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Synthesis of Pentapeptide FWKVV (Phe-Trp-Lys-Val-Val) and Its Activity as Antioxidants

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Abstract

Antioxidant pentapeptides are pentapeptide compounds that have antioxidant activity. One of the pentapeptide compounds that have antioxidant activity is FWKVV. FWKVV is a linear pentapeptide with the amino acid sequence phenylalanine-tryptophan-lysine-valine-valine, which was first isolated to hydrolyzate the muscle protein of Miiuy croaker (Miichthysmiiuy). In addition to isolation, FWKVV compounds can be produced by the peptide synthesis method because this method requires a shorter time than the isolation method from natural materials. Synthesis methods commonly used are solution-phase peptide synthesis and solid-phase peptide synthesis (SPPS). However, the SPSS method is more efficient because it does not require purification in every process. The purpose of this study was to synthesize FWKVV compounds using the SPPS method and test their antioxidant activity. FWKVV has been synthesized using the SPPS method with HBTU/HOBt coupling reagent and Fmoc protective group. The FWKVV crud produced was 148.8 mg and had antioxidant activity against DPPH radicals with an IC50 value of 4.2 mg/mL.

Keywords: Antioxidant, pentapeptide, FWKVV, SPPS, DPPH

INTRODUCTION

Bioactive peptides are compounds that contribute to bioactive functions by influencing metabolism and preventing disease or modulating the physiological system after being absorbed by the body (Leticia Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014). Peptides have various bioactive effects depending on the sequence and number of amino acids that make up the peptide, including in the gastrointestinal system, peptides have anti-obesity activity, the cardiovascular system as an antihypertensive, antithrombotic, antioxidant and hypocholesterolemic, the immune system as an antimicrobial, sitomodulator and immune modulator, and in the nervous system as opioid peptides (L Mora, Aristoy, & Toldrá, 2016).

Antioxidant peptides are a group of peptide compounds that can neutralize free radicals, so they can prevent and treat chronic diseases (R. Maharani et al., 2019). One of the peptides that have antioxidant activity is the FWKVV (Phe-Trp-Lys-Val-Val) compound. FWKVV is a linear pentapeptide compound with the amino acid sequence phenylalanine-tryptophan-lysine-valine-valine, which has been isolated from the muscle protein hydrolyzate of Miiuy croaker (*Miichthysmiiuy*) with antioxidant activity against DPPH radicals which is relatively high, namely 0.85 mg/mL (He, Pan, Chi, Sun, & Wang, 2019). However, the information regarding FWKVV is still very limited. For further exploration, apart from the isolation method, the synthesis method can be used to develop information about FWKVV. The synthesis method also allows researchers to obtain information about an analog of FWKVV, which can later be developed in further research by providing information about the relationship between activity and structure.

Research on peptide synthesis has been widely developed, especially for linear pentapeptide compounds that have the potential for antioxidant activity. Sabana et al., (2020) has been synthesized the antioxidant pentapeptide compound SCAP1 (Leu-Ala-Asn-Ala-Lys) using the solid phase peptide synthesis (SPPS) method previously isolated from oyster protein hydrolyzate (Saccostrea cucullata). In addition, nine SCAP1 analogs have been successfully synthesized using the SPPS method. The nine analogs that have been synthesized are known that asparagine residue has an important role in the antioxidant activity of the SCAP1 compound and the replacement of lysine residue with valine can increase antioxidant activity (R. Maharani et al., 2020).

Peptide synthesis can be carried out by two methods, namely the solution phase peptide synthesis method and solid-phase peptide synthesis (SPPS) as used in the research of Sabana et al., (2020) and R. Maharani et al., (2020). In solution-phase peptide synthesis, a fully protected peptide segment is needed whereas in SPPS the use of solid supports other than as a place for the extension of the peptide chain also functions as a protective group so that the group that binds to the solid support is automatically protected (Walker & Rapley, 2008). In addition, in the solution phase synthesis method, all reagents and dissolved reaction products are in the same medium so that purification is required at each reaction stage, whereas in the solid phase synthesis method, impurities will be easier to separate through simple filtering. To synthesize SCAP1 peptide, the SPPS method was chosen in this study because it has several advantages over solution-phase synthesis, namely a faster reaction time (Merrifield, 1963). The SPPS method has been used successfully in synthesizing several peptide compounds, including the linear tetrapeptide compound DPAP (Asp-Pro-Ala-Pro) with 10.85% revision (Eka Yanti & Rani Maharani, 2020), PSSY with 13.8% yield (R. Maharani et al., 2019), and PADY (Pro-Ala-Asp-Tyr) with a yield of 12.6% (R. Maharani et al., 2019).

