E-ISSN : 2355-9926
http://rjls.ub.ac.id

Antioxidant Activity of Purified Active Peptide Derived from *Spirulina platensis* Enzymatic Hydrolysates

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ABSTRACT

The aim of this study is to isolate the antioxidative peptide from *Spirulina platensis.* Peptide was obtained by proteolytic digestion, ultrafiltration, fractionation by RP-HPLC, identified by LC-MS/MS—MASCOT Distiller and measured its antioxidant activity by DPPH (2.2-Diphenyl-1-picrylhydrazyl) assay. Results showed that thermolysin was the most effective enzyme to digest this algae. The active peptide Phe-Ser-Glu-Ser-Ser-Ala-Pro-Glu-Gln-His-Tyr (m/z 1281.51) was identified and synthetized, which exhibited 45.98 ± 1.7% at concentration 128.15 µg/mL. Therefore, *S. platensis* is indicated as a potential therapeutic source for combating oxidative stress.

Keywords: Antioxidant; Cyanobacteria; DPPH; LC-MS; RP-HPLC.

INTRODUCTION

A free radical can be defined as an existence of uninvited independent molecular species which contain an unpaired electron on its orbital (Lobo et al., 2010). Many radicals, including Reactive Oxygen Species (ROS), are unstable and highly reactive. They could behave as oxidants or reductants, capable to move in any biologically relevant molecules and attack these essential macromolecules, leading to cell damage and homeostatic disturbance (Cheeseman and Slater., 1993).

The imbalance within the body, generated by the increasing of free radical production, leading to oxidative stress condition, resulted in the damage of a wide range of molecular important species including DNA, protein, and lipid (Rao et al., 2006). The excess of ROS were also postulated to be the initiation of several human diseases, including inflammation, cancer, aging process and cardiovascular diseases (Ramalingam et al., 2016). These oxidatively induced injury is ubiquitous (Cadet et al., 2017) and all organic molecules are very susceptible to oxidative damage from reactive species. Therefore, antioxidant compounds are urgently needed to reduce any substantial damage which could be occur daily (Cadet and Davies., 2017).

According to Marine and Fisheries Statistics in Indonesia 2015 report, the total fisheries and aquaculture production in Indonesia increased from 11.66 million tonnes in 2010 to 20.8 million tonnes in 2015 (BPS KKP., 2015). Freshwater and marine organisms have an enormous bioactive molecules which already have known could be developed as drugs and food supplements (Glaser and Mayer., 2009; Balitbang KKP., 2014). Several algae, including macro and microalgae, contain high protein as a source of bioactive peptides (Fan et al., 2014). In fact, they also have been recognized as potential natural antioxidants due to their various secondary

metabolites coupled with antioxidant activity (Ngo et al., 2011).

Spirulina platensis is a blue-green cyanobacteria which classified into freshwater microalgae. It is generally cultured in Asia, Africa, South America and usually grows almost all the year (Ferreira-Hermosillo et al., 2011). It contains 60-70% of proteins by weight and exhibits a wide range of biological properties, including antioxidant activity (Yu et al., 2016). In this study, the active peptide derived from *S. platensis* was isolated and examined its bioactivity as free-radical inhibitor using DPPH (2.2-Diphenyl-1-picrylhydrazyl) assay. Peptide was identified by LC-MS/MS after fractionated by RP-HPLC. Finally, the synthetic peptide was used to measure the peptide's bioactivity.

MATERIALS AND METHODS

Materials and Enzymatic Hydrolysates Preparation.

Crude powder of *S. platensis* supplied by Far East Microalgae Industries, Co., Ltd. (FEMICO) Company (Taipei, Taiwan). 2.2-Diphenyl-1-picrylhydrazyl (DPPH), BHT (Butylated hydroxytoluene), thermolysin, pepsin, trypsin and α-chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The molecular weight cut off (MWCO) 3 kDA cut off were obtained from Millipore (Bedford, MA, USA). All other chemicals were analytical grade.

