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Synthesis of Killer T Cell Epitopes for Peptide-Based Vaccine of SARS-CoV-2

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Abstract

COVID-19 is a current global pandemic, which has prompted many countries to develop ways to deal with it. Peptides have many medicinal and diagnostic benefits, so recently, many researchers have been developing peptide-based vaccines against COVID-19. In peptide-based vaccines, peptides act as specific antigens that will provide a faster immune response because they do not go through the process of cutting proteins in the Major Histocompatibility complex (MHC) antigen-presenting cells (APC) and can be directly presented outside the cells so that they can be recognized by the host killer T cells (CTL). Vaccine development can be accelerated with the help of immunoinformatics to predict specific epitopes to induce CTL. We have predicted the CTL epitope through the immunoinformatic method. This study aims to synthesize candidate CTL epitopes as a candidate for the SARS-CoV-2 vaccine using the SPPS method with the Fmoc/t-Bu strategy. In this study, two CTL epitopes were synthesized through a conventional solid-phase peptide synthesis (SPPS) method, and another CTL epitope was synthesized using a semi-automated peptide synthesizer. The SPPS method is faster because the purification is only carried out at the final stage, while the Fmoc/t-Bu strategy was applied because it provides a mild reaction condition. Both synthetic approaches were compared. The semi-automated peptide synthesizer made the synthesis faster and more efficient due to using an inert gas (N_2) during the synthesis. The synthetic peptides were characterized by TOF-ESI-MS. The three peptides showed ion peaks at m/z 1137.5509 $(M+H)^+$, 1064.3468 $(M+H)^+$, and 916.5859 $(M+H)^+$, indicating correct molecular ion peaks for EILDITPCSF, IPIGAGICASY, and FIAGLIAIV, respectively.

Keywords: Peptide; SARS-CoV-2 Vaccine; peptide vaccine; CTL epitope

1. Introduction

Currently, the world is facing a global COVID-19 pandemic. According to WHO, on December 3, 2021, it was recorded that in 99 countries, 263,563,622 people were confirmed positive 5,232,562 of them died. Meanwhile, according to WHO, in Indonesia itself, on December 3, 2021, 4,257,243 people were confirmed positive for COVID-19, and 143,858 died. However, the good thing at this point is that as many as 234,857,452 people in Indonesia have received the vaccination, of which 95,483,061 people have received the full dose while the remaining 139,374,391 people have just received the first dose.

COVID-19 is a disease caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This virus has four structural proteins, namely spike protein (S), membrane (M), envelope (E), and nucleoprotein (N). Then the virus infects the human body by binding to the angiotensin-converting enzyme 2 (ACE2) receptor. Several reports state that the S protein in the receptor-binding domain (RBD) is what attaches to the ACE2 receptor (Priestnall, 2020; Naqvi et al., 2020).

One way to deal with the COVID-19 pandemic is to fight the virus through mass vaccination. Vaccination is a way to prevent various deadly infectious diseases. The vaccine used later is in the form of a specific antigen to induce an immune response against the virus (Li et al., 2014). Existing vaccines for COVID-19 have different intermediates in inducing an adaptive immune response, such as Sinovac, which uses an inactivated virus; AstraZeneca, which uses a viral vector; and Pfizer and Moderna, which uses mRNA to induce antibody production that is an adaptive immune response (Kaur & Gupta, 2020).

Furthermore, the induction of immune response can be described from the cell mechanism when infected with the virus. In this case, the major histocompatibility complex (MHC) protein is sufficient to play a role in presenting antigens that will later be recognized by killer T cells (CTL). Of these mechanisms, MHC class 1 has a role in the activation

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of the CTL response. The antigen presented is a specific peptide fragment from the virus. If the peptide fragment is directly inserted, the immune response will be faster because it does not pass through the MHC protein and is directly recognized by the CTL (Rock et al., 2016). The advantages of peptide-based vaccines include accurate immune response activation, reduced allergic effects, relatively low cost, and safe (Li et al., 2014).

