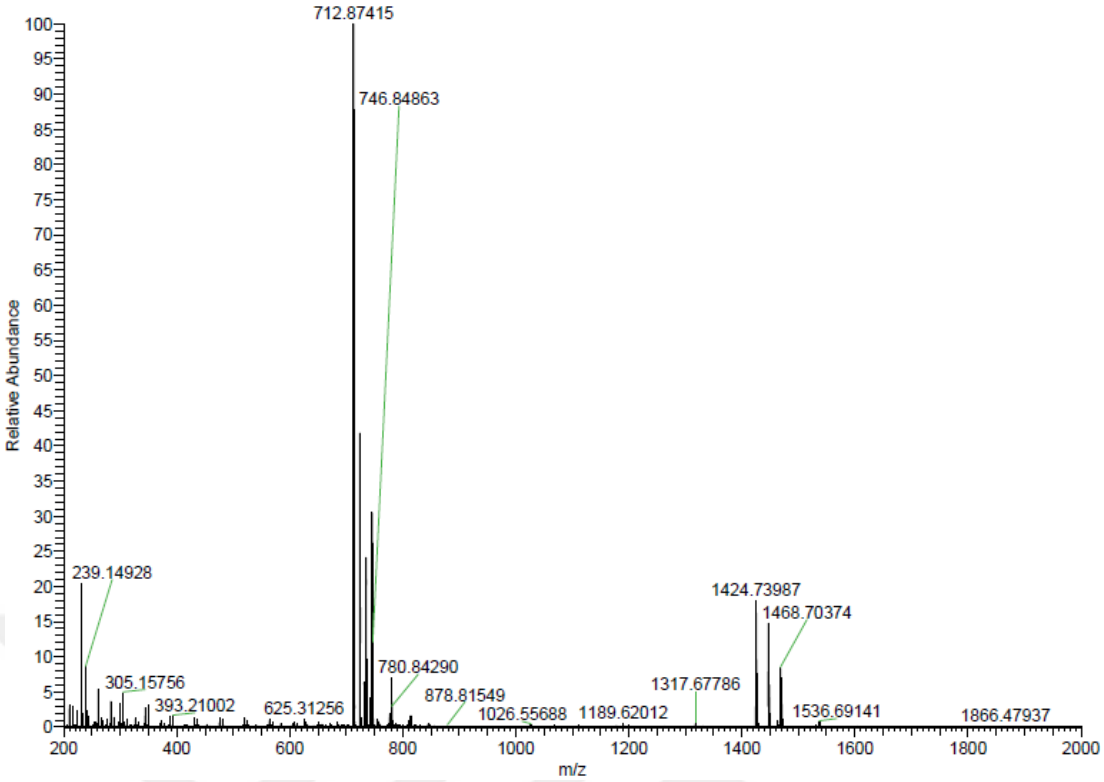

Figure 4.14 : The mass spectrum of EQYIKWPWYI.

Mw: Exact Mass= 1423.74 g/mol

[M+H] += 1424.73987 g/mol

The mass value of the peptide was obtained as 1424.73987 g/mol in the protonated stage from the spectrum in Figure 4.14. Then, the experimental value was calculated as 1,423.732045 g/mol. The result indicates that this peptide was synthesized successfully.

5. CONCLUSION AND FUTURE STUDIES

As a conclusion, based on the HPLC chromatograms of the peptides shown in Figures 4.1. to 4.7. and the calculations that were made under the mass spectrums which are shown in Figures 4.8 to 4.14., the peptides, RQIAPGQTGK, CYFPLQSYGF, PFAMQMAYRF, TEILPVSMTK, KWPWYIWLGF, LQYGSFCTQL, and EQYIKWPWYI, were synthesized successfully with a percent purity of 98%, 94%, 98%, 91%, 91%, 90%, and 85%, respectively.

In the scope of this thesis, the peptides designed by in-silico studies were synthesized and characterized by RP-HPLC and LC-HR/MS. Our future studies involve ELISA tests that determine the binding of peptides to targeted proteins, in conjunction with tests. Following these results, clinical studies will be conducted to access the use of these peptides as vaccines and recognition units in diagnostic kits. Then, a lateral flow test is going to be designed.

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