

Anticancer and Antioxidant Activities of the Peptide Fraction from Algae Protein Waste

I-CHUAN SHEIH,[†] TONY J. FANG,^{*†} TUNG-KUNG WU,^{*†} AND PENG-HSIANG LIN^{†,§}

[†]Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan, Republic of China, [‡]Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai street, Hsinchu 30068, Taiwan, Republic of China, and [§]Department of Food Science and Technology, Central Taiwan University of Science and Technology, 666 Buzih Road, Taichung City 40601, Taiwan, Republic of China

Algae protein waste is a byproduct during production of algae essence from *Chlorella vulgaris*. There is no known report on the anticancer peptides derived from the microalgae protein waste. In this paper, the peptide fraction isolated from pepsin hydrolysate of algae protein waste had strong dose-dependent antiproliferation and induced a post-G1 cell cycle arrest in AGS cells; however, no cytotoxicity was observed in WI-38 lung fibroblasts cells in vitro. The peptide fraction also revealed much better antioxidant activity toward peroxy radicals and LDL than those of Trolox. Among these peptides, a potent antiproliferative, antioxidant, and NO-production-inhibiting hendecapeptide was isolated, and its amino acid sequence was VECYGPNRPF. These results demonstrate that inexpensive algae protein waste could be a new alternative to produce anticancer peptides.

KEYWORDS: Algae; peptide; *Chlorella vulgaris*; antiproliferative

INTRODUCTION

Today cancer is the largest single cause of death in men and women (1), and chemoprevention has been a promising anticancer approach aimed at reducing the morbidity and mortality of cancer by delaying the process of carcinogenesis. A variety of compounds from nature sources have been shown to be beneficial for the inhibition of cancer, such as flavonoids, phenolic acids, carotenoids, etc. (2); the mechanisms which suppress tumorigenesis often involve inhibition of tumor cell mediated protease activity (3), attenuation of tumor angiogenesis (4), promotion of cell cycle arrest (5, 6), induction of apoptosis (7) and immunostimulation (8), etc. In addition, Chinery et al. (9) also reported their use with the chemotherapy agents 5-fluorouracil and antioxidants could cause complete remissions in colorectal cancer, where only partial remission is possible with chemotherapy agents only; therefore, antioxidants have been proposed to have potential for the prevention and treatment of diseases associated with active oxygen species, especially in cancer diseases (3, 10). Moreover, experimental and epidemiological evidence suggests that anti-inflammatory drugs may also decrease the incidence of mammary cancer, tumor burden, and tumor volume (11).

Recently, peptides from enzymatic hydrolysis of various food proteins exert quite different bioactivities (12), but there are few publications on anticancer peptides (7, 13–17). Marine proteins have wide sources and abundant content; therefore, activated peptides from marine food have been gaining attention in the past

few years. The applications of microalgae are as nutritional supplements, natural dyes, and skin care products, but there is no study reporting the anticancer activity of microalgae protein-derived peptides. Algae protein waste of the microalgae *Chlorella vulgaris*, which is normally discarded as low-cost animal feed, is a byproduct during production of algae essence from the microalgae in Taiwan. In previous investigations, we reported the angiotensin I converting enzyme (ACE) inhibitory property and antioxidant property of the peptide (18, 19) from *C. vulgaris* protein waste. In this study, we purified the anticancer oligopeptides from algae protein waste and the effects of peptides on cell proliferation, cell cycle arrest, antioxidant activity toward peroxy radicals, and low-density lipoprotein (LDL) were investigated.

MATERIALS AND METHODS

Materials. Algae protein waste containing over 50% protein content, the byproduct in algae essence manufacturing of Taiwan, was dried and kept at 20 °C prior to use. Cytochrome C, aprotonin, gastrin, Leu-Gly, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, Ham's F-12 medium fetal bovine serum (FBS), sulfanilamide, and *N*-naphthylethylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorescein, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), and 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) was purchased from Aldrich (Milwaukee, WI). Sephacryl S-100 HR, Q-sepharose Fast Flow, and Superdex peptide HR 10/30 was purchased from Pharmacia Biotech. Co. (Uppsala, Sweden). Pepsin was purchased from Nacalai Tesque (Kyoto, Japan). Human gastric cancer cell lines AGS, human normal lung cell WI-38, human colon adenocarcinoma cells C2BBel, human hepatoblastoma cell lines Hep G2 human cervical epithelioid carcinoma cells Hela, and mouse BALB/c macrophage RAW 264.7 cells were purchased from Food Industry Research Development Institute (Hsinchu, Taiwan). RPMI

