J. Microbiol. Biotechnol. (2016), 26(7), 1216–1223
http://dx.doi.org/10.4014/jmb.1601.01033

jmb

Purification and Identification of Antioxidant Peptides from Enzymatic Hydrolysate of *Spirulina platensis*

Jie Yu[1,2†], Yuanliang Hu[1,2†], Mingxiong Xue[3], Yaohao Dun[2], Shenao Li[2], Nan Peng[2], Yunxiang Liang[2,4], and Shumao Zhao[2,4*]

[1]*Hubei Key Laboratory of Edible Wild Plants Conservation & Utilization, College of Life Sciences, Hubei Normal University, Huangshi 435002, P.R. China*

[2]*State Key Laboratory of Agricultural Microbiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, P.R. China*

[3]*Beihai Shengbada Biotech Co., Ltd, Beihai 536000, P.R. China*

[4]*Hubei Collaborative Innovation Center for Industrial Fermentation, Wuhan 430068, P.R. China*

Received: January 15, 2016
Revised: April 6, 2016
Accepted: April 10, 2016

First published online
April 19, 2016

*Corresponding author
Phone: +86-27-87281040;
Fax: +86-27-87280670;
E-mail: shumiaozhao@mail.hzau.edu.cn

†These authors contributed equally to this work.

pISSN 1017-7825, eISSN 1738-8872

The aim of this study was to isolate antioxidant peptides from an enzymatic hydrolysate of *Spirulina platensis*. A novel antioxidant peptide was obtained by ultrafiltration, gel filtration chromatography, and reverse-phase high-performance liquid chromatography, with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay used to measure the antioxidant activity, and the sequence was determined to be Pro-Asn-Asn (343.15 Da) by electrospray ionization tandem mass spectrometry. This peptide was synthesized to confirm its antioxidant properties, and it exhibited 81.44 ± 0.43% DPPH scavenging activity at 100 μg/ml, which was similar to that of glutathione (82.63 ± 0.56%). Furthermore, the superoxide anion and hydroxyl free-radical scavenging activities and the SOD activity of the peptide were 47.84 ± 0.49%, 54.01 ± 0.82%, and 12.55 ± 0.75%, respectively, at 10 mg/ml. These results indicate that *S. platensis* is a good source of antioxidant peptides, and that its hydrolysate may have important applications in the pharmaceutical and food industries.

Keywords: Antioxidant peptide, free-radical scavenging activity, mass spectrometry, purification, *Spirulina platensis*

Introduction

Oxidation is an essential reaction in all living organisms. During the oxidative metabolic process, radicals (often referred to as free radicals) and reactive oxygen species are formed. Free radicals are atoms, molecules, or ions with unpaired electrons or an open-shell configuration, such as superoxide anion, and hydroxyl (•OH) [36, 38]. Under normal conditions, free radicals are effectively eliminated by the antioxidant defense system. However, when organisms age, their antioxidant defense systems weaken and the balance between the generation and elimination of free radicals is broken [29]. Excess free radicals can lead to cell injury and even cell death, which may result in diseases such as cancer, cardiovascular disease, diabetes mellitus, neurological disorders, and Alzheimer's disease [4, 15]. Thus, it is important for organisms to inhibit the excessive formation of free radicals.

Antioxidants are chemical compounds that can donate electrons or hydrogen atoms to the free radical to create a complex, protecting the human body from free-radical damage and retarding the progress of many diseases [14]. Generally, antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole are used for radical scavenging in biological systems. However, as synthetic chemicals, these antioxidants pose potential risks to human health and their use as food additives is restricted [18]. Therefore, over many years, the demand for safe and naturally occurring alternatives to synthetic antioxidants has grown steadily. In recent years, the antioxidant activity

of bioactive peptides generated from animal and plant proteins has attracted substantial attention. For example, peptides obtained from the digestion of proteins in animal products such as cod [11], eggs [22], dry-cured ham [9], and duck meat hydrolysates [34] have been found to possess antioxidant activity. In addition, plant proteins have also been demonstrated to be a good source of antioxidants; for instance, active peptides from walnuts [4], pulses [23], and corn gluten meal [39] have been assessed for their antioxidant capacities.

Functional peptides can be produced from enzymatic hydrolysis of various bioresource proteins [20]. *Spirulina* is a photosynthetic, helicoidal, filamentous, and multicellular blue green algae that contains 60–70% high-quality proteins by weight, essential amino acids, vitamins, essential fatty acids, provitamin A (β-carotene), and other nutritional components [19, 28]. *Spirulina* exhibits a wide range of biological activities and health care functions, which include anti-inflammatory [26], immune-enhancing [31], antivirus [1], fatty-liver-preventing [33], cardioprotective [16], and anticancer properties [17]. In addition, *Spirulina* is known for its antioxidant potential, which has been demonstrated in both in vitro and in vivo studies [5, 19, 32, 35].

The antioxidant activity of peptides is closely related to their amino acid composition, structure, and sequences [27]. In this study, *Spirulina platensis* was hydrolyzed by protein K to improve its quality and antioxidant properties. The antioxidant effects were then evaluated by assessing the capacity for scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. Ultrafiltration, gel filtration chromatography, and reversed-phase high-performance liquid chromatography (RP-HPLC) were used to purify the antioxidant peptides, and RP-HPLC-electrospray ionization tandem mass spectrometry (RP-HPLC-ESI-MS) was used to identify the sequence of the peptide with the highest antioxidative activity. Moreover, the peptide was synthesized according to the obtained amino acid sequences to confirm their antioxidant activities.