The dried powder was initially dissolved (1:3) in 20% trichloroacetic acid (TCA) for 12 h in 4°C and centrifuged 4000 rpm for 10 minutes. The TCA was removed using acetone three times and the pellet was lyophyilized. The dried protein then was hydrolyzed by trypsin (37°C), α-chymotrypsin (37°C), pepsin (37°C) and thermolysin (60°C) for 16h (E/S=1/20). The reaction was stopped by heating the mixture in a boiling water for 10 min. The hydrolysate then was fractionated into < 3 kDa MWCO. The filtrate was collected and lyophilized.

Hydrolysate Separation by RP-HPLC.

The enzymatic hydrolysates of *S. platensis* was fractionated by reverse-phase high performance liquid chromatography system (Chromaster, Hitachi, Japan). Fractions were eluted with distilled water containing 5% ACN & 0.1% TFA (buffer A) and distilled water containing 95% ACN & 0.1% TFA (buffer B). 20µL of < 3 kDa hydrolysates was loaded at a flow rate of 1 ml/min. Absorbance of fractions was monitored at 214 nm. Hydrolysates was run for 60 min, collected every 5 minutes, and lyophilized.

DPPH Free Radical Scavenging Activity.

DPPH-free radical scavenging activity was measured according to the method described by Yu et al (2016) with some modification. Fresh DPPH solution containing 0.1 mM DPPH in purified methanol were prepared daily. Fractions were diluted in methanol. The mixture, which comprised of 50 µL samples and 150 µL DPPH solution in 96-well plate, was agitated and incubated for 90 min in the dark at room temperature. The absorbance was measured by ELISA at 517 nm and calculated as = [(ΔControl-ΔBlank) - (ΔSample-ΔBlank)]/(ΔControl-ΔBlank) x 100%. Methanol (50 µL) diluted in DPPH (150 µL) was used as blank, whereas 200 µL of purified methanol was used as control.

Identification of Bioactive Peptide Sequence.

The most effective fraction was identified in liquid-chromatography tandem mass-spectrometry (LCQ DECA XP MAX system) using an electrospray ionization (ESI) source (Thermo Scientific Inc., USA).

Sample was loaded at flow rate 200 µl/min and scan range 100-1600 m/z. The MS-MS spectra were submitted to Mascot Distiller v2.3.2.0 (Matrix Science, London, UK) to find potential peptide.

Verification of The Antioxidant Activity.

The antioxidant peptide was synthesized by solid-phase peptide synthesizer (CEM Microwave Technology Ltd., Buckingham, England). The purity of the synthetized peptide was verified as > 80% by using RP-HPLC. The synthetized peptide was diluted in distilled water to a concentration range from 3.85-128.15 µg/mL.

Statistical Analysis.

Data was expressed as mean ± standard deviation. Results was analyzed using one way ANOVA in SPSS v.16 software (Chicago, SPSS Inc) followed by Tukey's range post hoc test after standardized the data, with statistical significance was set at $p < 0.05$ in three replication. The IC_{50} of the antioxidant peptide was examined using GraphPad Prism v6.0 (La Jolla, GraphPad Software Inc).

RESULTS AND DISCUSSION

Antioxidant activities of *S. platensis* purified protein.

The relative-stable DPPH-free radical scavenging was measured the antioxidant potential of compound by donating a hydrogen atom or scavenging the free-radical (Kedare and Singh., 2011). As shown in **Figure 1**, all samples exhibited DPPH radical scavenging activity at different concentrations. The observed scavenging activity of purified protein enhanced significantly as the concentration of the protein increased from 0.05 to 1 mg/mL.

Specifically, as the concentration were increased from 0.2 to 0.5 mg/mL, the scavenging activity also increased from 7.98 ± 0.4% to 11.80 ± 0.3%. Subsequently, the activity was increased rapidly with a scavenging activity of 16.42 ± 0.6% observed when the concentration reached 1 mg/mL.