*To whom correspondence should be addressed. T.J.F.: tel, +886-4-22861505; fax, +886-4-22876211; e-mail, tjfang@nchu.edu.tw. T.K.W.: tel, +886-3-5712121-56917; fax, +886-3-5725700; e-mail, tkwmll@mail.nctu.edu.tw.

medium (Roswell Park Memorial Institute) and DMEM medium (Dulbecco's modification of Eagle's medium) was purchased from JRH Biosciences (Lenexa, KS).

Preparation of Anticancer Peptides. Pepsin hydrolysate, the algae protein waste peptide (APWP), was prepared as follows: 10% (wt/vol) of APWP was digested by pepsin using enzyme to substrate of 2% (w/w) at pH 2.0 and temperature of 50 °C for 15 h. The digestion was adjusted to pH 7.0 and heated in a boiling water bath for 10 min at the end of reaction to inactivate the enzyme. The pepsin hydrolysate was then filtered through 0.45 μ m filter and collected for further study.

Isolation and Purification. The isolation and purification of peptides with anticancer properties was as described by Sheih et al. (18) with modification. Ammonium sulfate was added to a concentration of 40% saturation in the permeated solution from APWP, and the precipitated protein was removed by centrifugation (10 000g, 30 min). The concentration of ammonium sulfate in the supernatant was increased to 80% saturation successively, and the 40–80% precipitate was dissolved in a small volume of distilled water. The solution was fractionated using a Sephacryl S-100 high HR column (ψ 2.6 \times 70 cm) and detected the peptides at OD 210 nm. The active fraction with higher anticancer activity at the same total protein concentration in the AGS cells were subsequently loaded onto a Q-sepharose Fast Flow column (ψ 2.6 \times 40 cm), which pre-equilibrated with 20 mM Tris-HCl buffer solution (pH 7.8). The separation was performed with 1.0 M NaCl in the same buffer solution. The fractions with anticancer activity were then collected, dialyzed in deionized water, and lyophilized. The concentration of the peptides was determined by the Pierce micro BCA protein assay kit using the BSA as standard (Thermo Fisher Scientific Inc.), and the anticancer activity was assayed by means of a cytotoxicity assay at each purification step.

Cytotoxicity on AGS Cells. AGS cells were seeded in 96-well plates at about 3×10^3 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 different concentrations of the peptide fraction from APWP for 3 days. At the end of this period, the viability of AGS cells was measured using the MTT method, and the results were given as relative growth ratio of peptide-treated cells to untreated controls (designed as 1 or 100%).

Cell Cycle Kinetics Analysis. AGS cells were seeded in a 6 cm dish at about 5×10^5 cells/dish and treated with 36 μ g/mL peptide fraction from APWP for 24 and 48 h, respectively. After treatment, the cells were harvested with 0.25% trypsin at 24 and 48 h and washed with cold PBS buffer. The cells were collected by centrifugation (1000g, 5 min) and fixed in 70% ethanol. The cells were then stained with 20 μ g/mL propidium iodide containing 20 μ g/mL RNase (DNase free) for 2 h. The cells were analyzed with flow cytometry (BD FACSCalibur Flow cytometry system; BD Biosciences, San Jose, CA), and the results are expressed as a percentage of the cells in each phase (5).

Trolox Equivalent Antioxidant Capacity (TEAC) Assay. The total antioxidant activity of the peptide fraction from APWP was measured using a TEAC assay as described by Arts et al. (20). A 10 μ L portion of peptide solution was mixed with 200 μ L of the ABTS radicals, and the absorbance was monitored at 734 nm for 2 min. A decrease at 734 nm between the blank and a sample was used for calculating the ABTS radical scavenging capacity. The IC₅₀ value was defined as the concentration of peptides required to scavenge 50% of ABTS radicals. Trolox was used as a positive control.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ability of the peptide fraction from APWP to scavenge peroxy radicals using an ORAC assay was performed as described by Alberto et al. (21). The reaction was carried out in 75 mM phosphate buffer (pH 7.4). A 150 μ L portion of 96 nM fluorescein and 20 μ L of peptide solution were preincubated for 5 min at 37 °C, and the analysis was initiated by addition of 30 μ L of 320 mM AAPH. Fluorescein decay curves between the blank and the peptides were conducted. Trolox was used as the calibration solution, and all values were expressed as Trolox equivalents.

