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Synthesizing Cylic Peptides with Antioxidant Properties using Solid Phase Peptide Synthesis as an Alternative to Natural Product Isolation from Pork

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Abstract.

Tetrapeptides Ser-Leu-Tyr-Ala and Tyr-Leu-Tyr-Ala are two derivates that have been successfully isolated from the pork and synthesized using Solid Phase Peptide Synthesis (SPPS) method. Tetrapeptide Gly-Ala-Trp-Ala has also been successfully isolated from the Sardinella aurita fish found in Atlantic Ocean using SPPS. This study aims to synthesize tetrapeptide Ser-Leu-Tyr-Ala, Tyr-Leu-Tyr-Ala and Gly-Ala-Trp-Leu using the SPPS method, and to find the antioxidant properties of the synthesized tetrapeptides using DPPH test. The three tetrapeptides have been synthesized using 2-chlorotritilchloride resin as solid phase, Fmoc group protection, and coupling reagent HBTU/HOBt. HR-TOF-MS with m/z was [M+H]+ 453,23 and [2M+H]+ 905,49 for Ser-Leu-Tyr-Ala, [M+H]+114,61 for Tyr-Leu-Tyr-Ala and [M+H]+ 446,23 for Gly-Ala-Trp-Leu. The antioxidant properties of Ser-Leu-Tyr- Ala had an IC50 value of 11130,04 mg/ml, while the antioxidant properties of Tyr-Leu-Tyr-Ala had an IC50 value of 4319,522 mg/ml.

Keywords: solid phase peptide synthesis, antioxidant

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1. INTRODUCTION

Synthesis of organic compounds is one method to obtain chemical compounds that have certain activities other than through isolation methods from natural materials. The advantages of synthesis of organic compounds are that they can increase the efficiency and effectiveness of a process to obtain compounds, shorten time, save solvents, replace natural materials that were difficult to obtain, replace natural materials from prohibited (not *halal*) sources and increase the bioactivity of the resulting compounds [1]. Isolation of natural material compounds that have biological activities has been done extensively. However, the availability of the compounds is very limited, or the raw materials is difficult to obtain because they are available in remote locations. In addition,

there are many chemical compounds that have biological activity, but are originated from animals – for example from pigs – which cannot be consumed by Muslims.

Peptidomimetics is a field of chemistry that aims to improve the biological and mechanical properties of native proteins to increase bio-availability, bio-stability, bio-efficiency and bio-selectivity. Small protein-like molecules, designed to mimic a peptide, typically arise from structural modification of an existing peptide to enhance its stability and biological activity [2].

Based on the physiological properties of proteins, peptide-based therapeutics have received enormous attention from pharmaceutical companies due to their high potency, bio-reactivity, specificity, safety, and higher overall success rates. Peptides can be formulated to provide therapeutic agents which were stable and had effective bio-availability.

Peptides typically offer low toxicity and high specificity, and demonstrate fewer toxicology issues than other small- molecule drugs. Protein drugs also had chemical and biological diversity, low drug-drug interaction, low accumulation in organs and tissues, and low non-specific binding to non-target receptors [3,4].

Antioxidants are compounds that act as donor electrons or reducants that are able to inhibit, prevent or inactivate the development of oxidation reactions by preventing the formation of radicals. Antioxidants are important parameter for monitoring a person's health. The human body has an antioxidant system to ward off free radical reactivation radicals, which are continuously self-formed by the body [5,6].

Free radicals are highly reactive molecules because they have electrons that do not pair in their outer orbitals so that they can react with the body's cell molecules by binding to the electrons of those cell molecules [7]. Free radicals are produced continuously during normal metabolic processes, considered to be the cause of damage to the functioning of the body's cells that eventually trigger the onset of degenerative diseases. Free radical attacks on surrounding molecules will cause a chain reaction, which then results in new radical compounds. The impact of reactivity of free radical compounds ranging from cell or tissue damage, autoimmune diseases, degenerative diseases, to cancer. The risk of the disease could be reduced by the use of antioxidant compounds.

