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Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems

I.-Chuan Sheih [a], Tung-Kung Wu [b], Tony J. Fang [a,*]

[a] Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan, ROC
[b] Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsin-Chu 30068, Taiwan, ROC

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ABSTRACT

Microalgae have been a popular edible food, but there are no known reports on the antioxidative peptides derived from microalgae. The algae protein waste, which is normally discarded as animal feed, is a by-product during production of algae essence from microalgae, *Chlorella vulgaris*. Algae protein waste was hydrolyzed using pepsin, and a potent antioxidative peptide of VECYGPNRPQF was separated and isolated. The peptide could efficiently quench a variety of free radicals, including hydroxyl radical, superoxide radical, peroxyl radical, DPPH radical and ABTS radicals, and performed more efficiently than that observed for BHT, Trolox and peptides from marine protein sources in most cases. The purified peptide also has significant protective effects on DNA and prevents cellular damage caused by hydroxyl radicals. In addition, the peptide has gastrointestinal enzyme-resistance and no cytotoxicity observed in human lung fibroblasts cell lines (WI-38) *in vitro*. These results demonstrate that inexpensive algae protein waste could be a new alternative to produce antioxidative peptides.

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1. Introduction

In the normal cells, free radicals and reactive oxygen species (ROS) can be effectively eliminated by an enzyme-mediated system such as superoxide dismutase, peroxidase, glutathione peroxidase and non-enzymatic factors such as ascorbic acid, protein. When the homeostasis between the prooxidant formation and antioxidant capacity is disrupted, whereby prooxidant formation exceeds antioxidant capacity, oxidative damage will accumulate and result in patho-physiological events. Accumulating evidence indicates that active oxygen and free radicals would attack key biological molecules such as DNA, protein, and lipid that leading to many degenerative disease conditions (Suja et al., 2004), such as cancer (Leanderson et al., 1997), gastric ulcers (Debashis et al., 1997), Alzheimer's, arthritis and ischemic reperfusion (Vajragupta et al., 2000). To maintain the prooxidant–antioxidant balance, the removal of free radicals and ROS is probably one of the most effective defenses of a living body against various diseases (Yang et al., 2001; Butterfield et al., 2002; Fazlul and Li, 2002).

Many synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) have been used to retard the oxidation process; however, the use of synthetic antioxidants must be under strict regulation due to potential health hazards (Park et al., 2001). The search for natural antioxidants as alternatives is therefore of great interest among researchers.

Recently, bioactive peptides from enzymatic hydrolysis of various food proteins such as soy protein, casein, whey protein, gelatin and wheat gluten have been shown to possess antioxidative activity (Elias et al., 2008). However, antioxidative peptides from marine food sources are gaining attention as new antioxidative alternatives in the last few years (Je et al., 2005; Mendis et al., 2005; Rajapakse et al., 2005; Qian et al., 2008a,b). *Chlorella vulgaris* is a popular edible microalgae in Japan, so its safety is well established (Suetsuna and Chen, 2001). Presently, the commercial applications of microalgae are as nutritional supplements, natural dyes and skin care products (Spolaore et al., 2006), but there are no studies reporting the antioxidative activity of microalgae protein-derived peptides. Algae protein waste is a by-product derived from water-extraction process of microalgae, *C. vulgaris* during algae essence manufacturing. The underutilized algae wastes, containing above 50% protein, have low economical value to be used as animal feed. The pepsin hydrolysate from algae protein waste exhibited antioxidative activity in preliminary experiments, indicating that algae waste might become a new protein source for selection of novel antioxidative peptides. In this study, we used pepsin protease to produce biologically active hydrolysate from algae protein waste, and purified a bioactive peptide with free radical scavenging activity. We further investigated its antioxidative activity in

* Corresponding author. Tel.: +886 4 22861505; fax: +886 4 22876211.
E-mail address: tjfang@nchu.edu.tw (T.J. Fang).

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different oxidation systems in comparison with those of synthetic antioxidant BHT and natural antioxidants Trolox, and ascorbic acid. In addition, the protective effect of the purified peptide against oxidation-induced DNA and cellular damage also was determined.

2. Methods

2.1. Materials

Algae waste, the by-product in algae essence manufacturing of Taiwan, was dried and kept at 4 °C prior to use. ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, indoxyl-β-D-glucuronide (IBG), $FeSO_4 \cdot 7H_2O$, lucigenin, arginine, methylglyoxal, (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide, fetal bovine serum (FBS) and MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) were from Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) and H_2O_2 (30%, v/v) were purchased from Merck Co. (Darmstadt, Germany). Pepsin was from Nacalai Tesque (Kyoto, Japan). Fluorescein, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) and 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Aldrich (Milwaukee, WI, USA). PET-28a (+) was from Novagen (Madison, WI, USA). SYBR Green was purchased from Roche (Indianapolis, USA). Sephacryl S-100 HR and Q-sepharose Fast Flow was purchased from Pharmacia Biotech. Co. (Uppsala, Sweden). Human gastric cancer cell lines AGS and human normal lung cell WI-38 were purchased from the Food Industry Research Development Institute (Hsinchu, Taiwan). Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) medium were from JRH Biosciences (KS, USA).