Antioxidant Activity toward Copper-Mediated Oxidation of Human LDL. The copper-mediated oxidation of human LDL was measured according to the method of Shyu and Hwang (22). The oxidation of a reaction mixture containing 150 μ L of LDL (60 μ g/mL cholesterol) and 20 μ L of peptide fraction from APWP was initiated by addition of a

final concentration of 5 μ M CuSO₄ in PBS buffer. The formation of conjugated dienes was determined continuously by measuring absorbance at 232 nm at 10 min intervals over 24 h using a PowerWave spectrophotometer (BIO-TEK, Winooski, VT). The indices of antioxidant activity measured were lag times of LDL oxidation. Trolox was used as a positive control.

Molecular Weight Distribution. The molecular weight distribution of the peptide fraction from APWP was determined by a Superdex peptide HR 10/30 column at a flow rate of 0.5 mL/min. The standard molecular weight markers used were as follows: cytochrome C (MW 12 327 Da), apptinin (MW 6500 Da), gastrin (MW 2098 Da), and Leu-Gly (MW 188.2 Da) (23).

Peptide Determination. The peptide fraction from APWP was reloaded on an ODS C18 reverse-phase column (10 \times 250 mm) with a linear gradient from 0 to 40% acetonitrile in 0.1% TFA for 40 min at a flow rate of 2 mL/min. A single bioactive peak was collected and the purity was verified by an Agilent 6510 Q-TOF mass spectrometer (Agilent Technologies Inc. CA, USA). The amino acid sequence was determined by an automated Edman degradation with an Applied Biosystems Procise 494 protein sequencer (Foster City, CA, USA).

Protection Effect on Oxidation-Induced Cell Damage. AGS cells were seeded in 96-well plates at about 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 purified peptide for 2 h, 0.03 mM H₂O₂ was added to cells and incubated for another 2 h, and the effect of the peptide on the cytoskeletal morphology was investigated by phase-contrast micrograph.

Nitrite Effect on RAW 264.7 Cells. Nitrite accumulation was to be an indicator of NO synthesis. The RAW 264.7 cells were seeded in 96-well plates at about 5×10^4 cells/well and cultured in DMEM medium containing 10% fetal bovine serum (FBS) at 37 °C. After 4 h, the cells were treated with different concentrations of the peptide and LPS solution (1 μ g/well) for an additional 24 h. Then 80 μ L of cell culture medium was coinoculated with 160 μ L of Griess reagent (1% sulfanilamide, 0.1% *N*-naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) at room temperature for 20 min, and the absorbance at 540 nm was measured. The amount of nitrite in the sample was calculated from a sodium nitrite standard curve freshly prepared in culture medium.

RESULTS AND DISCUSSION

Separation of the Anticancer Peptides. Pepsin hydrolysates from APW were fractionated with ammonium sulfate, and the 40–80% fraction was collected. The 40–80% fraction precipitate was then dissolved in a small volume of distilled water, and two fractions were produced by a Sephacryl S-100 high HR column, according to the order of elution on a Sephacryl S-100 high HR column. The low molecular size fraction (designated as S2) was found to possess about 4-fold stronger cytotoxicity activity than that of the high molecular size fraction (designated as S1) (Figure 1a). The fraction S2 was further subjected to a Q-sepharose Fast Flow column. The results showed that two portions were obtained; the nonbinding fraction (designated as Q1) had higher cytotoxicity activity, and the binding fraction (designated as Q2) had no cytotoxicity activity against AGS cells (Figure 1b). The nonbinding peptides were collected, dialyzed, and lyophilized.