Previous research has shown that pork has antioxidant activity from tetrapeptide Ser-Leu-Tyr-Ala and Tyr-Leu- Tyr-Ala. However, peptides originated from pork are forbidden for Muslim. Another high antioxidant activity is also found from tetrapeptide Gly-Ala-Trp-Ala that is isolated from the fish *Sardinella aurita* from Atlantic Ocean that hard to catch. Therefore, alternatives should be sought to obtain peptide compounds with

the same sequence of amino acids that has biological activity in the form of the same antioxidants even tend to be better. Previous research have shown that the sequence of amino acids that has antioxidant activity is linear tetrapeptide and based on the literature it is known that linear peptides are usually unstable in vivo because they easily experience degradation reactions by enzymes in the digestive system. This could reduce effectiveness in some cases. To improve its stability it is necessary to do the cyclication [8,9].

This research aims to develop a generic, metathesis-driven process to synthesize those tetrapeptide, and modify the amino acid residue. The synthesis of three tetrapeptides (Ser-Leu-Tyr-Ala as tetrapeptide 1, Tyr-Leu-Tyr-Ala as tetrapeptide 2 and Gly-Ala-Trp-Leu – modification from the natural product Gly-Ala-Trp-Ala - as tetrapetide 3) are carried out by the method of synthesis of solid phase peptides (SPPS) due to the shorter synthesis time compared to the use of liquid phases, and purification can be done in the final stages only. Hence it can be concluded that the SPPS method is easier and faster [10].

In the SPPS method, Fmoc protective group strategies are used in unstable amino groups in alkaline atmospheres, and protective group protection strategies on side chains and the use of 2-chlorloratilchloride resins are chosen because they are unstable against acids [11]. The coupling reagents used in this study are *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*',*N*'- tetramethyluronium hexafluorophosphate (HBTU) / *N*-Hydroxybenzotriazole (HOBt) combinations that belong to the benzotriazole group which can make the amine group more reactive. The reagent serves to give an alkaline atmosphere to amino acid reactions which could interfere with the stability of amino acids so as to push the H^+ ions off and activate the carboxylic group so that the amine group in the next amino acid could react with the carboxylic group. This reagent can increase the reactivity of the amine group so that it was easier to react with subsequent amino acids.

Tetrapeptide synthesis products obtained are tested for purity using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC), then characterized using a mass spectrometer and tested its antioxidant activity using 1,1-Diphenyl-2-picrylhidrazil (DPPH) method. The synthesis of these tetrapeptide compounds is something that has never been done. This research is expected to provide information on new tetrapeptide compounds with antioxidant potential as well as know the role of amino acids against antioxidant activity.

2. MATERIALS, TOOLS AND METHODS

2.1. Materials

The materials used were amino acids Fmoc-Alanine-OH, Fmoc-Tyrosine(tBu)-OH, Fmoc-Leucine-OH, Fmoc- Serine(tBu)-OH, Fmoc-Tryptophan(Boc)-OH and Fmoc-Glycine-OH, resin 2-chlorotritilchloride, dichloromethane (DCM) solvent, dimethylformamide (DMF), *O*-(Benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HBTU) reagent, *N*-Hydroxybenzotriazole (HOBt), *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*- tetramethyluronium hexafluorophosphate (HATU), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*-Diisopropylethylamine (DIPEA), Trifluoroacetate (TFA), 1,8-

Diazabicyclo[5.4.0]undec-7-ene (DBU), chlorranyl solution 2%, ethanol, methanol, acetaldehide, acetonitril, piperidine, chloroform, 1,1-Diphenyl-2-picrylhidrazil (DPPH) and Vitamin C, aquadest.