Materials and Methods

Materials and Preparation of *S. platensis* Hydrolysates

Spray-dried, powdered *S. platensis* was obtained from Shengbada Biotech Co., Ltd. (China). Protease K was purchased from Xinyu Biotech Co., Ltd (China). DPPH and reduced glutathione (GSH) were purchased from Sigma-Aldrich (USA). Acetonitrile, trifluoroacetic acid (TFA), and methanol were HPLC-grade and purchased from Fisher Scientific (USA). All other chemicals and reagents, unless otherwise specified, were of analytical grade.

Spirulina platensis was mixed with deionized water (1:8 (w/v)), which was adjusted to pH 8.5 by adding 0.1 M NaOH. Protease K (700 U/g) was then added to hydrolyze the *S. platensis* for 3 h at 55°C. Subsequently, the hydrolysates were heated in a boiling water bath at 100°C for 10 min to inactive enzyme activity. They were then centrifuged at 10,000 ×*g* for 10 min at 4°C, and the supernatants were lyophilized and stored at -20°C.

DPPH Radical Scavenging Assay

DPPH free-radical scavenging activity was measured according to the method described by Chen *et al.* [4] with some modifications. Fresh DPPH solutions containing 0.1 mM DPPH in 95% alcohol were prepared daily. The mixture, which comprised 100 µl samples with 100 µl of DPPH solution in a 96-well plate, was agitated and incubated for 30 min in the dark at room temperature. The absorbance was measured by using a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, USA) at 517 nm (A_S). Ethanol was used as the blank (A_b), whereas distilled water was used as the control (A_C). The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left[1 - \frac{A_s - A_c}{A_b}\right] \times 100\% ,$$

where A_b is the absorbance of the blank, A_S is the absorbance of the sample solution, and A_c is the absorbance of the control.

Preliminary Extraction of Peptides by Ultrafiltration

Spirulina platensis hydrolysate (SH) was ultrafiltered through a membrane with a molecular weight (MW) cut-off of 3 kDa. SH was primarily categorized into two fractions: retentate (*i.e.*, fraction SHR, MW > 3 kDa) and permeate (ultrafiltrate, fraction SHP, MW < 3 kDa). *Spirulina platensis*, SH, and SHP were dissolved respectively in distilled water to a concentration of 10 mg/ml for the DPPH radical scavenging test.

Lyophilized SHP was dissolved in distilled water at 0.05–20 mg/ml for study of DPPH radical scavenging activity.

Gel Filtration Chromatography

The fraction with the highest antioxidant activity after ultrafiltration separation was separated by gel filtration chromatography on a Superdex Peptide HR 10/30 gel filtration column furnished with an AKTA purifier system (GE Healthcare, UK). It was then eluted with 0.1 M phosphate (pH 7.0) at a flow rate of 0.5 ml/min and monitored at 280 nm. The fractions with the desired peaks were pooled and lyophilized. All lyophilized fractions were re-dissolved in distilled water to a concentration of 0.365 mg/ml for the antioxidant activity test.

RP-HPLC Analysis

Fraction F2 (see Results and Discussion) was subjected to preparative RP-HPLC on a COSMOSIL MS-II C18 column (4.6 mm × 250 mm; Agilent Technologies Co., USA). The column was eluted with a linear gradient of acetonitrile (5–50% in 20 min)

containing 0.1% TFA at a flow rate of 1.0 ml/min. The fraction exhibiting the least antioxidant activity after gel filtration chromatography was used as the control to eliminate the same eluted peaks. Eluted peaks were detected at 214 nm and then lyophilized. All lyophilized fractions were re-dissolved in distilled water at 200 μg/ml for testing the antioxidant activity.

Identification of Peptides by ESI-MS

To identify the molecular mass and amino acid sequence of the purified peptide, the sample was loaded into an Agilent 6540 UHD Q-TOF system (Agilent Technologies Co.) with an ESI source. Ionization was performed in positive mode with a capillary voltage of 3 kV and at a flow rate of 0.4 ml/min. The ESI nebulizing gas and auxiliary gas used high-purity nitrogen. Spectra were recorded over a mass/charge (*m/z*) range from 0 to 2,000. Data were processed using Mass Hunter (Agilent Technologies Co.) and via manual calculations.

Verification of Antioxidant Activity

The antioxidant peptide derived from *S. platensis* was synthesized by the solid-phase procedure by Qiang Yao Biotech Co., Ltd. (China). The purity of the synthesized peptide was verified as >95% by using analytical HPLC coupled with MALDI-TOF MS. The purified peptide, synthesized peptide, and reduced GSH were dissolved in distilled water to a concentration of 100 μg/ml for the DPPH radical scavenging test.

The superoxide anion and hydroxyl free-radical scavenging activities and SOD activity of the synthesized peptide and reduced GSH were determined according to the manufacturer's instructions using the Superoxide Anion Free Radical Detection kit, Hydroxyl Free Radical Detection kit, and Superoxide Dismutase (SOD) Detection Kit, respectively, from Jiancheng Bioengineering Institute, China.

