Separation, Antitumor Activities, and Encapsulation of Polypeptide from Chlorella pyrenoidosa

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DOI 10.1002/btpr.1725
Published online April 18, 2013 in Wiley Online Library (wileyonlinelibrary.com)

Chlorella pyrenoidosa is a unicellular green algae and has been a popular foodstuff worldwide. However, no reports on the antitumor peptides from such a microalgae are available in the literature. In this study, using low-temperature high-pressure extraction, enzymatic hydrolysis, ion exchange, and gel filtration chromatography, we separated a polypeptide that exhibited inhibitory activity on human liver cancer HepG2 cells, and named the polypeptide CPAP (C. pyrenoidosa antitumor polypeptide). Furthermore, the micro- and nanoencapsulation of CPAP were investigated by using two methods: complex coacervation and ionotropic gelation. The in vitro release tests revealed that CPAP was well preserved against gastric enzymatic degradation after micro/nanoencapsulation and the slowly controlled release in the intestine could be potentially achieved. These results suggest that CPAP may be a useful ingredient in new functional and pharmaceutical applications. © 2013 American Institute of Chemical Engineers

Keywords: Chlorella pyrenoidosa, polypeptides, antitumor, encapsulation, controlled release

Introduction

Cancer is the largest cause of death in humans although there has been great progress in cancer treatments such as chemotherapy, surgery, and radiation. Recently, a variety of dietary compounds from natural sources have been shown to inhibit cancer cell growth, including flavonoids, isothiocyanates, curcumin, resveratrol, and so on. In particular, some proteins and peptides from foods have been reported to show anticancer or antiproliferative activities. For example, lunasin, a novel peptide identified in soybean and other seeds, was illustrated to suppress in vitro and in vivo chemical carcinogen-induced tumorigenesis. The peptides prepared from enzymatic hydrolysates of tuna dark muscle possessed antiproliferative activity on human breast cancer cells. A novel polypeptide (Mere15), purified from Meretrix meretrix Linnaeus, was shown to significantly suppress the growth of human lung adenocarcinoma A549 xenograft in nude mice.

Generally, to separate these bioactive peptides from protein hydrolysates, a simple method is to use column chromatography (ion exchange chromatography or gel filtration chromatography). Ion exchange chromatography allows the separation of ions and polar molecules based on their charge and size, respectively. After separation of polypeptides, for the successful delivery of them to the gastrointestinal tract, the use of encapsulation technology is essential to protect their degradation in the stomach owing to the existence of complex enzymes and gastric acids. Several microencapsulation technologies have been developed for use in the food and pharmaceutical industry, such as hydrogels, nanoemulsions, and nanoparticles. To prepare them, two typical methods including complex coacervation and ionotropic gelation are frequently used. The former refers to the phase separation of a liquid precipitate/phase when solutions of two hydrophilic colloids are mixed under suitable conditions, and the latter is based on the ability of polyelectrolytes counter ions to crosslink to form hydrogels.

Microalgae have the potential to become a novel source of bioactive molecules to enhance the nutritional and functional quality of foods. Chlorella, a type of unicellular green algae, has been a popular foodstuff worldwide. It has been reported to have certain beneficial physiological effects such as antihypertensive, antioxidative, hypcholesterolemic, and antitumor activities in animal and human studies. However, the antitumor activity of peptides derived from Chlorella pyrenoidosa is not extensively studied. In this article, we separated C. pyrenoidosa antitumor polypeptide (CPAP) and then the micro/nanoencapsulation and in vitro release properties of CPAP were investigated.

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Materials and Methods

Materials and chemicals

C. pyrenoidosa powder (48.2% of total protein contents) was obtained from Yuejian Bioengineering Co. Ltd., China. Bio-Rad Protein Assay Kit was purchased from Bio-Rad Laboratories Inc. Papain (800,000 U/g, M0009) and trypsin (1:250 U/g, M0022) were obtained from Guangzhou Qiyun Biotech Co., Ltd., China. Alcalase 2.4 L (P4860) was obtained from Sigma, USA. DEAE-52 (M10068) was from Whatman and Sephadex G-50 (M10097) from Pharmacia, Liluo Science & Technology Instrument Co., Guangzhou, China. Chitosan (Q10022), alginate (Q10073), and sodium tripolyphosphate (Q10045) were from Puboxin Biotech Co. Ltd., Beijing. Other reagents were of analytical grade and commercially available.