The FWKVV compound was synthesized using the SPPS method because this method has several advantages over the solution phase method, namely the faster reaction (Chan and White, 2000). The synthesized FWKVV compound was characterized using a mass spectroscopy method and tested for its antioxidant activity using the DPPH radical. Synthesis of FWKVV compounds using the SPPS method has not been reported, so it is hoped that it can provide new information about antioxidant compounds from the peptide group that is easier to synthesize.

METHODOLOGY

Tools and Materials

The tools used in this research are solid-phase peptide synthesis tube, rotary evaporator, freeze dryer, rotary suspension mixer, Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) Waters 1500-Series brand, Photo Diode Array (PDA) detector, Mass. Waters Spectrometer QTof MS with Electron Spray (ES) system, Perkin Elmer UV-Vis Spectrophotometer, and glass tools commonly used in laboratories.

The materials used were Fmoc-Phe-OH, Fmoc-Trp-OH, Fmoc-Lys-OH, Fmoc-Val-OH, 2-chlorotritylchloride resin, dichloromethane (DCM), dimethylformamide (DMF), DIPEA (diisopropylethylamine), TFA, methanol, p-chloranyl, acetaldehyde, piperidine, HBTU (2- (1H-Benzotriazole-1-il)-1,1,3,3-tetramethiluronium) and OBT (Hydroxybenzotriazole).

Procedures

First amino acid C terminal binding (*loading resin*)

A total of 250 mg of 2-chlorotritylchloride resin was added with 5 mL DCM, then shook for 30 minutes and filtered. In a round bottom flask prepared a solution of Fmoc-Val-OH (0.375 mmol), 5 mL DCM and DIPEA (0.75 mmol). The amino acid solution was added to the resin and shaken at room temperature for two hours. The resin was filtered, then washed using DCM. Furthermore, the calculation of resin loading was carried out, by sampling some resin grains weighed then put in an Eppendorf tube, then added 3 mL of 20% piperidine and let stand for 30 minutes. Then the absorption of the solution was measured with a UV-Vis spectrophotometer at a wavelength of 290 nm and with a 20% piperidine blank. The resulting loading resin value serves to determine the number of amino acids and the coupling reagent will be used.

A total of 5 mL of a mixture of methanol: DCM; DIPEA (15: 80: 5) was added to the resin which was then shaken for 15 minutes, this treatment was repeated twice. The resin was filtered and washed using DCM and dried for ± 15 minutes, resulting in a dry Fmoc-Val-resiny. Removing the protective group of Fmoc using 4 mL of 20% piperidine in DMF and shaking for 5 minutes. Furthermore, the resin was filtered and washed using DMF and DCM. Piperidinewas added and resin washes were added twice. Control of release of the protective Fmoc group using the chlorine test.

Second amino acid coupling (Fmoc-Val-OH)

In a round bottom flask prepared Fmoc-Val-OH (3ek), added with HBTU (3 ek) and HOBt (3 ek), added DIPEA (6 ek.) And dissolved in 4 mL DMF, then sonicated for 5 minutes. The yellow solution was added to the dry peptide resin (resin-Val-NH_2) and shaken overnight. The resin was filtered and washed with DMF and DCM, then dried. Control of the second amino acid coupling was carried out using the chlorine test. Removing the protective group Fmoc using 20% piperidine in DMF (4 mL) and shaking for 2 x 5 minutes. Furthermore, the resin was filtered and washed using DMF and DCM. The release control of the protective group Fmocwas carried out using the chlorine test.

Third amino acid coupling (Fmoc-Lys (Boc) -OH)

In a round bottom flask prepared Fmoc-Lys (Boc) -OH (3 ek) added with HBTU (3ek) and HOBt (3 ek), added DIPEA (6 ek.) And dissolved in 4 mL DMF, then sonicated for 5 minutes. The yellow solution was added to the dry peptide resin (resin-Val-Val-NH_2) and shaken for 4 hours. The resin was filtered and washed with DMF and DCM, then dried. The third amino acid coupling control was carried out using the chloranil test. Removing the protective group Fmoc using 20% piperidine in DMF (4 mL) and shaking for 2 x 5 minutes. Furthermore, the resin was filtered and washed using DMF and DCM. The release control of the protective group Fmocwas carried out using the chlorine test.