Results and Discussion

Hydrolysis of *Spirulina* and Antioxidant Activity

The relatively stable DPPH radical has been widely used to test the ability of compounds to act as free-radical scavengers or hydrogen donors and thus to evaluate antioxidant activity [21]. As shown in Fig. 1, all samples exhibited DPPH radical scavenging activity. The highest was SHP, and the higher was SH. The scavenging activity of SHP was 85.21 ± 1.59% at 10 mg/ml, which was significantly higher than that of the hydrolysates without ultrafiltration or phycocyanin without hydrolysis. These results indicate that the oxidation resistance of *S. platensis* can be improved by enzymolysis, and that the antioxidation was mainly derived from components with a MW < 3 kDa. This finding is in agreement with previous studies that suggested hydrolysates or peptides with lower MWs show higher free-radical scavenging activity [6, 39].

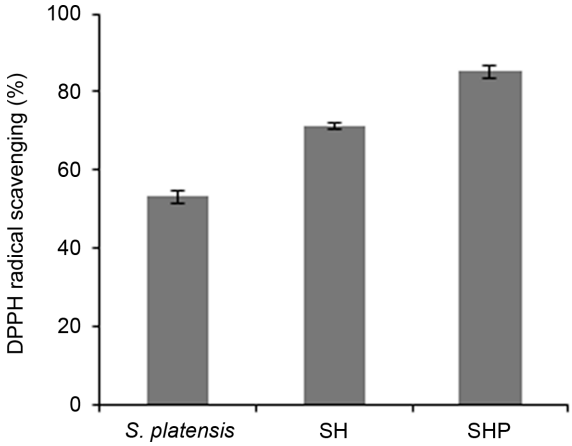

Fig. 1. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *S. platensis*, *S. platensis* hydrolysate (SH), and *S. platensis* hydrolysate ultrafiltrate (SHP).
The protein concentration was 10 mg/ml for the radical scavenging test. All data represent the mean ± standard deviation.

As shown in Fig. 2, the observed scavenging activity increased significantly as the concentration of the SHP fraction (MW < 3 kDa) increased from 0.05 to 20 mg/ml. Specifically, as the concentration increased from 0.5 to 2.5 mg/ml, the scavenging activity increased rapidly from 31 ± 0.6% to 78.80 ± 1.7%; subsequently, the activity increased more gradually, with a scavenging activity of 88.95 ± 1.3% observed when the concentration reached 20 mg/ml.

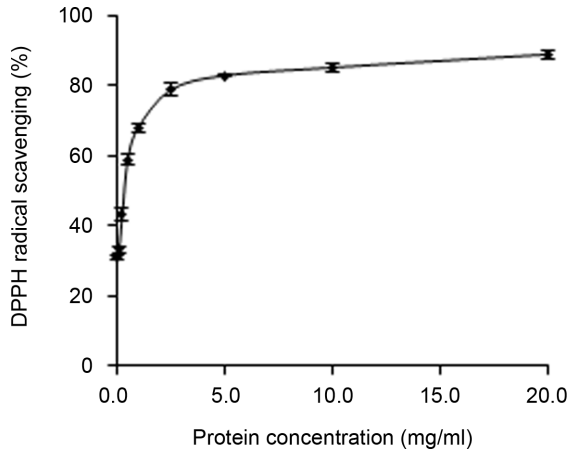

Fig. 2. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging (%) of *S. platensis* hydrolysate ultrafiltrate (SHP).
The lyophilized SHP was dissolved in distilled water to obtain protein concentrations of 0.05–20 mg/ml. All data represent the mean ± standard deviation.