2.2. Preparation of enzymatic hydrolysate

Algae protein waste (10%, w/v) was digested with pepsin at an enzyme to substrate ratio of 2% (w/w) and a temperature of 50 °C for 15 h. At the end of the reaction, the digestion was adjusted to pH 7.0 in order to inactivate the enzyme. The pepsin hydrolysate was then collected, freeze-dried and the molecular size distribution profile was analyzed by 20% SDS–PAGE. The product yield was defined as the weight ratio of the enzymatic hydrolysate over the algae protein waste without enzyme hydrolysis.

2.3. Purification of an antioxidative peptide

Ammonium sulfate was added to a concentration of 40% saturation in the permeate from the pepsin hydrolysates, and precipitated protein was removed by centrifugation (10,000g, 20 min). The concentration of ammonium sulfate in the supernatant was increased to 80% saturation. The 40–80% precipitate was collected and dissolved in distilled water, and the solution was fractionated using a Sephacryl S-100 high HR column ($\varnothing 2.6 \times 70$ cm) with 20 mM Tris–HCl buffer solution (pH 7.8). The fraction exhibiting higher antioxidative activity was subsequently loaded onto a Q-sepharose fast flow column ($\psi 2.6 \times 40$ cm) and then eluted with a linear gradient of NaCl (0–1.0 M) in the 20 mM Tris–HCl buffer solution (pH 7.8). The lyophilized fraction with higher antioxidative activity was reloaded on an Inertsil octadecylsilane (ODS)-3 semi-prep column (10×250 mm). The column was eluted with a linear gradient of acetonitrile (15–35% for 30 min) in 0.1% trifluoroacetic acid at a flow rate of 2.0 ml/min. Bioactive peaks were pooled and the purity of peptide was assayed by an Agilent 6510 Q-TOF mass spectrometer (Agilent Technologies Inc. CA, USA). The antioxidative activity was also assayed by means of a TEAC assay (as described in Section 2.6) at every purification step.

2.4. Determination of amino acid sequence

Amino acid sequence of the purified peptide was determined by an automated Edman degradation with an Applied Biosystems, Procise 494 protein sequencer (Foster City, CA, USA).

2.5. Stability against gastrointestinal proteases

The stability against gastrointestinal proteases was assayed *in vitro*. The purified peptide was digested with pepsin at an enzyme to substrate ratio of 1% (w/w) in the KCl–HCl buffer solution (pH 2.0) and a temperature of 37 °C for 3 h. The pepsin solution was neutralized to pH 7.8 before boiling for 10 min, and then subjected to successive digestion tests. The peptide solution was further digested by 1% (w/w) porcine pancreatin for 4 h at 37 °C, and the sample was boiled for 10 min followed by centrifugation (10,000*g*, 10 min) (Chiang et al., 2006). The sample was assayed for residual antioxidative activity by means of TEAC assay at every digestion step.

2.6. Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay was measured according to the method of Arts et al. (2004). A volume of 10 μl of purified solution was mixed with 200 μl of the ABTS radicals, and absorbance was monitored at 734 nm for 2 min. The decrease at 734 nm between the blank and a sample was used for calculating the scavenging activity. The IC_{50} value was defined as the concentration of peptide required to scavenge 50% of ABTS radicals. Trolox, BHT and ascorbic acid were used as positive controls. Results were presented as means of experiments performed in triplicate ± standard deviation.

2.6.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals scavenging activity assay

The DPPH radicals scavenging activity assay was measured according to the method of Huang and Mau (2006). An aliquot of 4 ml of purified peptide was mixed with 1 ml of methanol solution containing the 1 mM DPPH radicals. The mixture was allowed to stand for 40 min in the dark, and absorbance was monitored at 517 nm. The decrease at 517 nm between the blank and a sample was used for calculating the scavenging activity. The IC_{50} value was defined as the concentration of peptide required to scavenge 50% of DPPH radicals. Trolox, BHT and ascorbic acid were used as positive controls. Results were presented as means of experiments performed in triplicate ± standard deviation.

2.7. Hydroxyl radicals scavenging ability assay

Hydroxyl radicals scavenging activity was measured according to the method of Tsai et al. (2001). The hydroxyl radicals generating system was based on the Fenton reaction ($Fe^{2+} + H_2O_2$), so the reaction mixture included 1.0 ml of 3 μM IBG, 0.1 ml of 1.0 mM $FeSO_4$, 1.6 ml of 3% H_2O_2, 0.05 ml of 10 mM EDTA. The ultraweak photons were measured using BJL-ultraweak chemiluminescence (Jye Horn Co. Taiwan). A 10 μl aliquot of purified peptide was added to the reaction mixture, and the degree of reduction of the CL counts represented the scavenging abilities of the hydroxyl radicals. The IC_{50} value was defined as the concentration of peptide required to scavenge 50% of hydroxyl radicals. Trolox and BHT were used as positive controls. Results were presented as means of experiments performed in triplicate ± standard deviation.