2.2. Tools

The tools used in the study were SPPS tubes, freeze dryers, rotary evaporators, rotary suspension mixers, compressors, RP-HPLC analytics, mass spectrophotometers, UV-Vis spectrophotometers, hot plate stirrers, sonicators, analytical balance sheets, magnetic stirrer, desicator, eppendorf tubes and glassware commonly used in laboratories.

2.3. Synthesis Procedure

2.3.1. Coupling of the First Amino Acid Fmoc-Ala-OH for Tetrapeptide 1 and to, and Fmoc-Leu-OH for Tetrapeptide 3 to the Each Resin

A total of 0.3 g of 2-chlorotritilchloride resin was placed in the reactor, then added 5 mL DCM, then rotated in the SPPS tube using rotary suspension mixer for 10 minutes then dried using R-Compressor. Then into the each vial prepared Fmoc-Ala-OH (140.0985 mg) for tetrapeptide 1 and to and Fmoc-Leu-OH (159.03 mg), DCM (5 mL) and DIPEA (153.1 µL) then sonicated for 5 minutes and inserted into a SPPS tube containing resin and stirred using rotary suspension mixer for 4 hours at room temperature. The resin was filtered, then washed with DMF and DCM, then dried using R-Compressor. The dry resin was taken slightly for chloranil testing and observed discoloration to see if the coupling of amino acid C-terminal had been formed, then carried out resin loading.

To calculate the loading value of the resin, 0.6 mg Fmoc-Ala-OH-dry resin was weighed and added 2 mL piperidin 20% in DMF ad 10 mL in measuring glass, keep

for 1 hour, measured its absorbance with UV spectrophotometer at wavelength 290 nm.

Fmoc protecting group was removed using 10% DBU in DMF (8 mL), the mixture was stirred for 15 seconds. Furthermore, the resin was filtered and washed using DMF and DCM. Fmoc protecting group release control was performed using chlorranyl test. NH2-Ala-OH-resin and NH2-Leu-OH-resin are constructed and ready for the second coupling.

2.3.2. Second Amino Acid Coupling (Fmoc- Tyr(tBu)-OH for Tetrapeptide 1 and 2, and Fmoc-Trp(Boc)-OH for Tetrapeptide 3)

On the prepared vial, for tetrapeptide 1, Fmoc- Tyr(tBu)-OH **(**237.998 mg) then added with HBTU (196.4325 mg), HOBt (69.9854 mg), and DIPEA (176.1711 µL) dissolved in 5 mL DCM, the solution was then sonicated for 5 minutes. For tetrapeptide 2, Fmoc- Tyr(tBu)-OH **(**157.6287 mg) then added with HBTU (130.0994 mg), HOBt 46.3521 mg), and DIPEA (116.681 µL) dissolved in 5 mL DCM, the solution was then sonicated for 5 minutes. The solution was inserted into an SPPS tube containing dry resin peptides (NH2-Ala-OH-resin) and stirred for 24 hours on a rotary suspension mixer to produce Fmoc-Tyr(tBu)-Ala-resin for tetrapeptide 1 and 2. Meanwhile, for tetrapeptide 3, the prepared vial Fmoc- Trp(Boc)-OH **(**158.092 mg) then added with HBTU (140.592), HOBt (50.461 mg), and DIPEA (126 µL) dissolved in 4 mL DCM, the solution was then sonicated for 5 minutes. The solution was inserted into an SPPS tube containing dry resin peptides (NH2-Ala-OH-resin) and stirred for 4 hours on a rotary suspension mixer to produce Fmoc-Trp(Boc)-Ala-resin. The resin was filtered and washed with DMF and DCM then dried. Control of the coupling of the second amino acid was carried out using a chlorranyl test.

Fmoc protecting group for each peptide was removed using 10% DBU in DMF (8 mL), the solution was schoed for 15 seconds. Furthermore the resin was filtered and washed using DMF and DCM. Fmoc protecting group release control was performed using chlorranyl test. NH2-Tyr(tBu)-Ala-resin and NH2-Trp(Boc)-Leu-resin is constructed and ready for the third coupling.