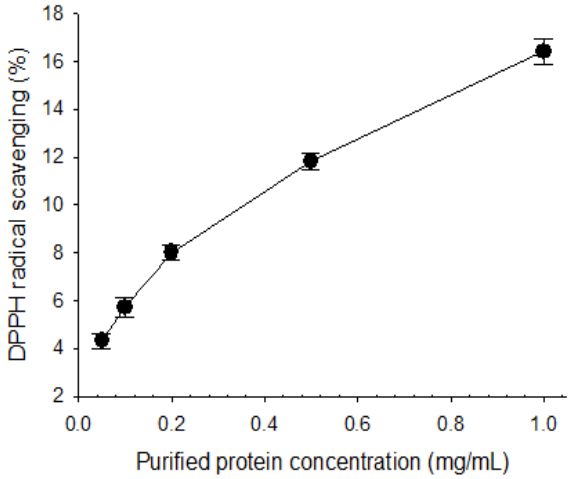

Figure 1. DPPH radical scavenging (%) of *Spirulina platensis* purified protein.

Antioxidant activities of *S. platensis* protein hydrolysates.

Generally, the scavenging activity of ultrafiltrated hydrolysates was significantly higher than without ultrafiltration or hydrolysis, which was indicated by the improvement of oxidation resistance compound by enzymatic hydrolysis (Yu et al., 2016). Therefore, the purified *S. platensis* protein in this study was directly digested by several proteolytic enzymes and filtrated to molecular weight cut off < 3 kDa. Several studies have previously reported that lower molecular weight shows higher free-radical scavenging activity due to their rich amino acid groups for donating electrons to DPPH radicals (Chi et al., 2015; Zhang et al., 2011). Moreover, Ngoh and Gan (2016) suggested that the peptide < 3 kDa from pinto bean protein hydrolysates exhibited the highest antioxidant activities than 100, 50, 30, and 10 kDa.

As shown in **Figure 2**, after digested by several proteolytic enzymes, thermolytic hydrolysate of *S. platensis* possessed the highest scavenging of DPPH radicals than other proteases, with the inhibition was 18.82 ± 1.7 mg/mL. This finding is in agreement with previous studies suggested that thermolysin is specifically catalyzes peptide bond containing hydrophobic and aromatic amino acid, which potential as antioxidant peptide (Keil., 1992).

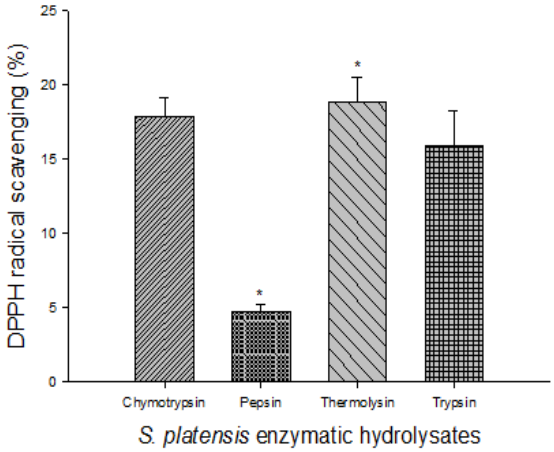

Figure 2. DPPH radical scavenging (%) of Spirulina platensis enzymatic hydrolysates, with the concentration 1 mg/mL. (*) symbol means significantly difference on p<0.05.

Fractionation and Purification of Antioxidant Peptides.

Reverse-Phase High Performance Liquid Chromatography was further employed to fractionate the antioxidant peptides and the *S. platensis* hydrolysate (1 mg/mL) was separated into 12 fraction (F1-F12; **Figure 3A**). Each fraction was collected, freeze-dried, and determined its antioxidant activity. As shown in **Figure 3B**, a clear difference was observed and fraction F4 exhibited the highest DPPH free radical scavenging activity with the inhibition 7.12 ± 0.28 mg/mL at concentration 83.33 µg/mL. Henceforth, this fraction was further characterized its amino acid sequence by mass spectrometry.