The total cell number was an indicator of the proliferative activity and cytotoxicity effects of the cell population. The peptide fraction showed a dose-dependent growth inhibitory effect on the AGS cell line, and the IC₅₀ value of 70.7 ± 1.2 μ g/mL against AGS cells was obtained from low-activity pepsin hydrolysates (IC₅₀ 1.74 ± 0.3 mg/mL). In addition, the inhibitive effect of the peptide fraction was not discovered with other cancer cells (such as human colon adenocarcinoma cells C2BBel, human hepatoblastoma cell lines Hep G2, and human cervical epithelioid carcinoma cells Hela) (data not shown); therefore, the effect of the peptides is cell type specific inhibition, with tumorous cells

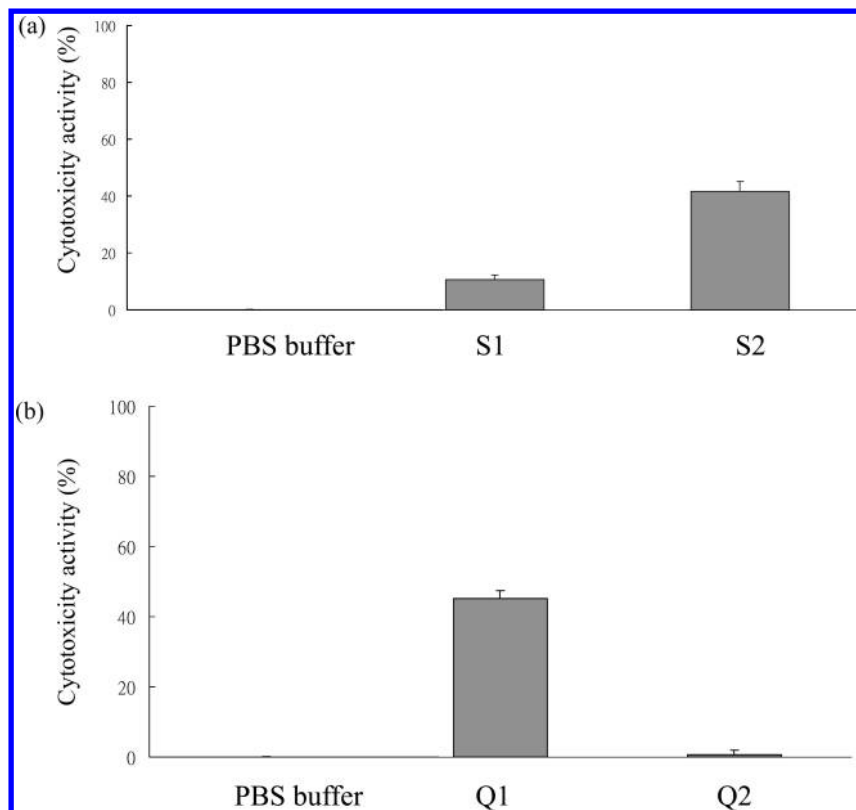


Figure 1. (a) Cytotoxicity activity on AGS cells of active fraction from 40 to 80% ammonium sulfate fraction on a Sephacryl S-100 HR column. The fractions are designated S1 and S2. (b) Cytotoxicity activity of active fraction S2 on a Q-sepharose Fast Flow column. The fractions are designated Q1 (nonbinding fraction) and Q2 (binding fraction). The values are represented as the mean \pm SD ($N = 3$) with the same total protein.

responding differently from their nontumorigenic counterparts (24). The purification factor was about 24.6, and the yield was approximately 2.9% from pepsin hydrolysates using a three-step purification procedure.

The function properties of peptides were highly influenced by molecular mass and molecular structure properties; therefore, the process using enzyme hydrolysis of the native protein in this study was important. Our study showed the peptide fraction from APWP had moderate cytotoxicity activity against AGS cells. In addition, no growth inhibitory effect in human normal lung WI-38 cell line by the peptide fraction was observed (data not shown). Numerous macroalgae have shown potent cytotoxic activities, and polysaccharides and terpenoids are considered as contributing factors for anticancer effects among those (25). There has been no study on anticancer peptides from microalgae protein, and we are the first to report AGS cell inhibition by peptides from algae protein waste, to the best of our knowledge.