The body's response that is formed before, after, or even during infection with SARS-CoV-2 was described by Cañete and Vinuesa in 2020. Their study found that these patients had a high abundance of T cells. So, T cells, especially CTL, can be used as a target in searching for candidate epitopes with the help of immunoinformatics. Furthermore, immunoinformatics can speed up the vaccine manufacturing process because the candidate can be predicted without going through laboratory experiments first (De Groot et al., 2020). In this study, we tried synthesizing two candidate CTL epitopes discovered by immunoinformatics with conventional solid-phase peptide synthesis (SPPS) and a semi-automated peptide synthesizer with the same SPPS approach.

Epitope candidate synthesis was synthesized using the SPPS method. This method was first discovered by Merrifield in 1963. SPPS is known for its advantages, such as short synthesis time and simple purification. However, this method still has many shortcomings in forming side reactions and aggregation. According to Albercio et al. in 2009, sensitive amino acid residues need to use proper side-chain protection. Semi-automated peptide synthesizer showed the same results as conventional SPPS. However, its use provides a faster and more efficient time. The procedure used is the same as SPPS because both synthesize peptides in the solid phase.

In addition, the use of cleavage reagents has a vital role in preventing the formation of aggregations. The possibility of aggregation during the synthesis, especially during the coupling of amino acids, can reduce the yield of the synthesis product. One way to prevent aggregation and increase yield is to reduce the resin loading value. For peptides with more than five amino acid chains, using a low resin loading value is recommended (0.2-0.6 mmol/g)(Chan & White, 2000; Sabana *et al.*, 2020; Maharani *et al.*, 2021). Peptide chains containing cysteine, methionine, tryptophan, and tyrosine residues have high aggregation potential. The use of type K cleavage reagents consisting of TFA and TIPS solutions is the best solution (Huang &Rabenstein, 1999; Alhassan et al., 2020). The CTL epitopes contain cysteine, methionine, and tyrosine residues. So, the use of a K-type cleavage reagent is the right choice.

In addition to that, the CTL epitope candidates also contain sensitive amino acid residues, such as glutamic acid, aspartic acid, glutamine, threonine, serine, cysteine, histidine, and tyrosine. So, the selection of protective groups and appropriate synthetic strategies are required. The residues of glutamic acid and glutamine have the potential to form pyroglutamate during synthesis, and the way to prevent this is to use HOBt coupling reagents and avoid using the benzyl (Bn) protecting group in the side chain. Meanwhile, in the peptide chain extension process, the side reactions can also include the formation of aspartimide at the aspartic acid residue, O-acylation, and O-N migration at the threonine and serine residues, enantiomerization at the cysteine and histidine residues, and the formation of phenolic ions at the tyrosine residue. This can be avoided by using t-Bu in the side chain and using HOBt as a coupling reagent (Chan & White, 2000; Albericio et al., 2009). Some potential difficult couplings can be overcome by using stronger coupling reagents such as HATU/HOAt.

The synthesis strategy carried out refers to the research of Maharani et al. in 2018, Rahim et al. in 2020, and Alhassan et al. in 2020. Based on the potential side reactions and aggregation that have been described, the synthesis strategy took advantage of the Fmoc/t-Bu strategy, HBTU/HOBt coupling reagent, 2-CTC resin, and TFA and TIPS solutions as cleavage reagents.

2. Experimental

Killer T cell epitope mining and conservancy analysis

In the beginning, we prepared a set of SARS-CoV-2 spike protein sequences as the reference in the epitope conservation analysis. The sequences of SARS-CoV-2 spike protein were retrieved from https://www.gisaid.org/. Duplicate and truncated sequences were discarded using a SeqKit tool (Shen et al., 2016). Next, the sequences containing the 'X' character were removed using an in-house script written in the python programming language. In the next step, the sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) (Nakamuka et al., 2018) to obtain the spike protein set as the reference in the epitope conservancy analysis (http://tools.iedb.org/conservancy/).