2.3.3. Third Amino Acid Coupling (Fmoc- Leu-OH for Tetrapeptide 1 and 2, and Fmoc-Ala-OH for Tetrapeptide 3)

On the prepared vial, for tetrapeptide 1, Fmoc-Leu-OH **(**183.048 mg) then added with HBTU (196.4325 mg), HOBt (69.9854 mg), and DIPEA (176.1711 µL) dissolved in 5 mL DCM, the solution was then sonicated for 5 minutes. For tetrapeptide 2, Fmoc-Leu-OH **(**121.235 mg) then added with HBTU (130.0994 mg), HOBt (46.3521

mg), and DIPEA (116.681 µL) dissolved in 5 mL DCM, the solution was then sonicated for 5 minutes. The solution was inserted into an SPPS tube containing dry resin peptides (NH2-Tyr-(tBu)-Ala-OH-resin) and stirred for 24 hours on a rotary suspension mixer to produce Fmoc-Leu-Tyr(tBu)-Ala-resin for tetrapeptide 1 and 2. Meanwhile, for tetrapeptide 3, the prepared vial Fmoc- Ala-OH **(**115.413 mg) then added with HBTU (140.592), HOBt (50.461 mg), and DIPEA (126 µL) dissolved in 4 mL DCM, the solution was then sonicated for 5 minutes. The solution was inserted into an SPPS tube containing dry resin peptides (NH2-Trp(Boc)-Ala-OH-resin) and stirred for 4 hours on a rotary suspension mixer to produce Fmoc-Ala-Trp(Boc)-Ala-resin. The resin was filtered and washed with DMF and DCM then dried. Control of the coupling of the second amino acid was carried out using a chlorranyl test.

Fmoc protecting group for each peptide was removed using 10% DBU in DMF (8 mL), the solution was schoed for 15 seconds. Furthermore the resin was filtered and washed using DMF and DCM. Fmoc protecting group release control was performed using chlorranyl test. NH2-Leu-Tyr(tBu)-Ala-resin and NH2-Ala-Trp(Boc)-Leu-resin is constructed and ready for the third coupling.

2.3.4. Fourth Amino Acid Coupling (Fmoc-Ser(tBu)-OH for Tetrapeptide 1, Fmoc-Tyr(tBu)-OH for Tetrapeptide 2, and Fmoc-Gly-OH for Tetrapeptide 3)

On the prepared vial, for tetrapeptide 1, Fmoc-Ser(tBu)-OH **(**198.6079 mg) then added with HBTU (196.4325 mg), HOBt (69.9854 mg), and DIPEA (176.1711 µL) dissolved in 5 mL DCM, the solution was then sonicated for 5 minutes. For tetrapeptide 2, Fmoc-Tyr(tBu)-OH **(**157.6287 mg) then added with HBTU (130.0994 mg), HOBt (46.3521 mg), and DIPEA (116.681 µL) dissolved in 5 mL DCM, the solution was then sonicated for 5 minutes. The solution was inserted into an SPPS tube containing dry resin peptides (NH2-Leu-Tyr-(tBu)-Ala-OH-resin) and stirred for 24 hours on a rotary suspension mixer to produce Fmoc-Ser(tBu)-Leu-Tyr(tBu)-Ala-resin for tetrapeptide 1 and Fmoc-Tyr(tBu)-Leu-Tyr(tBu)-Ala-resin for tetrapeptide 2. Meanwhile, for tetrapeptide 3, the prepared

vial Fmoc- Gly-OH **(**110.216 mg) then added with HBTU (140.592), HOBt (50.461 mg), and DIPEA (126 µL) dissolved in 4 mL DCM, the solution was then sonicated for 5 minutes. The solution was inserted into an SPPS tube containing dry resin peptides (NH2-Ala-Trp(Boc)-Ala-OH-resin) and stirred for 4 hours on a rotary suspension mixer to produce Fmoc- Gly-Ala-Trp(Boc)-Ala-resin. The resin was filtered and washed with DMF and DCM then dried. Control of the coupling of the second amino acid was carried out using a chlorranyl test.