Fourth amino acid coupling (Fmoc-Trp (Boc) -OH)

In a round bottom flask prepared Fmoc-Trp (Boc) -OH (3 ek) added with HBTU (3ek) and HOBt (3 ek), added DIPEA (6 ek.) And dissolved in 4 mL DMF, then sonicated for 5 minutes. The yellow solution was added to the dry peptide resin (resin-Val-Val-Lys (Boc)-NH_2) and shaken overnight. The resin was filtered and washed with DMF and DCM, then dried. The fourth amino acid coupling was controlled by using the chlorine test. Removing the protective group Fmoc using 20% piperidine in DMF (4 mL) and shaking for 2 x 5 minutes. Furthermore, the resin was filtered and washed using DMF and DCM. The release control of the protective group Fmocwas carried out using the chlorine test.

Fifth amino acid coupling (Fmoc-Phe-OH)

In a round bottom flask prepared Fmoc-Phe-OH (3 ek) added with HBTU (3ek) and HOBt (3 ek), added DIPEA (6 ek.) And dissolved in 4 mL DMF, then sonicated for 5 minutes. The yellow solution was added to the dry peptide resin (resin-Val-Val-Lys (Boc) -Trp (Boc) -NH_2) and shaken for 5 hours. The resin was filtered and washed with DMF and DCM, then dried. The fifth amino acid coupling control was carried out using the chlorine test. Removing the protective group Fmoc using 20% piperidine in DMF (4 mL) and shaking for 2 x 5 minutes. Furthermore, the resin was filtered and washed using DMF and DCM. The release control of the protective group Fmocwas carried out using the chlorine test.

Release of tetrapeptides from resins

Pentapeptide resin (resin-Val-Val-Lys (Boc) -Trp (Boc) - Phe-NH_2) was added with 5 mL of 95% TFA solution in water. The mixture was shaken for 1 hour at room temperature, this procedure was carried out 2 times. The release of peptides from the resin is indicated by the change in resin color to red. The resin is filtered then the filtrate is concentrated with a rotary evaporator. Furthermore, the pentapeptide solid was dried using a freeze dryer. The resulting peptide crud was analyzed for purity using analytical RP-HPLC using a LiChrospher RP-18 reverse phase column, and eluted using water: acetonitrile (0:70) gradient with 0.1% TFA buffer for 30 minutes, flow rate 2 mL/minute, and using a PDA detector with detection at a wavelength of 254 nm and characterized using a mass spectrometer.

Antioxidant Activity Test

Preparation a solution of 2,2-diphenyl-1-picry-hydazyl (DPPH) 160 ppm

An amount of 4 mg DPPH is placed in a brown vial bottle, then dissolved in 25 mL methanol. 800 µL of p.a methanol and 200 µL of DPPH solution were mixed and incubated for 30 minutes. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm (the absorbance of the DPPH should be at the susceptible 0.8-0.9).

Preparation of a stock sample solution of 8,000 ppm

An amount of 80 mg of sample was put into a 10 mL volumetric flask, then methanol was added to the limit mark and was sonicated until all samples were dissolved. The sample solution was centrifuged at a speed of 10,000 ppm for 10 minutes. The filtrate obtained is used for the activity test.

Testing samples

The stock solution that has been made is diluted to 1000 ppm, 2000 ppm, 3000 ppm, 4000 ppm, and 5000 ppm by adding DPPH and methanol solutions (the volume is made 1 mL). The solution was incubated for 30 minutes and its absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm.

Analysis

In determining the antioxidant activity of FWKVV crud the absorbance that has been obtained, the% inhibition at each concentration was calculated using Equation 1.

$$\%\, inh\ = \frac{A_{control} - A_{sample}}{A_{sample}} \times 100\% \qquad (1)$$

Percentage of inhibition at each concentration, then a linear equation (y = bx + c) is made, from the curve obtained from the relationship between concentration and% inhibition.