The sequences of killer T cell epitopes from experimental results were obtained from the Immune Epitope Database and Analysis Resources (https://www.iedb.org) (Vita et al., 2019). The sequences were processed using the R programming language (R Core Team, 2019) to filter epitopes binding to the dominant alleles in the Indonesian population.

2.1 Chemicals and instruments

The materials used are 2-Chlorotritylchloride (2-CTC) resin, O-benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyl-uronium-hexafluorophosphate (HBTU), N-hydroxy benzotriazole (HOBt), [bis(dimethylamino)methylene]-1H-1,2,3-

triazolo[4,5- b]pyridinium 3-oxide hexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), Fmoc-L-Glu(O*t*Bu)-OH, Fmoc-L-Ile-OH, Fmoc-L-Leu-OH, Fmoc-L-Asp(O*t*Bu)-OH, Fmoc-L-Thr(O*t*Bu)-OH, Fmoc-L-Pro-OH, Fmoc-L-Cys(Trt)-OH, Fmoc-L-Ser(O*t*Bu)-OH, Fmoc-L-Phe-OH, Fmoc-L-Gly-OH, Fmoc-L-Ala-OH, Fmoc-L-Tyr(O*t*Bu)-OH, Fmoc-L-Val-OH were purchased from GL Biochem (Shanghai, China). Trifluoroacetic acid (TFA), triisopropylsilane (TIPS), methanol, piperidine, acetaldehyde, p-chloranyl, dichloromethane (DCM), dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA) used in the synthesis were of analytical grade. Lastly, the N_2 gas for conditioning a semi-automated peptide synthesizer. The instrumentations used for synthesis were SPPS tubes, rotary evaporator, rotary suspension mixer, mass spectrometer (Waters TOF ESI-MS), UV-Vis spectrophotometer (TECAN Infinite pro 200), and semi-automated peptide synthesizer (Activo-P14).

2.2 General Procedures

a. Swelling Resin

A total of 250 mg of 2-CTC resin was added with 5 mL of DCM into the SPPS reactor. Then, it was shaken for 30 minutes using a rotary suspension mixer, then the resin was filtered using a compressor and washed with sufficient DCM.

b. Loading Resin

A mixture of C-terminal amino acid solution (0.375 mmol), two equivalents of DIPEA (0.375 mmol) (eq.), and 4 mL of DCM were added to the reactor. Then the reactor was shaken for 4 hours at room temperature using a rotary suspension mixer; then, the resin was filtered and washed with sufficient DCM.

The resin loading value was calculated by weighing 0.6 mg of resin in a vial and then adding 3 mL of 20% piperidine solution in DMF. The solution mixture was shaken until homogeneous and then allowed to stand for 30 minutes at room temperature. The absorbance of the solution was measured using a UV spectrophotometer at a wavelength of 290 nm with a 20% piperidine solution blank.

c. Capping Resin

A total of 5 mL of the MeOH:DCM:DIPEA mixture (15:80:5) was put into the SPPS reactor, then shaken for 15 minutes using a rotary suspension mixer. The resin was filtered using a compressor and washed with sufficient DCM. This procedure was carried out twice.

d. Fmoc Deprotection

A total of 4 mL of 20% piperidine solution in DMF was put into the SPPS reactor, then shaken for 5 minutes using a rotary suspension mixer. The resin was filtered using a compressor and washed with sufficient DCM and DMF; this procedure was carried out twice.

e. Chloranyl Test

Several resin grains were inserted into the microtube, then three drops of 2% acetaldehyde and 2% p-chloranyl were added to DMF each. The mixture was shaken and left at room temperature for 5 minutes. Then, the resin granules' color was observed. The color of the resin that changes to blue or green indicates the presence of free amines (positive); if the color of the resin does not change suggests the absence of free amines (negative).