Fmoc protecting group for each tetrapeptide was removed using 10% DBU in DMF (8 mL), the solution was schoed for 15 seconds. Furthermore the resin was filtered and washed using DMF and DCM. Fmoc protecting group release control was performed using chlorranyl test. NH2-Leu-Tyr(tBu)-Ala-resin and NH2-Ala-Trp(Boc)-Leu-resin is constructed and ready for removed the resin from the tetrapeptide.

2.3.5. Removed the Resin from the Tetrapeptide

The three tetrapeptide resin NH2-Ser(tBu)-Leu-Tyr(tBu)-Ala-resin, NH2-Tyr(tBu)-Leu-Tyr(tBu)-Ala-resin and NH2-Gly-Ala-Trp(Boc)-Leu-resin was added with 5 mL solution 95% TFA in water. The mixture was schoed for 10 minutes using a rotary suspension mixer at room temperature. The release of peptides from resin was characterized by the change in the color of the resin into red. This stage was done twice. Resin was filtered and washed with (2 x 5 mL) 95% TFA in water. Filtrate was tightened with rotary evaporator. Synthesized linear tetrapeptides were further analyzed using RP-HPLC analytics, purified and characterized using mass spectrophotometers.

2.3.6. Chlorranyl Test

The tetrapeptides were washed with 3 mL DCM, 3 mL DMF and 3 mL DCM, then dried using R-Compressor until they become dry resin. Then a few grains of resin were taken and then put in an eppendorf tube and added a 2% acealdehyde solution of 40 µL and a 2% chlorranyl solution of 40 µL and then stirred and observed discoloration that occurred in resin beads.

2.3.7. Evaporation Process

The tetrapeptide solution was inserted into a partial vial and then evaporated until dry, re-added tetrapeptide solution and then evaporated again. After that the vial was

washed with DMF, done twice washed, then put into the vial and then evaporated. Furthermore, the evaporation results were put into the decicator.

2.3.8. Analysing with RP-HPLC (Reverse Phase High Performance Liquid Chromatography)

The sample was taken 1 mg and in the sample added 1 mL of methanol in the eppendorf tube, the solution was analyzed using RP-HPLC with the C18 reverse phase column system (250 mm×4.6 mm), eluen A 7.75% acetone and TFA while eluen B miliq and TFA 0.1% for 20 minutes, with a flow rate of 1 mL/min with a temperature of 40˚C and seen at wavelengths of 210 nm and 240 nm.

2.3.9. Mass Spectrophotometer

The sample was taken 1 mg and in the sample added 1 mL of methanol in the eppendorf tube then the sample solution was analyzed using a mass spectrophotometer with observed peaks of its fragments.

2.3.10. Antioxidant Activity Analysis with DPPH Method

0.0039 grams of DPPH was added by 18.75 mL of methanol. Then in the tetrapeptide sample added 3ml methanol, after each the compound was sonicated until dissolved. Then a tetrapeptide sample was inserted into a 10 mL measuring tube, add methanol to the boundary mark and stirred until homogeneous. Furthermore, it was inserted into the eppendorf tube as much as 1.5 mL until it runs out. Then centrifuged for 15 minutes at a speed of 1000 rpm at a temperature of 4^{o}C. Blank solution was made using methanol 800 µL then added 200 µl DPPH into the test tube for 30 minutes in dark room. Then measure the absorbance using spectrophotometer at λ 517 nm.

Then dilution of tetrapeptide samples at 8000 ppm was conducted with several different concentrations (0.1700, 3400, 5100,6800 and 8500 ppm) as much as 1 mL (including 200 µL DPPH in them), then added methanol, and keep at room temperature for 30 minutes in the dark room and observed discoloration that occurred. Mixed absorbance was measured at a wavelength of 517 nm. Blank (solvent) was prepared with the same treatment, then calculated the % value of DPPH inhibition.