2.8. Superoxide radicals scavenging ability assay

Superoxide radicals scavenging activity was measured according to the method of Tsai et al. (2003). The superoxide radicals

were generated by lucigenin and methylglyoxal/arginine reagents in the BJL-ultraweak chemiluminescence. The reaction mixture included 1.0 ml of 2.0 mM lucigenin, 0.05 ml of 1.0 M arginine, 0.05 ml of 1.4 μM methylglyoxal and 1.0 ml of PBS buffer (pH 7.4). A 10 μl aliquot of purified peptide was added to the reaction mixture, and dropping degrees of the CL counts represented the superoxide radicals scavenging abilities. The IC_{50} value was defined as the concentration of peptide required to scavenge 50% of superoxide radicals. Trolox and BHT were used as positive controls. Results were presented as means of experiments performed in triplicate ± standard deviation.

2.9. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was measured according to the method of Alberto et al. (2004). The reaction was carried out in 75 mM phosphate buffer (pH 7.4). Sample solution (20 μL) and Fluorescein (150 μL, 96 nM) was pre-incubated for 5 min at 37 °C, and the ORAC analysis was initiated by adding 30 μL of 320 mM AAPH. Trolox was used as a calibration solution, and Fluorescein decay curves between the blank and a sample was performed. All values were expressed as Trolox equivalents. BHT and ascorbic acid were used as positive controls.

2.10. Protection effect of the purified peptide on oxidation-induced DNA damage

DNA damage was induced by hydroxyl radicals based on the Fenton reaction (Fe^{2+} + H_2O_2) as described in Qian et al. (2008a). The reaction system included 8 μl of PET-28a DNA, 3 μl of 2 mM $FeSO_4$, 4 μl of various concentrations of purified peptides and 4 μl of 0.06 mM H_2O_2. The mixture was incubated at 37 °C for 30 min, and then subjected to 1% agarose gel electrophoresis. DNA bands were stained with SYBR Green.

2.11. Protection effect of the purified peptide on oxidation-induced cell damage

AGS cells were seeded in 96-well plates at 4×10^3 cells/well, and cultured in RPMI medium containing 10% fetal bovine serum (FBS) at 37 °C. After an additional 24 h, the cells were treated with various concentrations of purified peptides for 2 h; 0.03 mM H_2O_2 was added to cells and incubated for another 2 h. The viability of oxidation-induced cells was measured using the MTT method. MTT (20 μl/well) was added at the end of this period and incubated for an additional 4 h. The absorbance was recorded at 570 nm with a 96 well plate reader (PowerWave XS, Biotek, Winooski, VT, USA), and the results were given as relative growth ratio of peptide-treated cells to untreated controls for treatments (designed as 1% or 100%) (Katayama and Mine, 2007).

2.12. Cytotoxicity of human normal lung WI-38 cells

WI-38 cells were seeded in 96-well plates at about 3×10^3 cells/well, and cultured in DMEM medium containing 10% fetal bovine serum (FBS) at 37 °C. After an additional 24 h, the cells were grown in the absence and presence of various concentrations of the peptides for 3 days. The viability of WI-38 cells was measured using the MTT method, and the results were given as relative growth ratio of peptide-treated cells to untreated controls for treatments (designed as 1% or 100%).

2.13. Statistical analysis

The student's *t*-test was used to determine the level of significance. A *p* value of less than 0.05 was taken as significant.

3. Results and discussion

3.1. Preparation of algae protein-derived bioactive peptides

The algae protein waste hydrolysate was prepared by pepsin hydrolysis at a temperature of 50 °C for 15 h and the product yield was 48.3%. As depicted in Fig. 1, the pepsin released a mixture of extremely small peptides with high antioxidative activity against ABTS radicals (IC_{50} 93.4 μg/ml) from algae protein, compared to no observable antioxidative effect in intact algae proteins (data not shown). The hydrolysis was necessary in order to release antioxidative peptides from an inactive, intact form of algae proteins, and the antioxidative activity in the hydrolysates was probably due to the presence of a range of different peptide sequences. Previous reports also have found that pepsin is capable of producing bioactive peptides from other food proteins (Suetsuna et al., 2000; Suetsuna and Nakano, 2000).