The fraction F4 of *S. platensis* thermolytic hydrolysate was further subjected to LC-MS/MS for peptide sequence identification. The amino acid sequence was identified as Phe-Ser-Glu-Ser-Ser-Ala-Pro-Glu-Gln-His-Tyr (FSESSAPEQHY / FY11) with m/z 1281.51 (the molecular weight = 1280.51 Da) (**Figure 4**). To confirm this result, peptide was synthesized chemically using Fmoc-protected amino acid synthesis. After examined its purity using RP-HPLC, the purified peptide value was 81.517%.

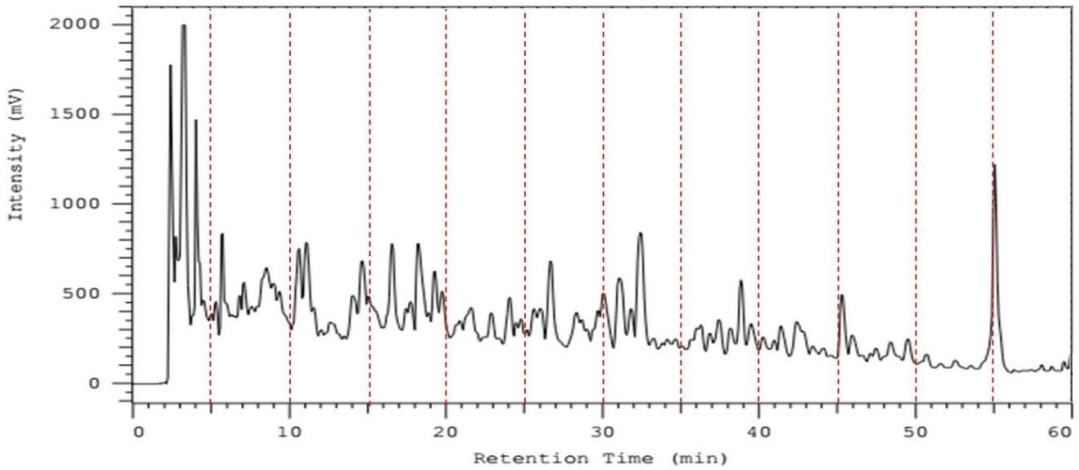

Figure 3A. Reverse-Phase HPLC separation of the selected pooled thermolysin enzymatic digestion.

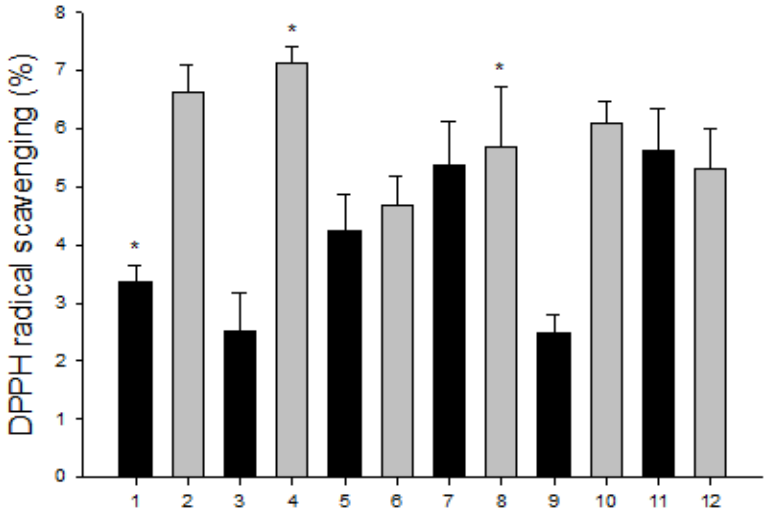

Figure 3B. DPPH radical scavenging (%) of Fraction F1-F12. (*) symbol means significantly difference on p<0.05.