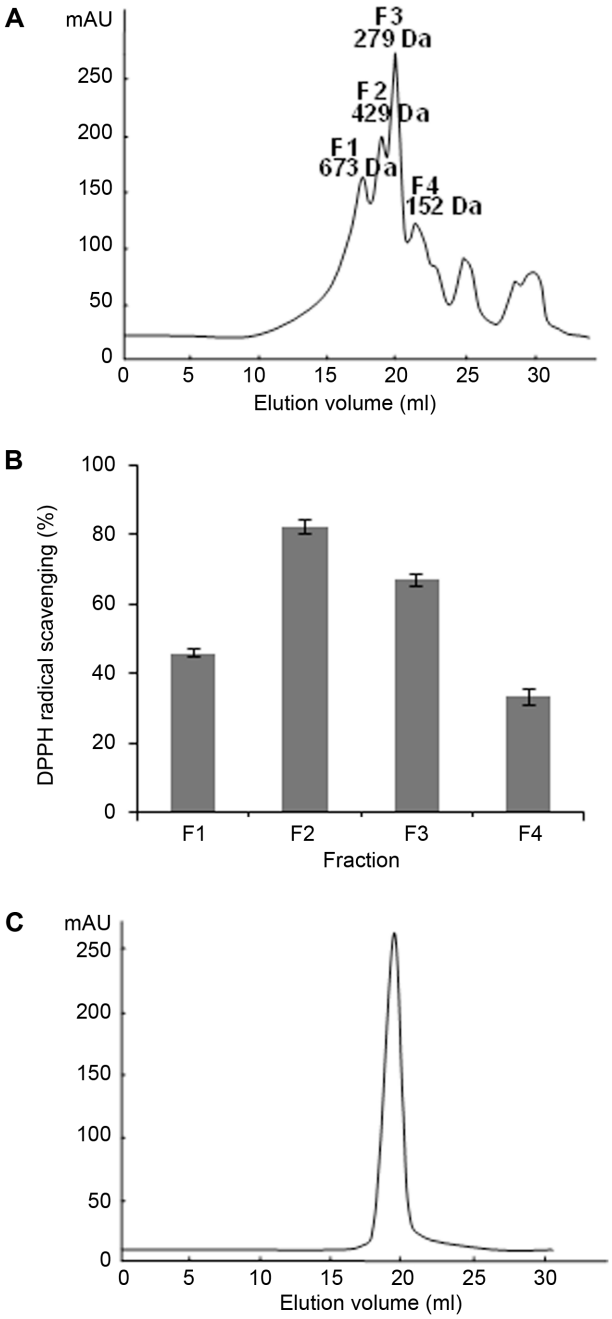

Fig. 3. Fractionation of *S. platensis* hydrolysate ultrafiltrate (SHP) on a Superdex Peptide HR 10/30 gel filtration column. (**A**) Elution curve. Markers F1–F4 represent the elution peaks. (**B**) DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging abilities of fractions F1–F4. (**C**) The fraction F2. All data represent the mean ± standard deviation.

Moreover, the SHP exhibited 92.25 ± 2.3% inhibition of DPPH radicals at 100 mg/ml (data not shown). These results indicate that, in comparison with larger peptides,

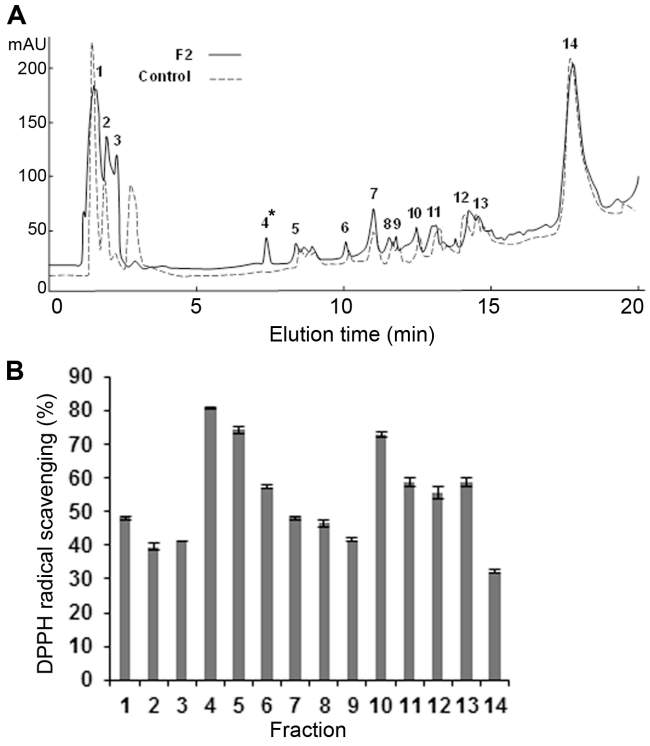

Fig. 4. Reverse-phase high-performance liquid chromatography separation of the selected pooled F2 fractions obtained from gel filtration chromatography.
(**A**) Elution curve. Numbers 1–14 represent the respective elution peaks of RF1–14. "Control" represents the fraction obtained by gel filtration chromatography that exhibited the least antioxidant activity; this was used as the control to eliminate the same eluted peaks. *represents the specific peak that existed in F2 but not in the control. (**B**) DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging of fractions RF1–14. All data represent the mean ± standard deviation values.

hydrolysates with MW < 3 kDa contained more amino acid groups that could readily donate electrons to DPPH radicals.

Purification of Antioxidant Peptides

Gel filtration chromatography was further employed to fractionate the antioxidant ingredients, and the SHP was separated into four fractions (F1–F4; Fig. 3A). Each fraction was pooled, concentrated, and freeze-dried, and the antioxidant activities were determined. As shown in Fig. 3B, fraction F2 exhibited the highest DPPH free-radical scavenging activity: 82.06 ± 2.16% at 0.365 mg/ml. Fraction F3 exhibited a similar, but lower, free-radical scavenging activity to fraction F2. These findings agreed with the ultrafiltration results, which showed that hydrolysates or peptides (obtained from *S. platensis*) with lower MW had