f. Amino Acid Coupling Mediated with HBTU/HOBt

Amino acid (3 eq.), HBTU (3 eq.), and HOBt (3 eq.) were dissolved with 4 mL of DMF, then DIPEA (6 eq.) was added to the vial. Then the mixture was added to the SPPS reactor after deprotection. The reactor was shaken using a rotary suspension mixer for 4 hours. The resin was filtered using a compressor and washed with sufficient DCM and DMF.

g. Amino Acid Coupling Mediated with HATU/HOAt

Amino acid (3 eq.), HATU (3 eq.), and HOAt (3 eq.) were dissolved with 4 mL of DMF, then DIPEA (6 eq.) was added to the vial. Then the mixture was added to the SPPS reactor after deprotection. The reactor was shaken using a rotary suspension mixer for 4 hours. The resin was filtered using a compressor and washed with sufficient DCM and DMF.

h. Cleavage Resin

A total of 5 mL of TFA:TIPS:H2O (95:2.5:2.5) was added to the SPPS reactor after deprotection. The reactor was shaken using a rotary suspension mixer for 2 hours. The resin is filtered using a compressor and then washed with DCM. Then the filtrate was collected using a round bottom flask. This procedure was repeated with a second shaking time of 1 hour. The filtrate is concentrated with a rotary evaporator.

i. Semi-automated Peptide Synthesizer

A total of 100 mg of 2-CTC resin was added with 2.5 mL of DCM into the reactor. Then the resin was shaken for 5 minutes and then dried with N_2 gas for 30 seconds. The first amino acid (0.375 mmol), DIPEA(2 eq.), and DCM (2.5 mL)were added to the reactor. Then, the reactor was shaken for 30 minutes at room temperature, then washed with DCM, and dried. The resin loading values were then measured and calculated. Capping resin was then carried out by the addition of a mixture of MeOH:DCM:DIPEA (15:80:5) for 2.5 mL to the resin. Then, the reaction mixture was shaken for 5 minutes. Furthermore, deprotection of Fmoc was undertaken by adding 20% piperidine in DMF (2.5 mL) to the resin, and the mixture was shaken for 3 minutes. This step was done twice. After the Fmoc deprotection step, the following amino acid (3 eq.) was coupled to the resin-amino acid-$NH_2$with the addition of HATU (3 eq.), HOAt (3 eq.), and DIPEA (6 eq.), in 2.5 mL of DMF. The reaction mixture was shaken for 1 hour. Then, it was washed with DCM and DMF and dried with N_2 gas. The deprotection and amino acid coupling steps were repeated until the last amino acid (N-terminal)was

attached to the resin. Lastly, the desired peptide was cleaved from the resin using a cleavage type K reagent (2.5 mL) for 1 hour, and the peptide was collected.

2.3 Procedures

EILDITPCSF

EILDITPCSF was produced, following the stages of resin swelling, resin loading, resin capping, Fmoc deprotection, subsequent amino acid coupling, then repetition of Fmoc deprotection and coupling to N-terminal amino acids, then resin cleavage. The Fmoc deprotection process and Amino acid coupling are always controlled by the chloranyl test. The amino acids used were Fmoc-L-Phe-OH, Fmoc-L-Ser(t-Bu)-OH, Fmoc-L-Cys(trt)-OH, Fmoc-L-Pro-OH, Fmoc-L-Thr(t-Bu)-OH, Fmoc-L-Ile-OH, Fmoc-L-Asp(t-Bu)-OH, Fmoc-L-Leu-OH, Fmoc-L-Ile-OH, and Fmoc-L-Glu(t-Bu)-OH.

IPIGAGICASY

IPIGAGICASY was produced, following the stages of resin swelling, resin loading, resin capping, Fmoc deprotection, subsequent amino acid coupling, then repetition of Fmoc deprotection and coupling to N-terminal amino acids, then resin cleavage. The Fmoc deprotection process and Amino acid coupling are always controlled by the chloranyl test. The amino acids used were Fmoc-L-Tyr(t-Bu)-OH, Fmoc-L-Ser(t-Bu)-OH, Fmoc-L-Ala-OH, Fmoc-L-Cys(trt)-OH, Fmoc-L-Ile-OH , Fmoc-L-Gly-OH, Fmoc-L-Ala-OH, Fmoc-L-Gly-OH, Fmoc-L-Ile-OH, Fmoc-L-Pro-OH, and Fmoc-L-Ile-OH.