3. RESULTS AND DISCUSSIONS

Three tetrapeptides Ser-Leu-Tyr-Ala, Tyr-Leu-Tyr-Ala and Gly-Ala-Trp-Leu had been successfully synthesized using solid phase synthesis methods using 2-chlorotritilchloride resin with Fmoc strategy and HBTU/HOBt coupling reagents. On the synthesis of tetrapeptide compounds, the first amino acid strapped to resins was an Alanine amino acid. Alanin amino acids had methyl side groups unprotected in C-terminals and amino groups in N-terminals protected by fmoc temporary protecting group. The extension of the peptide chain was done from the direction of C- terminal to N-terminal.

Conditioning using DCM solvents aims to adjust the atmosphere of the SPPS tube during the tetrapeptide synthesis process, resulting in an optimum and stable process. Washing was done to reduce other compounds that will be able to interfere with the reaction process. Before the process of synthesis of tetrapeptide begins, the resin 2-chlorrotritil chloride was calculated by comparison against the first amino acid with DIPEA then the resin was weighed 0.3 grams then inserted into the SPPS tube and added DCM solvent. This process was carried out to develop the resin so that the residue on the resin could be used and the active side of the resin could be more open and could optimize the coupling of the active side between the resin and the amino acids to be reacted at a later stage.

The coupling of Alanine amino acids in resins 2-chlorotritil chloride began by dissolving Fmoc-Ala-OH and Fmoc- Leu-OH with DCM and DIPEA. The use of DIPEA serves as an organic base and also as a reagent in the process of binding amino acids with resin. Furthermore, the reactor tube was inserted into the rotary suspension mixer for 24 hours of shaking. Shuffling was done to accelerate the distribution of molecules so as to enlarge the surface area of contact between amino acid molecules with the reagents. Furthermore, chlorranyl testing was carried out by the addition of acetaldehyde solution and chlorranyl solution in some dry resin beads then stirred. The absence of discoloration could be interpreted as the absence of a resin-free NH/NH2 groups.

Fmoc-Ala-OH's first amino acid coupling reaction to 2-chlorotritil chloride resin does not involve activation of carboxyl groups to prevent unwanted rasemization reactions. The reaction process that occurs was an alkaline acid reaction where hydrogen in the carboxy group in Fmoc-Ala-OH was taken by the base forms a carboxylic nucleophile. Then the nucleophiles formed attack and replace chloride atoms in quartile carbon atoms found in 2-chloromethyl chloride resin, then substitutes chloride atoms and form

bonds with resins through unimoleculic nucleophilic substitution reactions (SN1) thus forming Fmoc-Ala-resin [12].

Based on the reaction that occurs, the first reaction stage in was through the base acid reaction between Fmoc-Ala- OH and the alkaline DIPEA as a reagent that forms carboxy ions. Carboxyl ions then attack chloride ions on carbon atoms of 2-chloromethyl chloride resin. Thus, the first amino acid Fmoc-Ala-OH and Fmoc-Leu-OH was bound to resin. The chloride atom with the mark was an excellent go cluster, as it was influenced by three benzene clusters that could stabilize the intermediate. Chloride atoms could be removed by even carboxy weak nucleophiles [12].

The coupling reaction between the amino acid Fmoc-Ala-OH and resin was carried out for 24 hours while the amono acid Fmoc-Leu-OH and resin was carried out for 4 hours to optimize a reaction because not too many amino acids were bound to the resin and it will interfere during the extension of the peptide chain which will make it difficult for the amino acids that fall protectively to reach the free amino group.