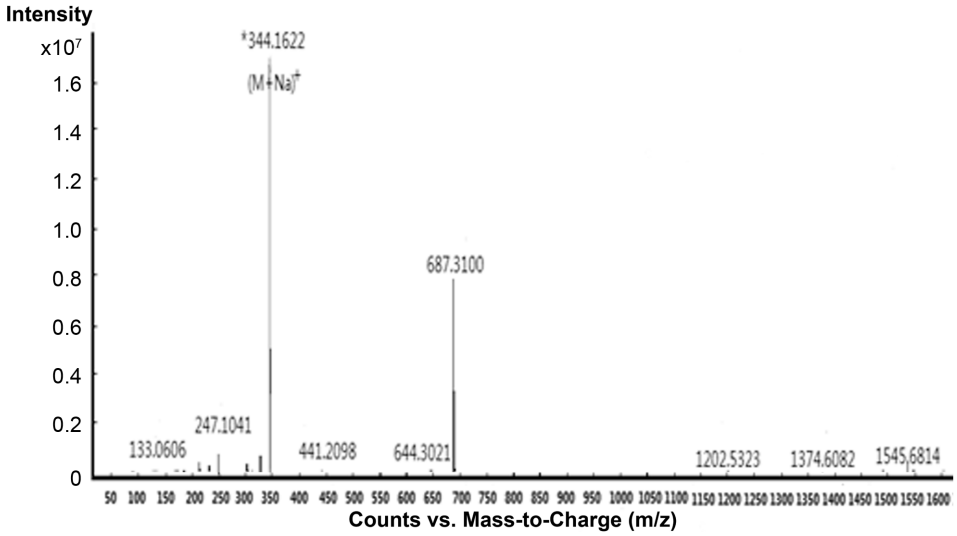

Fig. 5. Identification of the antioxidant peptide in fraction RF4 by reverse-phase high-performance liquid chromatography-electrospray ionization tandem mass spectrometry.

Ionization was performed in the positive mode with a capillary voltage of 3 kV and at a flow rate of 0.4 ml/min. The spectrum is derived from fragmentation of the ion at *m/z* 344.1622.

higher free-radical scavenging activity. Fraction F2 was subsequently subjected to gel filtration again, and we obtained only one eluted peak, suggesting that the F2 fraction was a mixture with a similar molecular weight range (Fig. 3C).

To purify the protein, the target fraction (F2) was further separated by RP-HPLC. As shown in Fig. 4A, fraction F2 was separated into 14 fractions (RF1–RF14). Each fraction was pooled, concentrated, and freeze-dried, and the fractions' DPPH radical scavenging activities were then measured (Fig. 4B). A clear difference was observed between the 14 fractions; fraction RF4 had the highest activity, with a DPPH radical scavenging activity of 80.74 ± 0.40% at 200 μg/ml. Furthermore, compared with the control, the RF4 fractions in the F2 sample were the characteristic peaks. Therefore, the RF4 fractions that showed antioxidant activity were further analyzed with mass spectrometry to identify and characterize the peptide sequences.

Identification of Antioxidant Peptides

The fraction RF3-RF6 that exhibited the highest antioxidant activity was subsequently subjected to LC–MS/MS for peptide sequence identification, and its amino acid sequence was identified as Pro-Asn-Asn (343.15 Da; Fig. 5). To confirm this result, the peptides derived from SH were chemically synthesized using Fmoc-protected amino acid synthesis methods and further evaluated for their antioxidant activity by assessing radical scavenging activity. As shown in Fig. 6A, the purified peptide effectively quenched DPPH radicals with a scavenging rate of 80.52 ± 0.67%, which was similar to that of the synthesized peptide (81.44 ± 0.43%) and GSH (82.63 ± 0.56%). These results suggest that the purification of SH greatly increased its antioxidant activity such that its DPPH radical scavenging activity was similar to that of GSH.

Superoxide anion and hydroxyl are two of the most important free radicals [30]. Superoxide anions are produced by the addition of an electron to molecular oxygen, and are harmful reactive oxygen species as they damage cellular components in biological systems. Hydroxyl radicals can be formed from superoxide anions and hydrogen peroxide in the presence of metal ions, and they can easily react with cellular biomolecules, including amino acids, proteins, DNA, and lipids, to exert a strong cytotoxic effect. Therefore, the scavenging of superoxide anions and hydroxyl free radicals was determined in the present study. The synthesized peptide exhibited effective antioxidant activity, and the superoxide anion and hydroxyl free-radical scavenging activities were 47.84 ± 0.49% and 54.01 ± 0.82%, respectively, at 10 mg/ml (Figs. 6B and 6C). However, GSH had higher superoxide anion and hydroxyl free-radical scavenging activities of 64.42 ± 0.70% and 92.64 ± 0.97%, respectively, at 10 mg/ml (Figs. 6B and 6C).

Antioxidant enzymes play an important role in protecting the human body from the harmful effects of oxygen radicals [12]. Thus, to further investigate the antioxidant