Effect of Peptides on AGS Cell Cycle. It is critical to maintain the homeostasis between cell proliferation and cell death in normal mammalian tissues; therefore, the process in which the rate of cell proliferation exceeds that of cell loss in tumor cells might be inhibited or perturbed (26). In this study, the cells were treated with the peptide fraction at a concentration of 36 $\mu\text{g}/\text{mL}$ for different time periods. After harvesting, the distribution of cell population in each phase of cell cycle was examined by flow cytometry. After 24 h of exposure to the peptides, the cells in the G1 phase decreased and a time dependent increase in the sub-G1 peak appeared. The peptide fraction arrested the cells in a post-G1 phase, and the numbers of sub-G1 cells gradually increased from 24 to 48 h after the peptide fraction administration (Figure 2), similarly to the antiproliferative activity against human lymphoma cell (U937) of peptide fraction from anchovy at

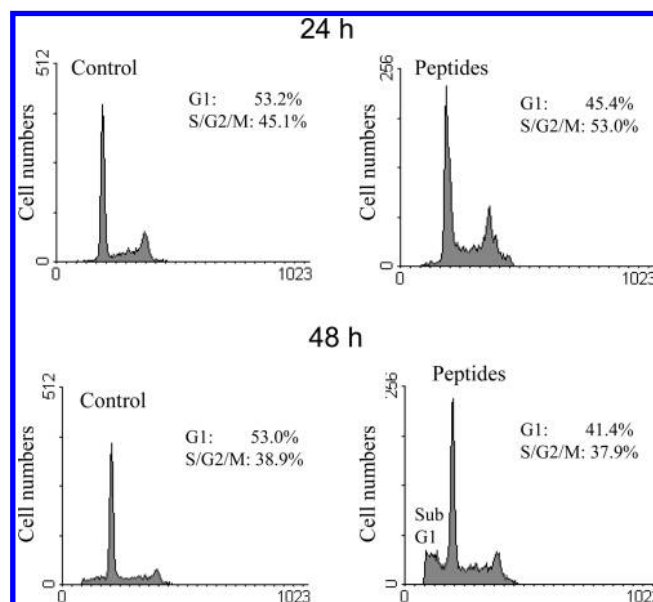


Figure 2. Effect of the peptide fraction from APWP on the cell cycle of AGS cells for 24 and 48 h.

500 $\mu\text{g}/\text{mL}$ (7). The results suggested that the peptides effectively induced cell death and inhibited the growth of AGS cells.

Antioxidant Characterization. The antioxidant activity of the peptide fraction from APWP was evaluated by means of TEAC, ORAC, and oxidation of human LDL assays. The TEAC assay was widely applied to the assay sum of the antioxidant capacity of the parent compound and that of the oxidation products of the parent compound (20), while the ORAC assay and anti-LDL-oxidation assay were used to test the lipid antioxidant ability

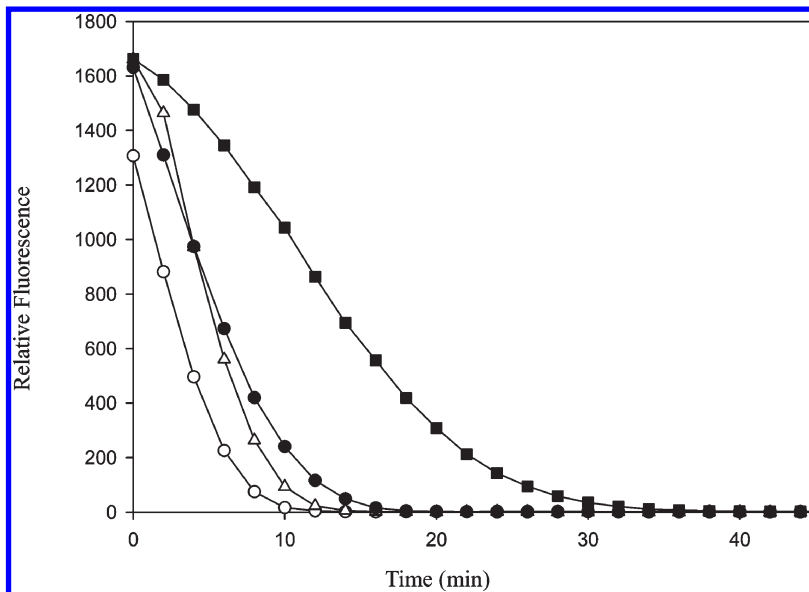


Figure 3. Peroxyl radical scavenging activity (ORAC assay) of the peptide fraction from APWP: (○) PBS buffer; (●) 7.6 ng/mL; (■) 76 ng/mL; (△) Trolox 156 ng/mL.