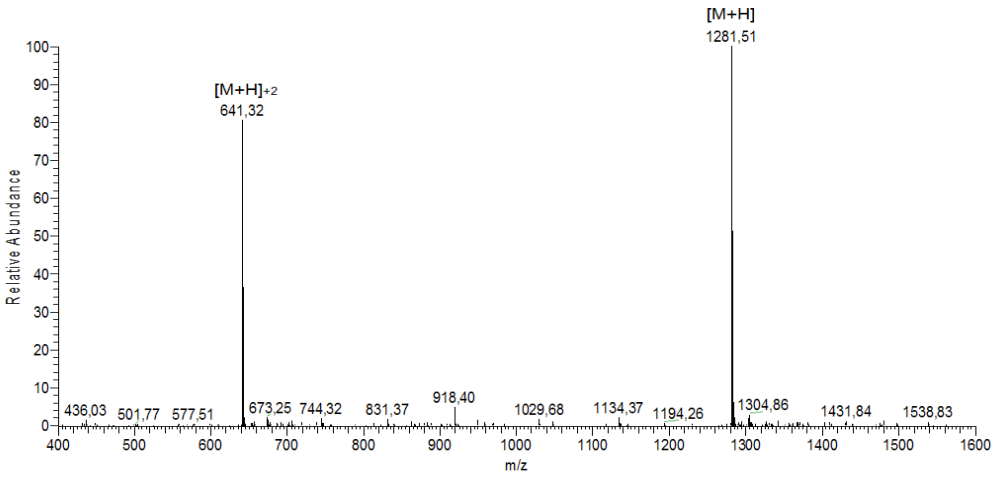

Figure 4. Identification of the antioxidant peptide in F4 by LC-MS/MS.

Antioxidant Activity of Purified Peptide.

Antioxidant enzymes are important for protecting the human body from the oxygen radicals destruction, thus it is important to investigate the antioxidant activity of purified peptide against free-radicals (Cai et al., 2015). After examined the antioxidant activity by assessing DPPH radical scavenging assay, the DPPH inhibition of FY11 peptide was 45.98 ± 1.7% at concentration 128.15 µg/mL, which was considered to possess an IC_{50} at the predicted concentration 171.47 µg/mL (**Figure 5**).

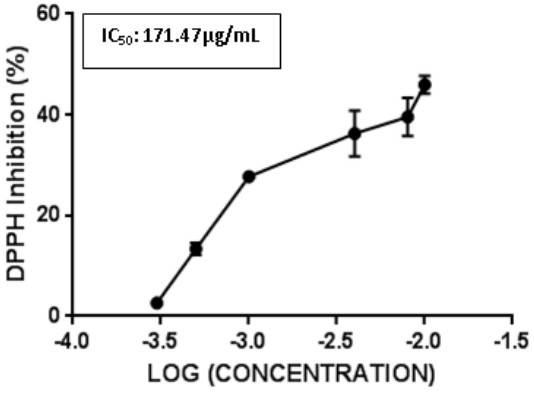

Figure 5. The predicted IC_{50} model of FY11 derived from *S. platensis* as DPPH free radical scavenging peptide.

Generally, the potential anti-oxidative peptide is highly influenced by their amino acid characteristics, which consists of 5-20 amino acids with the molecular weights are 400-2500 Da (Mora et al, 2014). In this study, the purified peptide contained 11 amino acids with the molecular weight 1280.51 Da. Peptides with lower molecular weight, 2-20 amino acids, assumed to cross the intestinal barrier more easily to produce biological effects (Kou et al., 2013). Similar to our results, peptide Val-Glu-Gly-Lys-Ser-Pro-Asn-Val (VEGKSPNV) from red scorpion fish *Scorpaena notata* using fungus penicilum digitatum enzyme possessed antioxidant IC_{50} at concentration 0.232 µg/mL. However, this peptide had shorter sequence thus showed better antioxidant potency (Aissaori, 2016). Together with this reason, peptide Met-Pro-Asp-Ala-His-Leu (MPDAHL) from egg white protein using trypsin protease was exhibited high antioxidant activity at IC_{50} 40.99 µg/mL (Liu et al., 2015). In addition, another potential antioxidant peptides from several natural products were also consists of 2-20 amino acids, such as microalgae (Kang et al., 2011; Ko et al., 2012; Power et al., 2012), macroalgae (Je et al., 2009; Wang et al., 2010); fish (Ngo et al., 2011), squid (Mendis et al., 2005) and soy (Moure et al., 2006).