Then the 50% inhibition (% IC50) is calculated using Equation 2. The value c is intersep and b is slope.

$$IC_{50} = \frac{\mathbf{50-c}}{\boldsymbol{b}} \quad (2)$$

RESULTS AND DISCUSSION

The FWKVV compound has been successfully synthesized using the solid support phase 2-chlorotrityl chloride resin. 2-chlorotrityl chloride resin is used because of its dense resin structure which reduces the occurrence of racemization and the formation of a by-product in the form of diketopiperazine (Chan & White, 2000). The resin development process aims to open the active site of the resin, allowing the first amino acids to bind to the active site of the resin (Sewald & Jakubke, 2002).

In this synthesis, the first amino acid of the FWKVV target compound is the amino acid valine (Val). The amino groups at the N-terminal are protected with the Fmoc group so as not to interfere with the reaction. The binding of the amino acid valine to the resin does not involve activation of the carboxyl group but begins with the binding of acidic hydrogen to the Fmoc-Val-OH carboxyl group by the base N, N-diisopropylethylamine (DIPEA) through an acid-base reaction, and nucleophiles are formed. The nucleophile will attack the quaternary carbon in 2-chlorotrityl chloride resin and substitute the chloride atom so that it binds to the resin. The reaction between the amino acid valine and resin through a unimolecular nucleophilic substitution reaction (SN1) to form Fmoc-Val-resin (Figure 1) (Chan & White, 2000).

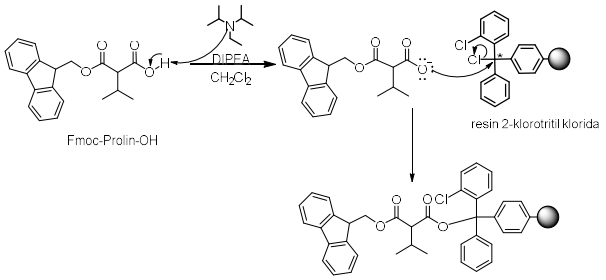

Figure 1.The binding reaction of the amino acid proline as a terminal C end to 2-chlorotrityl chloride resin (Chan & White, 2000; R. Maharani et al., 2020, 2019)

The reaction was carried out for 4 hours because the researchers wanted the resin loading value or the amount of the first amino acid that entered the resin not to be too large. After all, the more first amino acids bound to the resin would cause a reaction process that was prone to the formation of diketopiperazine by-products (Chan & White, 2000). The determination of the resin loading value was carried out using a UV spectrophotometer, and it was known that the FWKVV resin loading value was 0.3 mmol/g resin, the resin loading value was quite good because it was in a good resin loading value range of 0.2-0.8 mmol/g (Chan & White, 2000). Based on the resin loading value, we can see that not all amino acids are bound to the resin. Therefore it is necessary to capping resin using a mixture of methanol: DCM: DIPEA (15: 80: 5) which aims to seal the active site of the resin so that it does not bind to further amino acids (Figure 2).

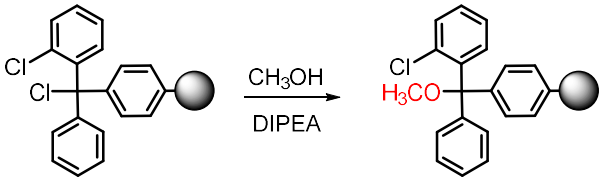

Figure 2. Resin capping reaction using methanol (R. Maharani et al., 2019)

The next step is the release of the protective group Fmoc (deprotection), deprotection serves to provide the active site of the first amino acid which can react with the second amino acid. The protective group Fmoc was chosen because it is a protective group that is unstable in alkaline conditions so that it can be released using a base. Deprotection was carried out by adding 20% piperidine solution in DMF. The Fmoc release reaction starts from the hydrogen atoms in the fluorene ring which will be bound by piperidine to form free amino acid groups and an intermediate type of aromatic cyclopentadiene compound (Chan & White, 2000). These intermediate compounds are easily broken down into dibenzofulven compounds and carbon dioxide (Figure 3).

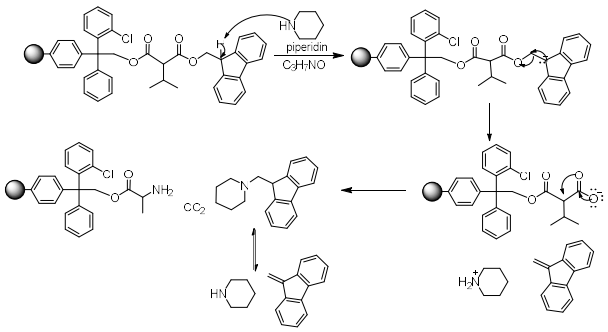

Figure 3. The reaction mechanism for the release of the protective Fmoc group (Chan & White, 2000; Eka Yanti & Rani Maharani, 2020).