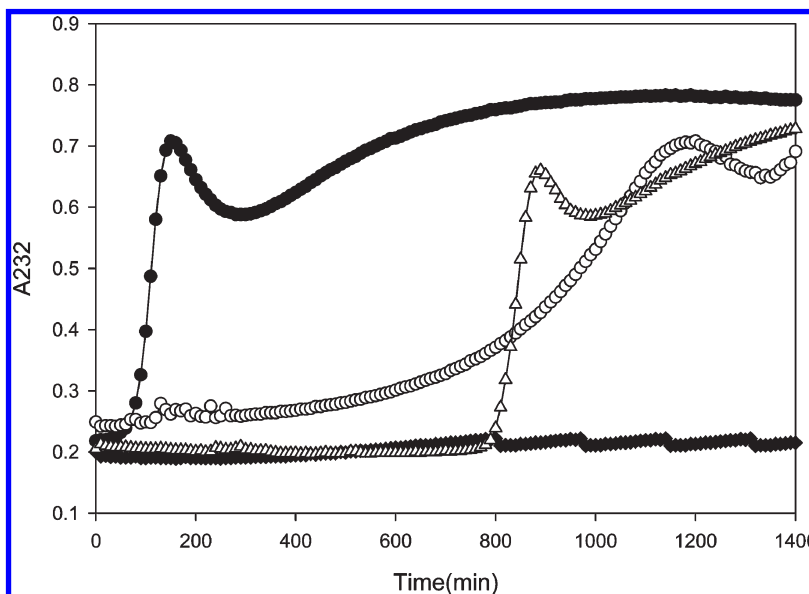


Figure 4. Effect of the peptide fraction from APWP on the Cu^{2+} -induced oxidation of human low-density lipoprotein (LDL): (●) PBS buffer; (△) the peptides 1.52 $\mu\text{g/mL}$; (◆) no Cu^{2+} ; (○) Trolox 10 $\mu\text{g/mL}$.

of the peptide fraction. The peroxy radicals and oxidation of human LDL were more likely to be produced *in vivo*; thus, they were physiologically more relevant in the antioxidant assay system.

The ABTS radicals were stable radical sources; therefore, they also could provide a ranking order for antioxidants. The IC_{50} value of the peptide fraction from APWP was $42.5 \pm 0.8 \mu\text{g/mL}$, in comparison to zero antioxidant activity from unhydrolyzed algae protein. The hydrolysis of the algae protein waste was necessary in order to release different peptide sequences with bioactivity from intact algae protein.

Lipid peroxidation is responsible for various pathological phenomena in biological systems. The antioxidant activity was then confirmed in lipid oxidation by using the ORAC assay, and **Figure 3** depicts the time-dependent decay of fluorescein induced by AAPH for the peptide fraction from APWP at different concentrations. The results showed the peptide fraction had a concentration-dependent increase in the antioxidant activity

against peroxy radicals, where the antioxidant activity of the peptide fraction at a concentration of 7.6 ng/mL (197.0 ± 2.1 ng/mL Trolox equivalent) was about 26-fold stronger than that of Trolox. The peroxy radicals ($\text{LOO}\cdot$) could be formed in the lipid peroxidation process; therefore, the peptides could directly scavenge the peroxy radicals to terminate the free radical chain reaction of lipid peroxidation.

LDL is particularly susceptible to lipid peroxidation, due to its high content of polyunsaturated fatty acids, and copper is a prooxidative transition metal in the lipid system and is typically found in food ingredients. The protective effect of the peptide fraction from APWP on LDL oxidation against copper ion was tested in that regard. **Figure 4** illustrates the LDL oxidation kinetics induced trace copper ion in the absence and in the presence of the peptides and Trolox; the peptide fraction could inhibit Cu^{2+} -mediated oxidation of LDL by lengthening the time to 776 ± 2.3 min, compared to 74 ± 1.2 min for the control

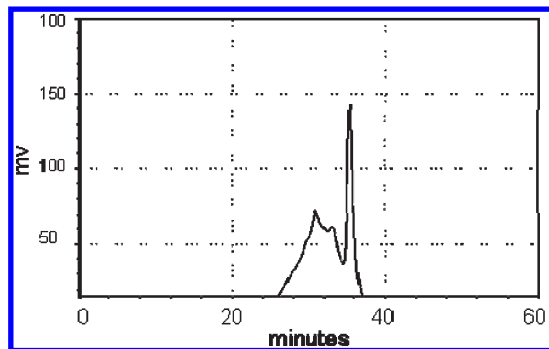


Figure 5. Determination of molecular weight of the peptide fraction from APWP on HPLC with a Superdex peptide HR 10/30 column. HPLC was carried out with 0.2 M phosphate buffer as the mobile phase at a flow rate of 0.5 mL/min.

sample, for conjugated diene formation at a peptide concentration of 1.52 $\mu\text{g/mL}$. The antioxidant activity of the peptide fraction was about 6.5-fold higher than that of Trolox (10 $\mu\text{g/mL}$ over 803 ± 2.1 min). One of the protection mechanisms against LDL oxidation might be related to the strong capacity of the peptides to chelate the Cu^{2+} (27).