FIAGLIAIV

FIAGLIAIV was produced, following the stages of resin swelling, resin loading, resin capping, Fmoc deprotection, subsequent amino acid coupling using HATU/HOAt, then repeating Fmoc deprotection and coupling to N-terminal amino acids, then cleavage resin. The amino acids used were Fmoc-L-Val-OH, Fmoc-L-Ile-OH, Fmoc-L-Ala-OH, Fmoc-L-Ile-OH, Fmoc-L-Leu-OH, Fmoc- L-Gly-OH, Fmoc-L-Ala-OH, Fmoc-L-Ile-OH, and Fmoc-L-Phe-OH.

3. Results and Discussion

Based on the immunoinformatic study, EILDITPCSF, IPIGAGICASY, and FIAGLIAIV, shown in Table 1, were found to have good conservation of new variants of COVID-19. In addition, the three sequences have conservation of spike protein (S) in each of their variants, especially in the origin, delta, and omicron variants, which have recently received the attention of the WHO. S protein is one of the virus's proteins that is often used for vaccine development.

Synthesis of EILDITPCSF (Scheme 1) was undertaken using a conventional SPPS with Fmoc/t-Bu-based strategy., The peptide elongation process was carried out from C-terminal to N-terminal. The synthesis begins with resin swelling, which aims to develop the resin so that the amino acids to be bonded are easily bound. Then, the resin loading was carried out, and its loading values were calculated to give 0.56 mmol/g. The active site of the resin that does not bind to the first amino acid needs to be covered through the capping process. This resin capping can reduce the potential for binding the second amino acid to the resin that would otherwise be attached to the first amino acid.

Fmoc deprotection was carried out after resin capping and before subsequent amino acid coupling. The release of the protective Fmoc on the amine group provides the active site for subsequent amino acid coupling so that a peptide bond is formed. In this study, deprotection using 20% piperidine in DMF for 5 minutes was carried out twice. The chloranyl test that gave a change in the color of the resin from yellow to green or blue indicated the presence of free amines. When the resin did not change color, it indicated the absence of free amines.