3.2. Purification of antioxidative peptide from pepsin hydrolysate of algae protein waste

The peptides present in pepsin hydrolysates were fractionated with ammonium sulfate. The 40–80% fraction exhibited higher antioxidative activity against ABTS radicals as compared with 0–40% fraction, where the antioxidative activity was about 2.4-fold stronger than that of the 0–40% fraction. The 40–80% fraction precipitate was subsequently fractionated into two portions (designated as G1 and G2), according to the order of elution on a Sephacryl S-100 high HR column (Fig. 2a). The low molecular weight fraction G2 was found to possess around 2.5-fold stronger antioxidative activity against ABTS radicals than that of fraction G1. The fraction G2 was successively subjected to a Q-sepharose Fast column and the antioxidative portion (non-adsorption faction) was pooled. The peptides in eluate were lyophilized and separated with Inertsil octadecylsilane (ODS)-3 semi-prep reverse phase column (10 × 250 mm) (Fig. 2b). The bioactive peak (designated as P1) was pooled and the purity was verified by an Agilent 6510 Q-TOF mass spectrometer (above 98%). The peptide then was subjected to Edman degradation. The primary sequence of amino acids of the purified peptide in this study was VECYGPNRPQF and the molecular weight was 1309 Da. In order to validate the antioxidative activity of the purified peptide, a synthetic hendeca-peptide with the same sequence was synthesized and tested. The synthetic peptide exhibited the same antioxidative activity as the purified peptide from algae protein hydrolysate (data not shown). The antioxidative peptide with an IC_{50} of 9.8 μg/ml was purified about 9.5-fold from pepsin hydrolysates (IC_{50} 93.4 μg/ml) using a four-step purification procedure, and the yield of purified peptide was approximately 0.94% from pepsin hydrolysates.

3.3. Digestive stability against gastrointestinal proteases

To understand the digestive stability against antioxidative peptide purified from algae protein hydrolysate, the purified peptide was first incubated with pepsin and then was subjected to successive digestion with pancreatin. The results showed that the antioxidative activity of the purified peptide could be preserved after treatment with these gastrointestinal enzymes *in vitro* ($p > 0.05$) suggesting that the peptide might be resistant to digestion in the gastrointestinal tract.

3.4. Antioxidative characterization of purified peptide

In the present study, we evaluated the protective effect of purified peptide from the algae protein waste hydrolysate against

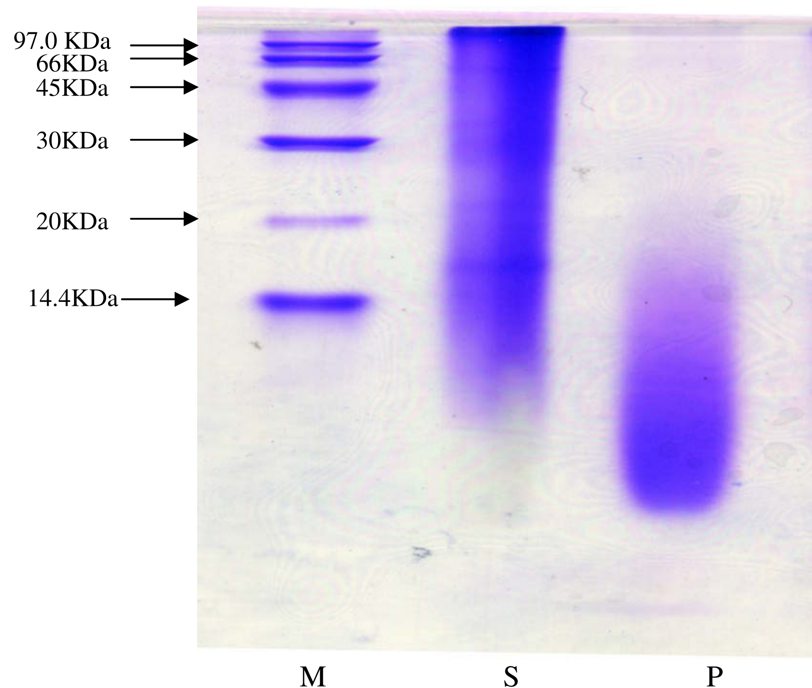

Fig. 1. Hydrolysis pattern of algae protein waste digested with pepsin enzyme on 20% SDS–PAGE. M: Marker, S: non-hydrolyzed algae protein waste, P: pepsin hydrolysate.

different sources of free radicals including the ABTS radicals, DPPH radicals, the peroxyl radicals, the hydroxyl radicals, and the superoxide radicals. The peroxyl radicals, superoxide radicals, and hydroxide radicals were more likely to be produced *in vivo*, hence, were physiologically more relevant than ABTS radicals and DPPH radicals. However, ABTS radicals and DPPH radicals were stable radical sources, so they also could provide a ranking order for antioxidants. Synthetic antioxidant BHT, natural antioxidant Trolox, and ascorbic acid were used as positive controls in this study.