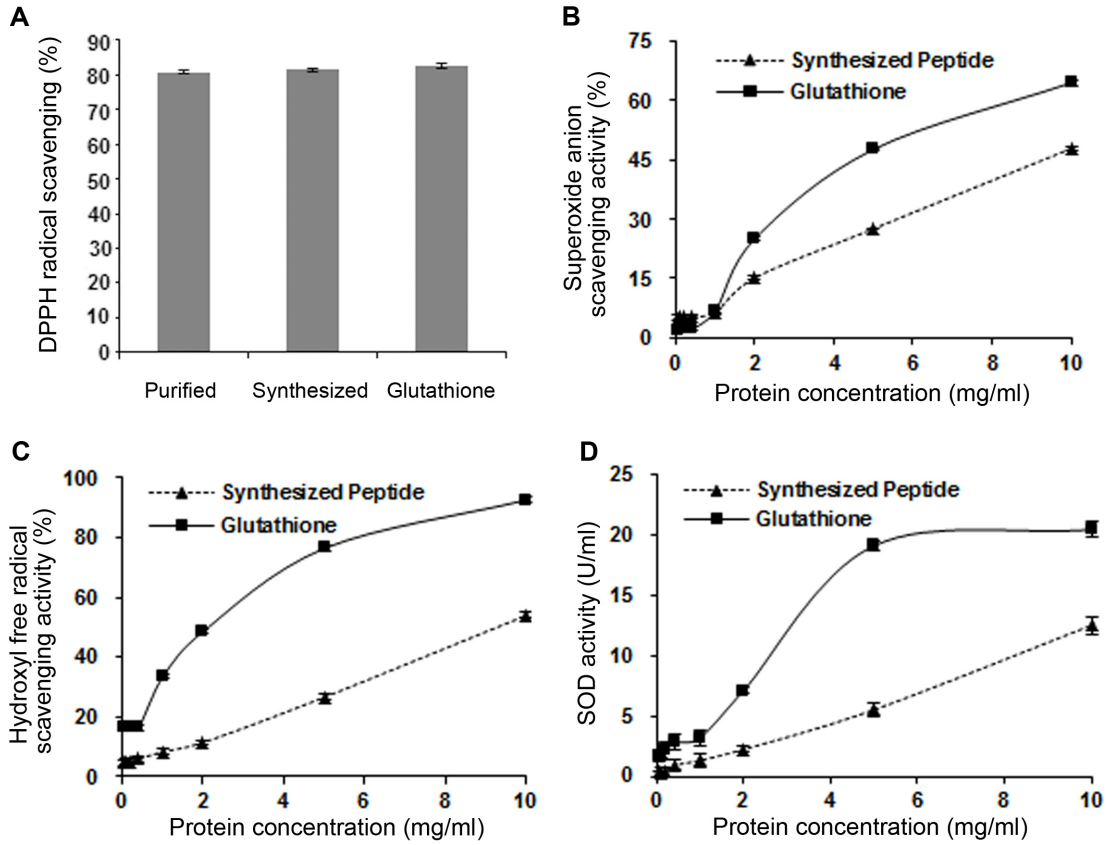

Fig. 6. Radical scavenging activities of the purified peptide, synthesized peptide, and reduced glutathione.
(**A**) DPPH radical scavenging activity; (**B**) Superoxide anion scavenging activity; (**C**) Hydroxyl free-radical scavenging activity. (**D**) SOD activity. All data represent the mean ± standard deviation.

ability of the peptide in the present study, the SOD activity was determined. As shown in Fig. 6D, the SOD activities of the synthesized peptide and GSH were 12.55 ± 0.75% and 20.48 ± 0.68%, respectively, at 10 mg/ml.

In general, the activity of antioxidative peptides is highly influenced by their molecular mass, and peptide fractions with MW from 200 to 3,000 Da have higher antioxidant activity [18, 21, 24]. In this study, the purified antioxidative peptide contained three amino acids (343.15 Da). Previous studies have shown that peptides with lower MW are the most efficient antioxidants [18]. Peptides with lower MW are more likely to cross the intestinal barrier; in a recent study, peptides that contained 2–20 amino acid residues generally crossed the intestinal barrier more easily to exert biological effects [18]. Many previous studies suggest that antioxidant peptides from protein hydrolysates obtained from products such as walnuts [4], eggs [22], corn gluten meal [39], tilapia [37], and sheep cheese whey [7] contain 2–20 amino acid residues. The purified peptide of the present study possessed high antioxidation activity in radical scavenging, which was similar to that of GSH (307 Da). GSH is a powerful antioxidant that has been commercialized and is widely used in many fields. It plays important roles in detoxification, the immune response, and protection against reactive oxygen species [2]. The DPPH scavenging activity of the purified peptide from the present study was much higher than that of peptides identified from eggs (50%, 60 mM) [22], Spanish dry-cured ham (50%, 1.5 mg/ml) [9], and corn gluten meal (72.84%, 0.5 mg/ml) [39], and was similar to that of a peptide from the enzymatic hydrolysates of duck meat [34].

It is well known that the amino acid composition and sequence are important factors in the activity of bioactive peptides [9, 20]. In this study, the sequence of the antioxidant peptide was identified as Pro-Asn-Asn. Some studies have shown that hydrophobic amino acid residues, such as Pro, His, Met, Cys, Tyr, Trp, Phe, and Met, may enhance the activity of antioxidant peptides [3, 8, 13]. In

particular, it is generally accepted that Pro is especially important to the radical scavenging activity of peptides because of its unique structure [18]. In addition, Asn also appears to play a vital role in such peptides' antioxidant activity [10]. Several studies have previously reported Asn as a constituent of antioxidant peptides, for example in Asn-Tyr-Asp-Glu-Tyr [10], Arg-Gln-Ser-His-Phe-Ala-Asn-Ala-Gln-Pro [18], Asn-Arg-Tyr-His-Glu [19], and Asn-Gly-Leu-Glu-Gly-Leu-Lys [25]. The purified peptide Pro-Asn-Asn has several active groups, including amino, carboxyl, and hydroxyl groups. Further investigation is required to analyze and confirm the antioxidant mechanisms of Pro-Asn-Asn, but the findings of this study provide a solid foundation for future research.