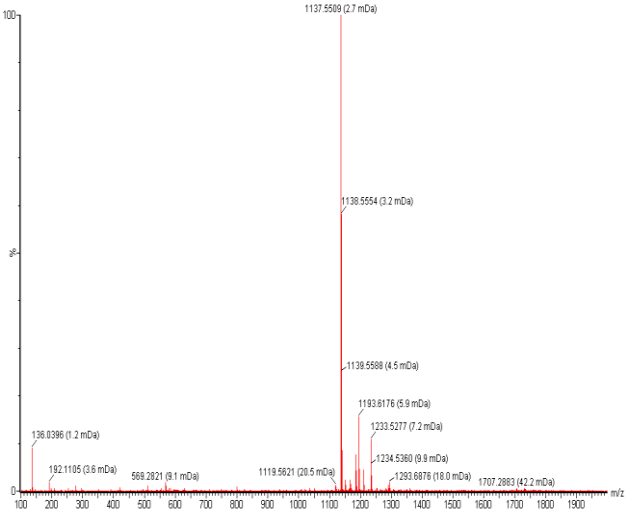
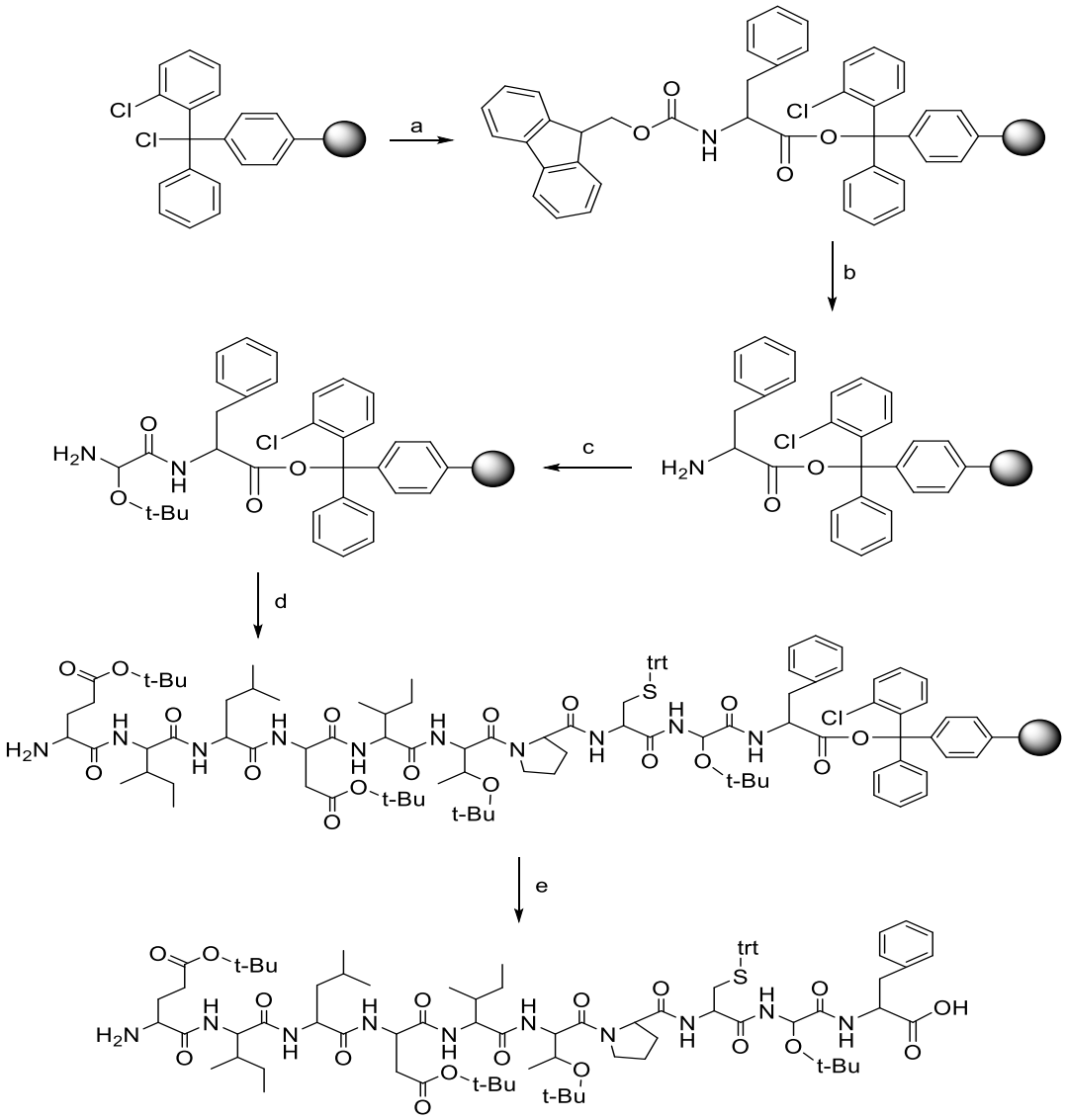


Fig. 1. Chromatogram of Mass Spectrometry of EILDITPCSF

In the SPPS method, the reactions that occur are heterogeneous. Heterogeneous reactions allow collisions between molecules at low reaction times. This weakness can be minimized by increasing the amount of reagent in excess. In this opportunity, reactions were carried out with excess amino acids (3 eq.), coupling reagents and their respective additives (3 eq.), and DIPEA (6 eq.). The mol amount depends on the value of the resulted resin loading value calculated in the early stage of the synthesis.