The release of the Fmoc protective group was monitored by the chlorine test. In the chlorine test, the success of deprotection was indicated by a change in the color of the resin to purple or red which indicates the presence of a free amine (-NH_2) group. The reaction of amino group testing with chlorine can be seen in Figure 4.

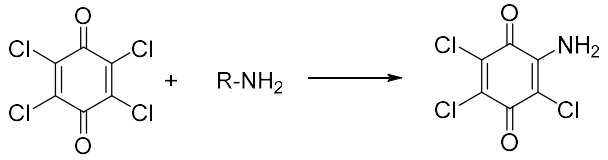

Figure 4. Test reaction of the protective group Fmoc with chlorine (R. Maharani et al., 2019)

After Fmocis released and a free NH_2 group is produced, the next step is the coupling reaction. The coupling reaction is a reaction to form an amide bond between one amino acid and the second amino acid using activating reagents. In this study, the activating reagent used was HBTU/HOBt. HBTU/HOBt is a series of coupling reagents for the uronium/aminium salt group, where the combination of these coupling reagents can increase yield and reduce the formation of epimerization (Valeur & Bradley, 2009).

The success of the second amino acid coupling reaction Fmoc-Val-OH was monitored using the chlorine test. In the chlorine test, the resin color did not change (yellow) indicating the absence of free NH_2 groups which meant that the coupling reaction was successful. The next step is the deprotection of the Fmoc protective group. The coupling and deprotection stages of the Fmoc protective group were repeated until the desired peptide chain was obtained, namely Val-Val-Lys-Trp-Phe. The stages of the synthesis reaction are shown in Figure 5.

After obtaining the target peptide compound, the next step is to release the peptide from the resin (cleavage). The release of the peptides from the resin used 95% TFA in water for 1 hour. This process is carried out twice to ensure that all peptides are removed from the resin. The very high concentration of TFA and the release time are long enough so that the side chain protective groups (Boc) on the amino acids lysine and tryptophan are also released. Boc group serves to protect the side chains that react easily with carboxyl groups. The reaction of releasing peptides from the resin is shown in Figure 6.

The FWKVV peptide crude obtained was a brownish-yellow solid of 148.8 mg. then the FWKVV crude was analyzed using a mass spectrometer and gave the molecular ion peak $[M + H]^+$ at 678.4. This corresponds to the relative molecular mass of FWKVV of 677.4 g/mol.

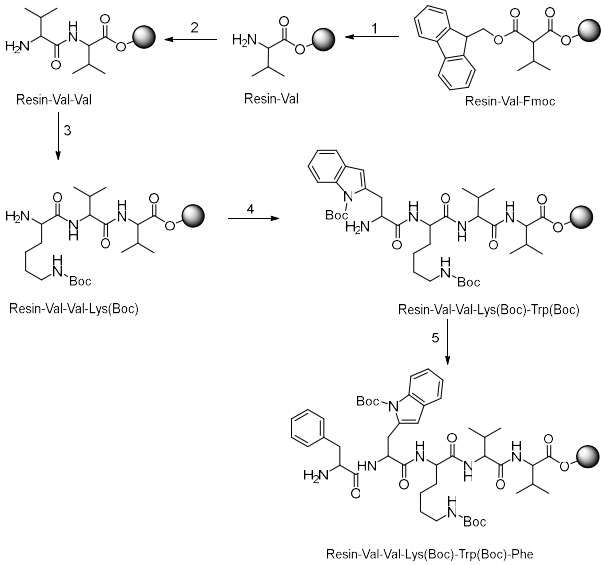

Figure 5. Schematic of FWKVV synthesis, (1) 20% piperidine in DMF, (2) a. Fmoc-Val-OH, HBTU/HOBt, DIPEA b. 20% piperidies in DMF, (3) a. Fmoc-Lys (Boc) -OH, HBTU/HOBt, DIPEA b. 20% piperidine in DMF, (4) a. Fmoc-Trp (Boc) -OH, HBTU/HOBt, DIPEA b. 20% piperidine in DMF, (5) a. Fmoc-Phe-OH, HBTU/HOBt, DIPEA b. 20% piperidine in DMF