The TEAC assay, which was the ABTS radical cation decolorization assay, was widely applied to evaluate the total antioxidative activity in both lipophilic and hydrophilic samples (Kong and Xiong, 2006). Fig. 3a illustrated the correlation between the percentage inhibition toward ABTS radicals and tested samples, including purified peptide, BHT, Trolox and ascorbic acid. Reaction of all samples in this study with ABTS radicals was concentration-dependent, and the 50% inhibition concentration (IC_{50}) value of the purified peptide was 9.8 ± 0.5 μM in comparison with the standard antioxidants Trolox, ascorbic acid, and BHT with IC_{50} values at 32.5 ± 1.3, 30.0 ± 0.8 and 57.4 ± 2.3 μM, respectively. The purified peptide from algae protein exhibited the highest ABTS radical scavenging activity by hydrogen or electron donation among them, and that of synthetic antioxidant BHT was the lowest.

The DPPH radical was one of the few stable radical sources and widely used to test the free radical-scavenging ability of various samples (Shimoji et al., 2002; Benvenuti et al., 2004; Sakanaka and Tachibana, 2006). As shown in Fig. 3b, the purified peptide showed a concentration-dependent increase of antioxidative activity for concentrations up to 60 μM, and that of the purified peptide was close in value to that observed for natural antioxidant ascorbic acid. Trolox had the highest antioxidative activity against DPPH radicals (IC_{50} 23.0 ± 1.8 μM), and that of synthetic antioxidant BHT was the lowest (IC_{50} 968.3 ± 2.6 μM) (data not shown). The IC_{50} values of LEELEEELEGCE from bullfrog skin was 16.1 μM (Qian et al. (2008b)), and YFYPEL from casein protein was 98 μM (Suetsuna et al., 2000), so the purified peptide (IC_{50} 58.0 ± 1.2 μM) does have moderate antioxidative activity against DPPH radicals.

The hydroxyl radical is considered the most reactive free radical in biological tissues; it easily reacts with molecules such as amino acids, proteins, and DNA, resulting in cell damage (Cacciuttolo et al., 1993). It was also believed to be an active initiator for peroxidation of lipids (Rajapakse et al., 2005; Elias et al., 2008). The hydroxyl radicals generating system was based on the Fenton reaction ($Fe^{2+} + H_2O_2$) in this assay. Increased concentrations of all tested samples exhibited dose-dependent scavenging activities with varying capacity. In addition, the hydroxyl radicals scavenging effect of the purified peptide (IC_{50} 8.3 ± 0.15 μM) showed a slightly lower radical scavenging activity than that of the natural antioxidant Trolox (IC_{50} 2.0 ± 0.3 μM), but much more effective than that of the synthetic antioxidant BHT (IC_{50} 44.4 ± 1.6 μM). The purified peptide LPHSGY from Alaska Pollock protein hydrolysate scavenged 35% of hydroxyl radicals at 53.6 μM (Je et al., 2005), and LKQELEDLLEKQE from oyster protein hydrolysate could quench hydroxyl radicals at IC_{50} values of 28.76 μM (Qian et al. (2008a)). The IC_{50} values of FDSGPAGVL, NGPLQAGQPGER, NADFGLNGLEGLA and NGLEGLK from giant squid protein hydrolysate were 90.90, 100.72, 497.32 and 428.54 μM, respectively (Mendis et al., 2005; Rajapakse et al., 2005). The results suggested that the highly active purified peptide having the lowest IC_{50} values (IC_{50} 8.3 ± 0.15 μM), was an effective antioxidant in quenching hydroxyl radicals.

Superoxide radicals could promote oxidative reactions due to its ability to reduce transition metals, release protein-bound metals and form perhydroxyl radicals which initiate lipid oxidation (Aikens and Dix, 1991; Elias et al., 2008). For cytoprotection against this reactive oxygen, superoxide dismutase (SOD), which catalyzes the neutralization of superoxide anion to hydrogen peroxide, is one of the defense mechanisms in the living cell. The scavenging effect of the purified peptide for superoxide radicals was investigated, and the antioxidative activity drastically increased with increasing concentration of the purified peptide. The purified peptide could effectively scavenge superoxide radicals (IC_{50} 7.5 ± 0.12 μM), and it was far superior to Trolox (IC_{50} 24.8 ± 0.4 μM) and BHT (IC_{50} 218.1 ± 2.1 μM). Suetsuna et al. (2000) reported that the peptide