Scheme 1. Synthesis of EILDITPCSF. Reagents: (a) 1. Fmoc-L-Phe-OH, DIPEA, DCM, 2. Methanol; (b) 20% piperidine in DMF (c) 1. Fmoc-L-Ser(t-Bu)-OH, HBTU/HOBt, DIPEA, DMF; 2. 20% piperidine in DMF; (d) repeated step (c) until N-terminal; (e) TFA:TIPS:H_2O (95:2.5:2.5)

The coupling reagent used in the synthesis is HBTU/HOBt. A HATU/HOAt reagent was also employed for any difficult coupling, especially for any coupling involving β-branched amino acids such as isoleucine and valine. The use of stronger coupling reagents like HATU/HOAt can increase the yield of the synthesis product and make the reaction more efficient. This is due to applying a single coupling protocol instead of double or multiple coupling. Repetition of the reaction usually results in aggregation and reduces the efficiency of the reaction.

Cleavage of the resin was carried out after the N-terminal amino acid coupling was successful and the Fmoc deprotection process had been carried out. In this study, the cleavage resin used TFA:TIPS:H_2O (95:2.5:2.5), commonly called the cleavage cocktail type K reagent. This process makes the peptides released from the resin, together with the protective amino acid side chains. TIPS and water have functioned as scavengers that can capture the released carbocations, so they will not react with the peptide. The peptide fraction was collected and then concentrated using a rotary evaporator, eventually analyzed by mass spectrometry to confirm the desired ion peak at*m/z* 1137.5509 $(M+H)^+$ (Figure 1).

Table 1. Conservation of killer T cell epitopes of SARS-CoV-2 spike protein

No	Peptide	Conservation degree
1	EILDITPCSF	99.52%
2	IPIGAGICASY	99.89%
3	FIAGLIAIV	98.92%

The synthesis of IPIGAGICASY was carried out with a similar protocol as applied for EILDITPCSF. The obtained resin loading value is 0.48 mmol/g. The coupling reagents used are the same, namely HBTU/HOBt and HATU/HOAt. The cleavage resin step also took advantage of the type K cocktail reagent. The synthesis results were confirmed by mass spectrometry with the presence of a molecular ion peak of *m/z* 1064.3468 $(M+H)^+$.

The synthesis of FIAGLIAIV was synthesized using a different technique than before. A semi-automated peptide synthesizer was used to prepare the peptide. In the synthesis, the reaction time for each step was reduced, and all reactions were undertaken under inert nitrogen gas. The resin loading was only carried out in 30 minutes, which was shorter than the four-hours loading applied in the conventional synthesis. Using a shorter loading resin found that the resin loading value is 0.60 mmol/g, which still gives a good resin loading value. The coupling reaction was also undertaken in a very short time, which was one hour instead of 4 hours as applied in the conventional SPPS. HATU/HOAT reagent was employed to facilitate the amide bond formation. The resin cleavage step took advantage of a type K cocktail reagent and was carried out for 1 hour. The peptide was then collected, evaporated, and analyzed by mass spectrometry. The mass spectra showed a correct molecular ion peak at*m/z* 916.5859 $(M+H)^+$. It seems likely that the inert condition applied for the SPPS reaction has made the synthesis process faster and more efficient even though the shorter reaction time was used.

Peptides synthesized in this study will be tested in-vitro for their strong binding with MHC-I molecule using the Surface Plasmon Resonance method. A stable binding with MHC-I will indicate a potency to be further studied ex-vivo and in-vivo as a peptide-based vaccine for SARS-CoV-2 that protects against broader concerns variants.

4. Conclusion

EILDITPCSF, IPIGAGICASY, and FIAGLIAIV were successfully synthesized using conventional SPPS and semi-automated peptide synthesizers. However, using a semi-automated peptide synthesizer gives the same results with a faster and more efficient reaction time, so its use is highly recommended. In addition, a semi-automated peptide synthesizer will provide enough peptides for further studies in developing a peptide-based vaccine of SARS-CoV-2.

5. Conflicts of interest

There are no conflicts to declare

6. Funding sources

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