Accumulating evidence indicated that active oxygen and free radicals would attack key biological molecules such as DNA, protein, and lipid that lead to many degenerative disease conditions. Many studies have demonstrated low antioxidant activity in the tumor cells of the injured organ (pulmonary, hepatic, pancreatic cells, gastric cancer, etc.) (28). Dietary kelp could inhibit mammary carcinogenesis by enhancing antioxidant enzyme activity and reducing lipid peroxides in livers of treated rats; therefore, the algal antioxidant mediated mechanism had been hypothesized as a contributing factor for anticancer activity (29). Agents that can protect cells against reactive oxygen species by scavenging free radicals are suitable candidates as powerful chemopreventive agents (15). Gastric cancer is one of the most frequent cancers in the world, and chemotherapy together with surgery is the mainstay in the treatment of gastric cancer. The peptide fraction herein has excellent antioxidant properties and it might be useful as an adjuvant for gastric cancer modality.

Molecular Weight Distribution. Gel permeation chromatography (GPC) with an HPLC system was used to study the molecular weight distribution of the peptide fraction from APWP. Molecular weight standards, including cytochrome C (MW 12 327 Da), apurotonin (MW 6500 Da), gastrin (MW 2098 Da), and Leu-Gly (MW 188.2 Da), were used, and their retention times were found to be 18.2, 22.5, 29.1, and 41.5 min, respectively, in our assay system. The results (Figure 5) showed that the bioactive peptides from APWP were all located at a lower molecular weight pool (< 6500 Da), and most of them ($> 90\%$) had a molecular weight above about 200 Da and below 2000 Da in this study by comparing the sample elution peak time to the elution peak time for standards. Our results showed that small peptides were generated by adequate control of the enzyme hydrolysis process, which might be more effectively absorbed than intact protein.

Peptide Determination. The peptide fraction from APWP was further separated successively by a reversed-phase HPLC column, and a purified peptide was attained. The purity was verified by an Agilent 6510 Q-TOF mass spectrometer ($> 95\%$), and the peptide was subjected to Edman degradation experiments for amino acid sequence determination. The determined sequence was obtained as Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe, with a molecular mass of 1309 Da and a dose-dependent inhibition effect of the AGS cells with an IC_{50}