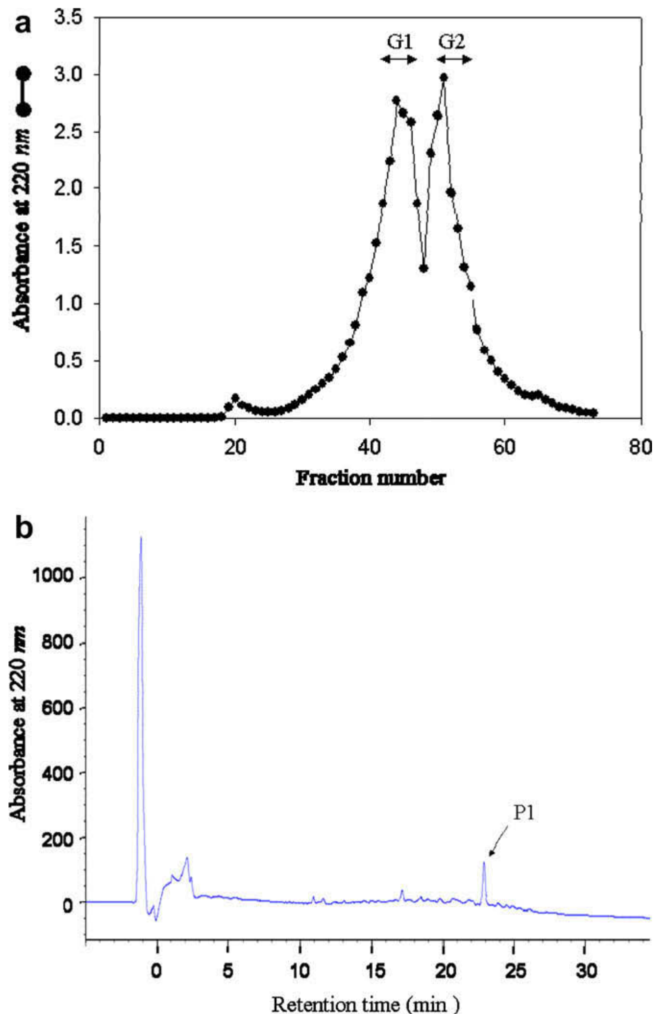

Fig. 2. (a) Elution profile of the active fraction from 40% to 80% ammonium sulfate fractions on a Sephacryl S-100 HR column (designated as G1 and G2). Separation was performed at a flow rate of 90 ml/h and collected at a fraction volume of 6 ml. (b) Reverse-phase HPLC pattern of antioxidative fraction from Q-sepharose Fast column on an ODS-3 semi-prep column (10 × 250 mm), and the separation was carried out with a linear gradient from 15% to 35% acetonitrile in 0.1% TFA for 30 min at a flow rate of 2 ml/min. The P1 fraction represented the purified peptide.

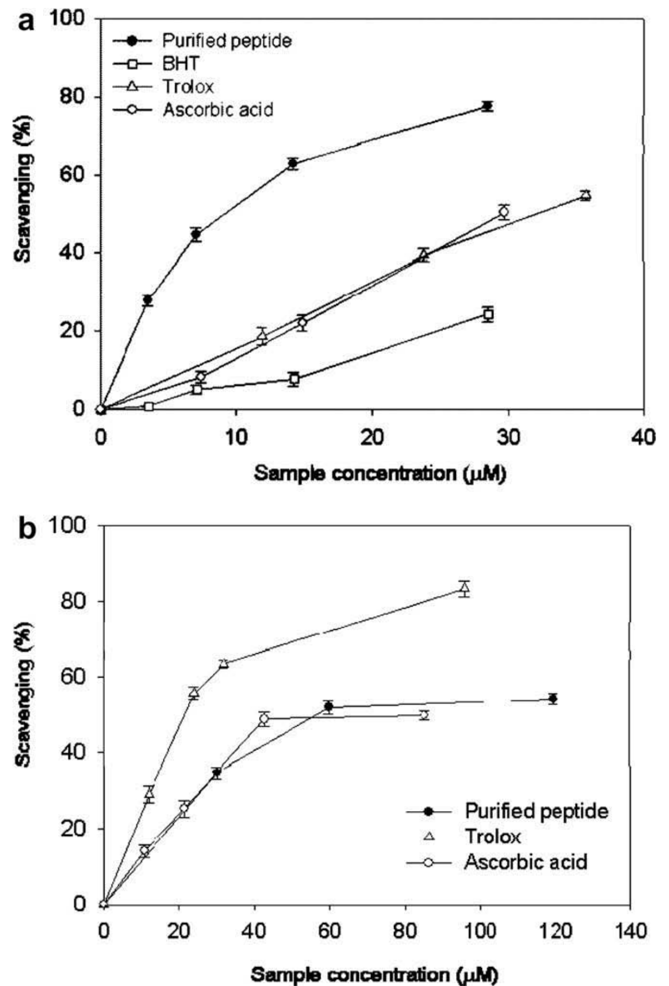

Fig. 3. Radical scavenging activity of the purified peptide and selected compounds toward (a) ABTS radicals (TEAC test); (b) DPPH radicals.