In conclusion, to the best of our knowledge, this paper is the first to report the antioxidant activity of peptides derived from *S. platensis* hydrolysates. A novel antioxidant peptide, Pro-Asn-Asn (343.15 Da), was purified and identified by using ultrafiltration, gel filtration chromatography, and RP-HPLC-ESI-MS/MS. Furthermore, the peptide was synthesized to confirm its identity and ascertain its properties. The DPPH radical scavenging activity of the purified peptide (which reached 81.44 ± 0.43% at a concentration of 100 μg/ml) was similar to that of GSH, and the synthesized peptide exhibited good antioxidant ability as indicated by its superoxide anion and hydroxyl free-radical scavenging activities and SOD activity. Our findings suggest that *S. platensis* is a good source for the preparation of antioxidant peptides, and that hydrolysates from *S. platensis* could have important applications in the pharmaceutical and food industries.

Acknowledgments

This work was supported by the Project of National Science & Technology Support Program of China (Grant 2013BAD10B02); the Fundamental Research Funds for the Central Universities (Grant 2014JC004); and the Nature Science Foundation of Hubei Province (Grant 2015CFC797).

References

1. Ayehunie S, Belay A, Baba TW, Ruprecht RM. 1998. Inhibition of HIV-1 replication by an aqueous extract of *Spirulina platensis* (*Arthrospira platensis*). *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **18:** 7-12.
2. Baek M, Choy J-H, Choi S-J. 2012. Montmorillonite intercalated with glutathione for antioxidant delivery: synthesis, characterization, and bioavailability evaluation. *Int. J. Pharm.* **425:** 29-34.
3. Cai L, Wu X, Zhang Y, Li X, Ma S, Li J. 2015. Purification and characterization of three antioxidant peptides from protein hydrolysate of grass carp (*Ctenopharyngodon idella*) skin. *J. Funct. Foods* **16:** 234-242.
4. Chen N, Yang H, Sun Y, Niu J, Liu S. 2012. Purification and identification of antioxidant peptides from walnut (*Juglans regia* L.) protein hydrolysates. *Peptides* **38:** 344-349.
5. Chen X-M, Yang F, Bai Y, Zhou Y-H, Zheng W-J. 2008. Effects of tellurium stress on the growth and antioxidase system of *Spirulina platensis* and *S. maximum*. *Acta Hydrobiol. Sin.* **32:** 148-153.
6. Chi C-F, Hu F-Y, Wang B, Li Z-R, Luo H-Y. 2015. Influence of amino acid compositions and peptide profiles on antioxidant capacities of two protein hydrolysates from skipjack tuna (*Katsuwonus pelamis*) dark muscle. *Mar. Drugs* **13:** 2580-2601.
7. Corrêa APF, Daroit DJ, Fontoura R, Meira SMM, Segalin J, Brandelli A. 2014. Hydrolysates of sheep cheese whey as a source of bioactive peptides with antioxidant and angiotensin-converting enzyme inhibitory activities. *Peptides* **61:** 48-55.
8. Elias RJ, Kellerby SS, Decker EA. 2008. Antioxidant activity of proteins and peptides. *Crit. Rev. Food Sci.* **48:** 430-441.
9. Escudero E, Mora L, Fraser PD, Aristoy M-C, Toldrá F. 2013. Identification of novel antioxidant peptides generated in Spanish dry-cured ham. *Food Chem.* **138:** 1282-1288.
10. Fan J, He J, Zhuang Y, Sun L. 2012. Purification and identification of antioxidant peptides from enzymatic hydrolysates of tilapia (*Oreochromis niloticus*) frame protein. *Molecules* **17:** 12836-12850.
11. Girgih AT, He R, Hasan FM, Udenigwe CC, Gill TA, Aluko RE. 2015. Evaluation of the in vitro antioxidant properties of a cod (*Gadus morhua*) protein hydrolysate and peptide fractions. *Food Chem.* **173:** 652-659.
12. Gonzalez-Burgos E, Carretero ME, Gomez-Serranillos MP. 2013. Kaurane diterpenes from *Sideritis* spp. exert a cytoprotective effect against oxidative injury that is associated with modulation of the Nrf2 system. *Phytochemistry* **93:** 116-123.
13. Hernández-Ledesma B, Dávalos A, Bartolomé B, Amigo L. 2005. Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *J. Agric. Food Chem.* **53:** 588-593.
14. Ji K, Jang N, Kim Y. 2015. Isolation of lactic acid bacteria showing antioxidative and probiotic activities from kimchi and infant feces. *J. Microbiol. Biotechnol.* **25:** 1568-1577.
15. Karthikeyan R, Manivasagam T, Anantharaman P, Balasubramanian T, Somasundaram S. 2011. Chemopreventive effect of *Padina boergesenii* extracts on ferric nitrilotriacetate (Fe-NTA)-induced oxidative damage in Wistar rats. *J. Appl. Phycol.* **23:** 257-263.
16. Khan M, Shobha JC, Mohan IK, Naidu MUR, Sundaram C, Singh S, *et al.* 2005. Protective effect of *Spirulina* against doxorubicin-induced cardiotoxicity. *Phytother. Res.* **19:** 1030-1037.
17. Konickova R, Vankova K, Vanikova J, Vanova K, Muchova L,