After the first coupling of amino acids in resins, it was necessary to do resin capping. The purpose of capping resin was to close the active side of the resin 2-chlorotritil chloride so that the next amino acid does not bond with the active side of the resin that does not bond with the first amino acid (Figure 1). Resin capping was done by adding a mixture of dichloromethane:methanol:DIPEA with a ratio of 80:15:5 to the resin.

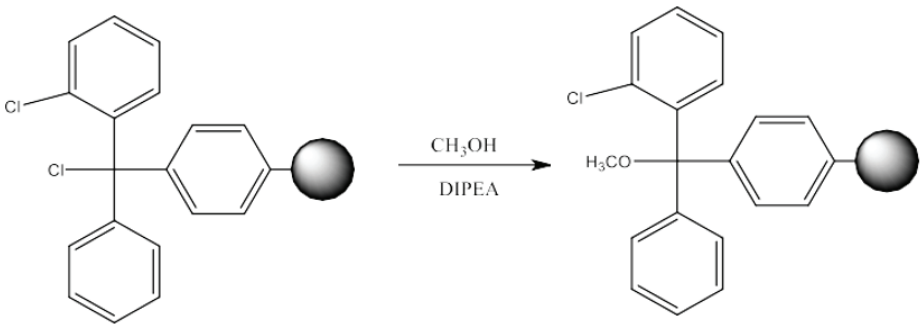

Figure 1: Reaction of Capping Resin Using Methanol and DIPEA [11].

After capping the resin, the release of temporary Fmoc protecting groups (deprotection) was carried out. The release of the Fmoc protecting group aimed to free the active side of the amino acid first so that it could interact with subsequent amino acids through a coupling reaction. Fmoc protecting group was a protecting group that was lame against bases, so when deprotection was used alkaline solution, in this study used a 10% DBU solution in DMF. The release of the Fmoc protecting group begins with the attack of hydrogen atoms on the fluorine group found in Fmoc by piperidins thus forming the intermediate compound cyclopentadiene [11]. The success of deprotection could be

proven through chlorranyl test. Positive test of deprotection success was characterized by the discoloration of resin from yellow to green.

Once a free amino group was produce, a coupling was performed with a second amino acid. The preparation of linear peptide fragments was done by adding the next amino acid to the resin. Amino acids form peptide bonds through condensation bonds between amino groups and carboxylic groups activated by coupling reagents. Solid phase peptide synthesis was a heterogeneous reaction so that the interaction between molecules in the solid phase and solution phase becomes limited. Therefore, the increase in the amount of amino acids was done 2 times as much as it was supposed to increase the concentration and rate of reaction. In general, amino acids were added as much as 2 equivalents of the loading value of resin.

The second amino acid residue, Fmoc-Tyr(tBu)-OH for tetrapeptide 1 and 2, and Fmoc-Trp(Boc)-OH for tetrapeptide 3 was performed using a combination of HBTU/HOBt coupling reagents. The second residue of amino acids was performed by dissolving 2 equivalents of Fmoc-amino acids, 2 equivalents of HBTU, 2 equivalents of HOBt and 8 of the base equivalents of DIPEA in DCM solvents. The coupling reagent was sonicated for 5 minutes so that the reagent dissolves perfectly and could improve reaction kinetics. The second residue of amino acids was carried out for 24 hours so that not too many amino acids were bound to the resin and it will interfere with the extension of the peptide chain which will make it difficult for the protective-falling amino acids to reach the free-amino group. The success of the second amino acid coupling was shown by a chlorine test characterized by the no discoloration of resin. After the second amino acid coupling was performed, fmoc protecting group was removed using a 10% DBU base solution in DMF for 15 seconds. The next stage was the third and fourth amino acid residue coupling starting with the third amino acid residue with fmoc coupling and deprotection sequence followed by the fourth amino acid residue with the same procedure until the tetrapeptide was constructed in the resin.