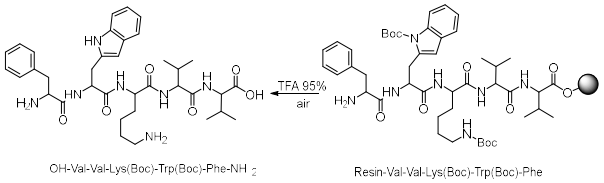

Figure 6. The reaction of the release of FWKVV peptide from the resin

The presence of the FWKVV peak was reinforced by the appearance of the ½ $[M + H]^+$ peak at 339.7. However, the FWKVV crude obtained is not pure, that is, there are still many peaks of impurities or other peptides that are formed, for example, the $[M + H]^+$ peak at 531.3 which is the peak of the tetrapeptide (Trp-Lys-Val-Val) Figure 7. The crude FWKVV The impure obtained was also strengthened by analytic RP-HPLC analysis using a LiChrospher RP-18 reverse phase column, and eluent using a water-acetonitrile (0-70) gradient eluent with 0.1% TFA buffer for 30 minutes and a flow rate of 2 mL/minute. The results of the purity analysis show that there are many peaks, which means that the results of the FWKVV synthesis are still not pure Figure 8. Based on the results of the

analysis showing that the synthesized FWKVV compound is impure.

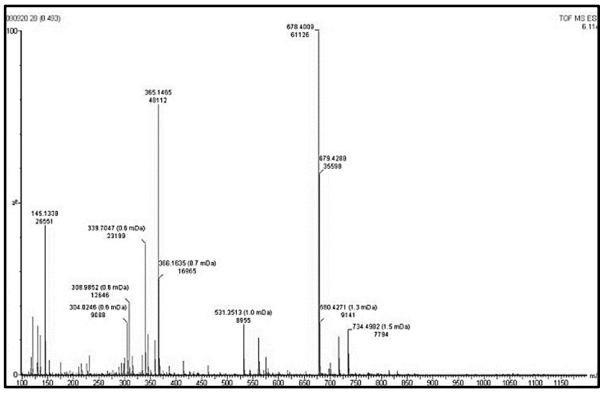

Figure 7. The results of the mass spectrophotometer analysis (TOF ES-MS) of the FWKVV compound, namely the peak of molecular ion $[M + H]^+$ at 678.4.

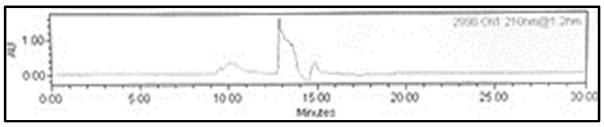

Figure 8. RP-HPLC chromatogram of FWKVV compound.

Antioxidant activity of FWKVV crude

The crude FWKVV was tested for its antioxidant activity using the DPPH radical inhibition method. This method is used because this method is relatively simple, fast, and effective in testing the anti-radical activity of a compound (Waode Rustiah & Nur Umriani, 2018). DPPH radicals react with antioxidant compounds by donating hydrogen atoms, resulting in a change in the color of DPPH (purple to yellow) and the absorbance is measured at a wavelength of 516 nm (Robby Mahardika & Occa Roanisca, 2018). The concentration of the solution used in the test was five variations, namely 1000, 2000, 3000, 4000, and 5000 ppm.

Based on the DPPH radical inhibition test, the IC50 value will be obtained. The crude of FWKVV has an IC50 value of 4.2 mg/mL. The test results showed that the FWKVV crude had antioxidant activity, but was not more active than the isolated FWKVV compound. This is evidenced by the IC50 FWKVV value of the isolation results, which is greater, namely 0.85 mg/mL (He et al., 2019) compared to the IC50 FWKVV value as a result of the synthesis. This difference is since the synthesized FWKVV crud is not yet pure so that there are still some compounds or impurities that affect the antioxidant activity of FWKVV.

CONCLUSION

FWKVV pentapeptide compounds were successfully synthesized using the solid phase peptide synthesis method (SPPS) with a crud mass of 148.8 mg. FWKVV crude has antioxidant activity against DPPH radicals with an IC_{50} value of 4.2 mg/mL.

Anknowledgement

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