YFYPEL obtained from casein protein had a potent superoxide radical scavenging activity at IC_{50} values of 79.2 μM. Qian et al. (2008a) indicated that LKQELEDLLEKQE from oyster protein hydrolysate could quench superoxide radicals at IC_{50} values of 78.97 μM. For NADFGLNGLEGLA and NGLEGLK from giant squid muscle protein hydrolysate, the IC_{50} values were 669.34 and 573.83 μM, respectively (Rajapakse et al., 2005). These results suggested that the purified peptide from the algae protein waste hydrolysate in this study has the lowest IC_{50} value (IC_{50} 7.5 ± 0.12 μM). Therefore, it is possible that the purified peptide might have a high SOD-like activity thereby scavenging superoxide radicals in living cells. In addition, the scavenging effect of the purified peptide was also compared with the synthetic fragment-peptides. The results suggested that the C-terminal PNRPQF, with the highest superoxide radicals scavenging activity (IC_{50} of 6.36 ± 0.2 μM), was far superior to VECYG (IC_{50} 394.72 ± 1.5 μM) and CYGPNR (IC_{50} 38.81 ± 0.8 μM), so it played an important role in SOD-like activity (data not shown). Suetsuna et al. (2000) also reported the C-terminal sequence was important for the antioxidative activity.

The ORAC assay was further used to test the antioxidative capacity of the peptide to quench peroxyl radicals, and the ORAC value was usually reported as a Trolox equivalent (Elias et al., 2006). The protective effect of an antioxidant was measured by assessing the fluorescence decay curve (AUC) of the sample compared to a blank in which no antioxidant was presented. Fig. 4 depicted the time-dependent decay of fluorescein induced by AAPH for purified peptide from algae protein, and the results showed the purified peptide had a concentration-dependent increase in the inhibition of fluorescein decay. The antioxidative activity against peroxyl radicals was in the order of purified peptide > ascorbic acid > BHT, indicating that purified peptide was more active than the three reference antioxidants. The peroxyl radicals ($LOO^{\cdot}$) could be formed in the lipid peroxidation process, so the purified peptide could directly scavenge the peroxyl radicals to terminate the free radical chain reaction of lipid peroxidation. The purified peptide in the study showed the highest antioxidative activity against peroxyl radicals, which might be attributed to the presence of more residues of proline and tyrosine. The phenoxy radicals were much more stable, and had longer lifetimes than peroxy radicals, so the propagation of the radical-mediated peroxidizing chain reaction could be inhibited. The phenomenon was also found in the antioxidative peptides identified from milk proteins (Pihlanto, 2006).

The antioxidative properties of peptides were highly influenced by molecular mass and molecular structure properties (Suetsuna et al., 2000). Most of the reported peptides exhibiting antioxidative activity were those with low molecular weights (Rajapakse et al., 2005; Pihlanto, 2006) and were usually more effective in intestine

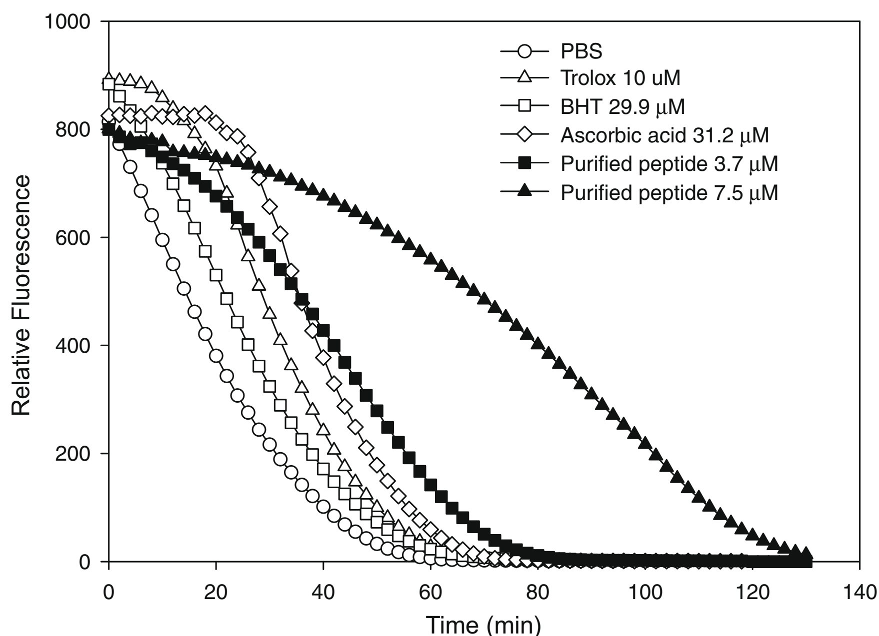

Fig. 4. Radical scavenging activity of the purified peptide and selected compounds toward peroxyl radicals (ORAC assay).