After tetrapeptide target was constructed, then resin was removed from the tetrapeptides. The removed of target peptides from resins was carried out using a 95% TFA acid solution in water. TFA was 95% used because in addition to remove target peptides from resins, high concentrations of TFA reagents were also intended to remove tBu side

chain protecting groups. Water acts as a cation scavenger that forms during the peptide release reaction. Brownish- red filtrate formed after the removed of peptides from resin was accommodated in vials and then tightened using rotary evaporators.

Peptides filtrate that had been tightened using rotary evaporators and produce tetrapeptides brownish-yellow jam as much as 145.5 mg for tetrapeptide 1, 173.3 mg

for tetrapeptide 2 and 110.21 mg for tetrapeptide 3. Crude of the tetrapeptides then analyzed using RP-HPLC analytics using reverse phase columns illusionized using eluen gradien 7.75% acetone:air with 1% TFA buffer for 20 minutes, flow rate of 1mL/min using PDA detector with wavelength detection of 210 nm and 240 nm. The results of RP-HPLC analysis showed that the synthesized tetrapeptide was not pure, indicated by the absence of several peaks suspected the protecting groups still occure, so it is necessary to deprotect again.

Furthermore, the characterization of tetrapeptides using a mass spectrophotometer indicates the present of peak ions of the terapeptide 1, Ser-Leu-Tyr-Ala molecules at m/z $[M+H]^+$ 453.23 and m/z $[2M+H]^+$ 905.49, tetrapeptide 2, Tyr-Leu-Tyr-Ala molecules at m/z $[M+H]^+$ 528.61 and tetrapeptide 3, Gly-Ala-Trp-Leu molecules at m/z $[M+H]^+$ 446.23.

Those three tetrapeptide compounds are then tested for antioxidant activity using DPPH method. For tetrapeptide 1, Ser-Leu-Tyr-Ala, antioxidant tests is conducted using a microscale with concentration variations of 0, 2300, 4600, 6900, 9200 and 11500 ppm. The solvent stored for 30 minutes to make a reaction between free radical DPPH and the tetrapeptide. The result for tetrapeptide 1 shows that the number of IC50 is 11130,04 µg/mL. That means the tetrapeptide Ser-Leu-Tyr-Ala has a weak antioxidant activity because the IC50>150 µg/mL. For tetrapeptide 2, Tyr- Leu-Tyr-Ala, antioxidant tests is conducted using a microscale with concentration variations of 0, 1600, 3200, 4800, 6400 and 8000 ppm. The solvent stored for 30 minutes to make a reaction between free radical DPPH and the tetrapeptide. The result for tetrapeptide 2 shows that the number of IC50 is 4319.522 µg/mL. That means the tetrapeptide Tyr-Leu-Tyr-Ala also has a weak antioxidant activity because the IC50>150 µg/mL. Antioxidant activity of tetrapeptide 3, Gly-Ala-Trp-Leu was not conducted because the tetrapeptide is unpure.

4. CONCLUSION

Three tetrapeptides compounds, Ser-Leu-Tyr-Ala as tetrapeptide 1, Tyr-Leu-Tyr-Ala as tetrapeptide 2 and Gly- Ala-Trp-Leu had been successfully synthesized using the SPPS method resulting products as much as 145.5 mg for tetrapeptide 1, 173.3 mg for tetrapeptide 2 and 110.21 mg for tetrapeptide 3.

The result of characterization with HR-TOF-MS which gives a value of *m/z* $[M+H]^+$ of the terapeptide 1, Ser-Leu- Tyr-Ala molecules at m/z $[M+H]^+$ 453.23 and m/z $[2M+H]^+$ 905.49, tetrapeptide 2, Tyr-Leu-Tyr-Ala molecules at m/z $[M+H]^+$ 528.61 and tetrapeptide 3, Gly-Ala-Trp-Leu molecules at m/z $[M+H]^+$ 446.23.

Tetrapeptide Ser-Leu-Tyr-Ala and Tyr-Leu-Tyr-Ala have weak antioxidant activity with the IC50>150 µg/mL.

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