Subhanova I, *et al.* 2014. Anti-cancer effects of blue-green alga *Spirulina platensis*, a natural source of bilirubin-like tetrapyrrolic compounds. *Ann. Hepatol.* **13:** 273-283.
18. Kou X, Gao J, Xue Z, Zhang Z, Wang H, Wang X. 2013. Purification and identification of antioxidant peptides from chickpea (*Cicer arietinum* L.) albumin hydrolysates. *LWT Food Sci. Technol.* **50:** 591-598.
19. Kulshreshtha A, Zacharia JA, Jarouliya U, Bhadauriya P, Prasad G, Bisen PS. 2008. *Spirulina* in health care management. *Curr. Pharm. Biotechnol.* **9:** 400-405.
20. Lee JK, Li-Chan EC, Byun H-G. 2015. Characterization of β-secretase inhibitory peptide purified from skate skin protein hydrolysate. *Eur. Food Res. Technol.* **240:** 129-136.
21. Li Y, Jiang B, Zhang T, Mu W, Liu J. 2008. Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food Chem.* **106:** 444-450.
22. Liu J, Jin Y, Lin S, Jones GS, Chen F. 2015. Purification and identification of novel antioxidant peptides from egg white protein and their antioxidant activities. *Food Chem.* **175:** 258-266.
23. Lopez-Barrios L, Gutierrez-Uribe JA, Serna-Saldivar SO. 2014. Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients. *J. Food Sci.* **79:** R273-R283.
24. Moure A, Domínguez H, Parajó JC. 2006. Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochem.* **41:** 447-456.
25. Rajapakse N, Mendis E, Byun HG, Kim SK. 2005. Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *J. Nutr. Biochem.* **16:** 562-569.
26. Remirez D, González R, Merino N, Rodriguez S, Ancheta O. 2002. Inhibitory effects of *Spirulina* in zymosan-induced arthritis in mice. *Mediat. Inflamm.* **11:** 75-79.
27. Sarmadi BH, Ismail A. 2010. Antioxidative peptides from food proteins: a review. *Peptides* **31:** 1949-1956.
28. Shimamatsu H. 2004. Mass production of *Spirulina*, an edible microalga, pp. 39-44. *Asian Pacific Phycology in the 21st Century: Prospects and Challenges*. Springer, Berlin.
29. Stadtman ER. 2006. Protein oxidation and aging. *Free Radic. Res.* **40:** 1250-1258.
30. Tümay M, Hatungil R, Berköz M, Yalin S, Erdogan S. 2013. Role of free radicals on retinitis pigmentosa. *Asian J. Chem.* **25:** 1561.
31. Tayag CM, Lin YC, Li CC, Liou CH, Chen JC. 2010. Administration of the hot-water extract of *Spirulina platensis* enhanced the immune response of white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. *Fish Shellfish Immun.* **28:** 764-773.
32. Tobon-Velasco JC, Palafox-Sanchez V, Mendieta L, Garcia E, Santamaria A, Chamorro-Cevallos G, Limon ID. 2013. Antioxidant effect of *Spirulina* (*Arthrospira*) *maxima* in a neurotoxic model caused by 6-OHDA in the rat striatum. *J. Neural Transm.* **120:** 1179-1189.
33. Torres-Durán P, Miranda-Zamora R, Paredes-Carbajal M, Mascher D, Blé-Castillo J, Dýaz-Zagoya J, Juárez-Oropeza M. 1999. Studies on the preventive effect of *Spirulina maxima* on fatty liver development induced by carbon tetrachloride, in the rat. *J. Ethnopharmacol*. **64:** 141-147.
34. Wang L-S, Huang J-C, Chen Y-L, Huang M, Zhou G-H. 2015. Identification and characterization of antioxidant peptides from enzymatic hydrolysates of duck meat. *J. Agric. Food Chem.* **63:** 3437-3444.
35. Yang F, Wong KH, Yang YF, Li XL, Jiang J, Zheng WJ, *et al.* 2014. Purification and in vitro antioxidant activities of tellurium-containing phycobiliproteins from tellurium-enriched *Spirulina platensis*. *Drug Des. Dev. Ther.* **8:** 1789-1800.
36. Zhang T, Li Y, Miao M, Jiang B. 2011. Purification and characterisation of a new antioxidant peptide from chickpea (*Cicer arietium* L.) protein hydrolysates. *Food Chem.* **128:** 28-33.
37. Zhang Y, Duan X, Zhuang Y. 2012. Purification and characterization of novel antioxidant peptides from enzymatic hydrolysates of tilapia (*Oreochromis niloticus*) skin gelatin. *Peptides* **38:** 13-21.
38. Zheng XF, Sun XJ, Xia ZF. 2011. Hydrogen resuscitation, a new cytoprotective approach. *Clin. Exp. Pharmacol. Physiol.* **38:** 155-163.
39. Zhuang H, Tang N, Yuan Y. 2013. Purification and identification of antioxidant peptides from corn gluten meal. *J. Funct. Foods* **5:** 1810-1821.