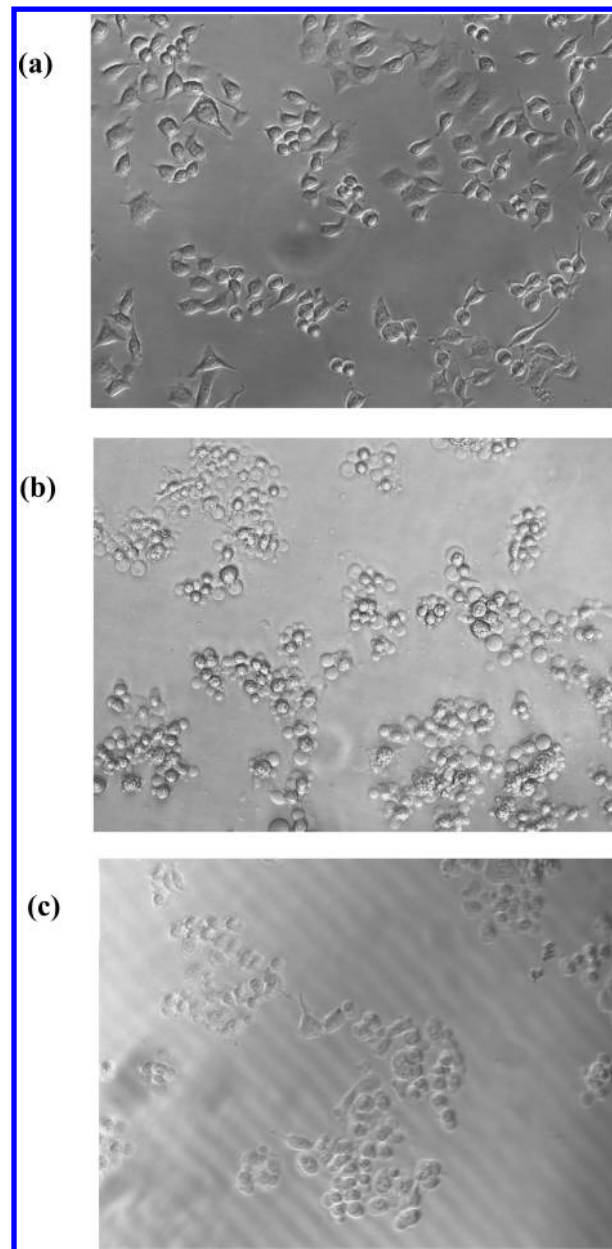


Figure 6. Phase-contrast micrographs of H_2O_2 -induced oxidation of AGS cells: (a) cells without H_2O_2 treatment; (b) cells incubated with 30 μM H_2O_2 ; (c) cells coincubated with 30 μM H_2O_2 + purified peptide (0.15 mM).

value of 256.4 ± 1.2 μM . The yield was approximately 0.94% from pepsin hydrolysates using the aforementioned four-step purification.

Protection Effect of Hendecapeptide on Oxidation-Induced Cell Damage. Hydroxyl radical is one of the major causative factors in stress-induced gastric ulceration (30); thus, we assessed the protective effect of hendecapeptide on radical-mediated cellular injuries using AGS cells exposed to H_2O_2 -induced oxidative damage. The microscopic pictures in Figure 6 show that the control cells have an intact morphology, and the H_2O_2 -treated cells showed significant bubbles in cell morphology. Hydroxyl radical caused alternation in the structure of biological membranes and further destroyed the cellular integrity. However, the cells that were treated with purified peptide 2 h prior to H_2O_2 treatment had a protective effect against H_2O_2 injury.

Nitrite Effect of Hendecapeptide on RAW 264.7 Cells. It is well-known that macrophages play an important role in the immune response and in host defense against bacteria and tumors;

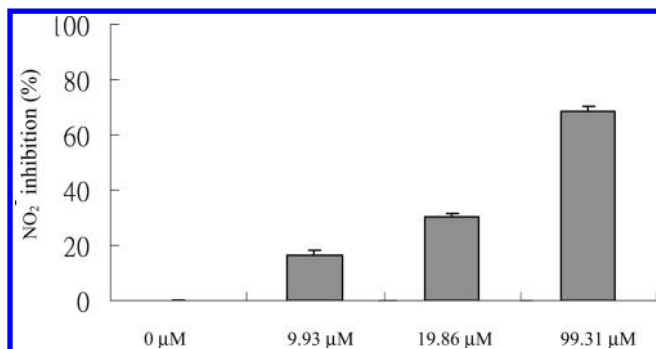


Figure 7. Inhibition of NO₂⁻ production by RAW 264.7 cells stimulated with LPS (1 µg/well) for 24 h with and without the purified hendecapeptide derived from algae protein algae. The values are represented as the mean ± SD (N = 3).

furthermore, inflammatory macrophage products, such as nitric oxide (NO), may result in cytotoxicity. The effect of the hendecapeptide on NO production was also investigated, and inhibition of LPS-induced NO production was observed at increasing concentrations of peptide and was found to be concentration dependent, with an IC₅₀ value of 42.4 µM (Figure 7).

Anticancer peptides have attracted concern recently due to their characteristics of multifunction, high sensitivity, stability, and so on. There have been a few publications on anticancer peptides from food protein, such as fish sauce (7), soy protein (13, 14), mollusk protein (15), milk protein (16), and beef protein (17); however, no study had used algae protein waste as the protein source for anticancer peptides. The results of our study demonstrate the effect of oligopeptides from APWP on growth inhibition and promotion of cell cycle arrest in human gastric cancer cells, and these species also exhibit antioxidant activity much better than that of natural antioxidants. This result suggests that the peptides could be potentially useful adjuncts in the treatment of gastric cancer. Hence, its easily accessible source makes algae protein waste attractive as a protein source in the future industrial production of functional peptides.

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