(Webb, 1986). The peptide in the study had a low molecular weight of 1309 Da, and it also was gastrointestinal enzyme tolerant. On the other hand, the characteristic amino acid composition of the peptide was also important for the antioxidative activity. The oxidative susceptibility of a given amino acid in the peptide to free radical attack was dictated in large part by its R groups (Elias et al., 2008). The most reactive amino acids tend to be those containing nucleophilic sulfur-containing side chains, aromatic side chains, or imidazole-containing side chains. The peptide sequence utilized was VECYGPNRPQF in this study. The Cys residue of the peptide was expected to protect lipid and other biomolecules by donating protons to peroxyl radicals and other free radicals in the cell**.** The aromatic amino acids (Tyr, Phe) could make active oxygen stable through direct electron transfer (Qian et al., 2008a,b). Three residues (Val, Pro, Phe) of these were hydrophobic amino acids, and hydrophobic amino acids might increase the affinity and reactivity to the cell membrane in the living cells (Mendis et al., 2005). The unique composition and sequence of amino acid residues might play a vital role in antioxidative activity of the purified peptide**.** Furthermore, the antioxidative activity was also limited by the structure of the polypeptide, and the solvent accessibility of the amino acids in the peptide was also important (Elias et al., 2008).

3.5. Protection effect of the purified peptide on oxidation-induced DNA damage

Further antioxidative study on oxidation-induced DNA damage was analyzed to elucidate the positive role of antioxidative peptide in prospective humans. In this study, the hydroxyl radicals generating system was based on the Fenton reaction ($Fe^{2+} + H_2O_2$). Fig. 5 showed that the super coil (SC) form in DNA was completely converted to the open circular (OC) form due to the hydroxyl radical damage (line 2). The purified peptide had the protective capacity increased with increasing peptide concentrations ranging from 10.6 to 84.9 μM in oxidation-induced DNA damage (lines 3–5). It further strengthened the ability of the purified peptide to protect hydroxyl radical induced damage.

3.6. Protection effect of the purified peptide on oxidation-induced cell damage

Free radicals caused alternation in the structure of biological membranes and further interfered with cellular integrity and metabolism leading to cellular toxicity (Butterfield et al., 2002). Debashis et al. (1997) also mentioned hydroxyl radicals were one of the major causative factors in stress-induced gastric ulceration. We assessed the protective effect of purified peptide on radical-mediated cellular injuries. The cell culture experiment was performed using the human gastric cancer cell lines (AGS) by exposing them to H_2O_2-inducing oxidative damage. The results showed hydrogen peroxide remarkably induced cell death in the AGS cell line by 80%, and pre-incubation with the various concentrations of the peptide had a dose-dependent protective effect in cell survival enhancement. The viability of AGS cells increased by 40% at 0.15 mM dosage of the peptide, compared with that of the cells without peptide pretreatment (data not shown). It follows that the purified peptide might protect cultured cell lines from H_2O_2 toxicity as assessed by the MTT reduction assay. However, the growth inhibitory effect in the human normal lung WI-38 cell line was not observed for the bioactive peptides (0.01–0.35 mM). Chemotherapy together with surgery was the mainstay in treatment of gastric cancer (Camp et al., 2004). Antioxidants had also been demonstrated to be effective in reducing the risk of carcinogenesis

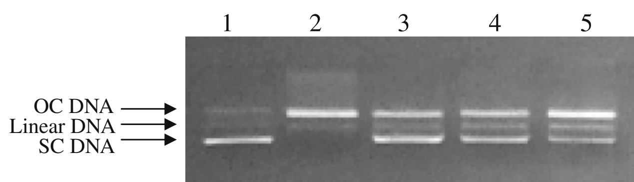

Fig. 5. Protective effect of purified peptide on hydroxyl radicals-induced oxidation of plasmid DNA. Lane 1, pET 28 plasmid DNA; Lane 2, $FeSO_4$ and H_2O_2 treatment (as DNA damage control); Lanes 3–5, $FeSO_4$ and H_2O_2 treatment in the presence of purified peptide at the concentrations of 84.9 μM; 42.4 μM and 10.6 μM, respectively.

partially due to their antioxidative activity (Yang et al., 2001; Fazlul and Li, 2002). The peptide developed in the study herein had excellent antioxidant properties and might also be employed as an adjuvant to the conventional therapeutic modalities for gastric cancer potency.

4. Conclusion

There are numerous reports on bioactive compounds in microalgae. There are no reports to date regarding the antioxidative effect of peptides from algae protein waste. We investigated the antioxidative potency of the peptide from algae in different mechanisms of oxidation *in vitro.* In addition, the protective abilities of the purified peptide in oxidation-induced cell death and DNA damage were also tested. We have shown that the peptide from algae protein has a greater efficiency in scavenging various free radicals compared to that observed for the other compounds we tested in this study. The peptide from the algae protein waste has the potential to be a good dietary supplement for prevention of oxidative stress-related diseases such as atherosclerosis, coronary heart disease and cancer. This study also proposes an alternative method for usage of the by-product of the algae essence extraction process, which is normally employed as a low-value animal feed.

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