As an antioxidative peptide, it is important to mark out that the amino acid sequence, especially containing hydrophobic and aromatic amino acids, possess high antioxidant activity, due to the presence of an indole/imidazole/pyrrolidine ring as an important proton donor to create a more stable products and terminate radical chain reactions (Torres-fuentes et al., 2015; Zou et al., 2016). Another study suggested that the neighboring amino acids residues and the three hydrophobic amino acids as the C-terminal, werealso influence free radical activity of peptides (Farvin et al., 2010; Kang et al., 2011; Elias et al., 2008). Peptide FY11 from *S. platensis* thermolytic hydrolysate in our study contained phenylalanine in the N-terminal position, alanine and proline in the middle as the neighboring residues and three hydrophobic amino acids (glycine-histidine-tyrosine) as the C-terminal position. It also contained two glutamic acids in the middle position, thus increased its antioxidant activity. In particular, the existance of imidazole and pyrrolidine ring in both histidine and proline are very important as hydrogen donor due to their unique structure, which act as hydroxyl radical scavengers (Kou et al., 2013; Girgih et al., 2014).

On the other hand, the antioxidative peptide Val-Glu-Cys-Ile-Gly-Pro-Asn-Arg-Pro-Glu-Phe (VECIGPNRPEF) obtained from a well—known microalgae *Chlorella vulgaris* digested with pepsin exhibited better IC_{50} inhibition, 0.756 µg/mL (Sheih et al., 2009). Although peptide contained the same number of sequence as FY11, it had more hydrophobic amino acid residues. The existance of sulfur-containing-amino acid within cysteine residue acted as stronger antioxidants, produce H_2O_2 in the presence of transition metal ions (Atmaca, 2004; Flora, 2009). Furthermore, this thiol antioxidant is able to protect the death of the cells by their proglutathione properties, rather than reactive oxygen direct scavenging (Kim et al., 2003; Sen et al., 1997). Based on QSAR method, cysteine was predicted as the most active antioxidant amino acid, especially in tripeptides structure (Tian et al., 2015).

The existence of two negatively charged glutamic acids in our peptide assumed to increase its antioxidant capability. The presence of excess electron on negative charged enhances its free radical quenching

activity (Zou et al., 2016). For instance, the rapeseed peptides using solid state fermentation method displayed high activities of scavenging free radicals, which exists glutamic acid (19.5%), lysine (7.6%) and proline (7.3%) as the dominant amino acids within sequences (He et al., 2012).

The purified peptide with various characteristics of chemical structure, such as small molecular weight, hydrophobicity, imidazole/pyrrolidine ring, the negatively charged of some residues, all show the influence of the antioxidant capacity FY11 peptide from *S. platensis* hydrolysate. Further investigation is required to confirm the antioxidant mechanisms, but this finding promotes the development for future research.

In conclusion, the antioxidative peptide FY11 derived from thermolysin hydrolysate of *S. platensis* was identified by LC-MS/MS. Peptide was synthetized and purified to confirm its bioactivity. Furthermore, the DPPH radical scavenging activity of the purified peptide (which reached 81.517%) was exhibited good antioxidant activity with an IC_{50} value was 171.47 µg/mL and assumed to have a moderate inhibition. Our findings suggest that *S. platensis* has beneficial health effects, especially prepared as an antioxidative peptides. These results show that the hydrolysate from *S. platensis* could be potentially used as functional foods with pharmaceutical functions associated with oxidative stress.

ACKNOWLEDGMENT

This project was funded by Far East Microalgae Industries Co., Ltd. (FEMICO). The first author would like to express her gratitude to double degree program (DIKTI-NPUST scholarship) of Brawijaya University (UB) and National Pingtung University of Science & Technology (NPUST) for supporting this research.

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