Extraction of proteins

Ten grams of C. pyrenoidosa powder was dissolved in 200 mL of distilled water and was subjected to protein extraction using a Low-Temperature High-Pressure Continuous Flow Cell Breakage machine (Juneng Biology & Technology Co., Ltd., Guangzhou, China) at 6°C. The resulting fraction with the highest antitumor activity from the ion exchange chromatography separation was further separated by gel filtration chromatography. Five microliters of bioactive fraction dissolved in distilled water at a concentration of 20 mg/mL were loaded onto a Sephadex G-50 (1.6 × 30 cm). The column was eluted with distilled water at a flow rate of 0.4 mL/min, respectively. The eluate was collected (4 mL/tube) and detected at 280 nm. The eluates at the same peak were combined, concentrated by a rotary evaporator, and freeze-dried. The fraction with the highest antitumor activity was subjected to the next separation step.

Hydrolysis of proteins

The extracted proteins were separately hydrolyzed with three proteases (papain, trypsin, and alcalase) under each optimal condition. For papain, the hydrolysis was conducted in a water bath shaker (SHA-CA, Taicang, China) with the ratio of enzyme to substrate (E/S) 3% w/w, pH 6, temperature 70°C, and reaction time 4 h. For trypsin, with the ratio of enzyme to substrate (E/S) 3% w/w, the following conditions were used: pH 7; temperature, 47°C; and reaction time, 8 h. For alcalase, with the ratio of enzyme to substrate (E/S) 3% w/w, the following conditions were used: pH 8; temperature, 50°C; and reaction time, 10 h. After hydrolysis, the enzyme was inactivated by placing the samples in boiling water for 10 min. The hydrolysates were then subjected to centrifugation at 8,000 r/min for 15 min. The resulting supernatants were lyophilized (FDU-1200, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and stored in a desiccator for further use.

Ultrafiltration

Each of lyophilized enzymatic hydrolysates was dissolved in distilled water and ultrafiltered using Vivaspin 10, 5 and 3 kDa molecular weight cutoff (MWCO) membranes, respectively (Sartorius Co., Goettingen, Germany). Each fraction (0–3, 3–5, 5–10, and >10 kDa) was collected, and its antitumor activity was measured. The active fractions were lyophilized (FDU-1200, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and stored at −20°C for further separation.

Ion exchange chromatography

The fraction with the highest antitumor activity among 0–3, 3–5, 5–10, and >10 kDa hydrolysates was separated by ion exchange chromatography. Ten microliters of bioactive fraction dissolved in distilled water at a concentration of 20 mg/mL were loaded onto a DEAE-52 column (1.6 × 30 cm). The column was eluted with distilled water, 0.05, 0.1, and 0.2 mol/L of NaCl at a flow rate of 0.4 mL/min, respectively. The eluate was collected (4 mL/tube) and detected at 280 nm. The eluates at the same peak were combined, concentrated by a rotary evaporator, and freeze-dried. The fraction with the highest antitumor activity was subjected to the next separation step.

Gel filtration chromatography

The fraction with the highest antitumor activity from the ion exchange chromatography separation was further separated by gel filtration chromatography. Five microliters of bioactive fraction dissolved in distilled water at a concentration of 20 mg/mL was loaded onto a Sephadex G-50 (1.6 × 30 cm). The column was eluted with distilled water at a flow rate of 0.4 mL/min. The eluate was collected (4 mL/tube) and detected at 280 nm. The eluates at the same peak were combined, concentrated by a rotary evaporator, and freeze-dried. The resulting fraction with the highest antitumor activity was used for further analysis.

Cell culture and assay of antitumor activity

Human liver cancer HepG2 cells, purchased from Animal Experimental Center of Zhongshan University, were cultured in a 37°C humidified atmosphere with 5% CO2 Dulbecco’s modified Eagle’s medium (Gibco Co., USA), supplemented with 10% fetal bovine serum.

The antitumor activity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Amresco Co., USA). Cells were plated at a density of 3 × 10^4 cells/well in a 96-well microtiter plate overnight, then treated with 1 mg/mL of hydrolysates, and varying concentrations of hydrolysate fractions by chromatography separation (0.1–0.5 mg/mL) or the chemotherapeutical agent 5-flurouracil (5-FU, a standard chemotherapeutic drug to treat cancers) as a positive control, and incubated for 72 h. About 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37°C. The supernatant was aspirated and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 100 µL of dimethyl sulfoxide for 10 min. Finally, the absorbance was read at 490 nm with a microplate reader (Model 550, Bio-Rad Co., USA). The hydrolysate concentration that gives 50% growth inhibition is referred to as the IC50, which was calculated as the percentage of inhibition by the formula: Inhibition (%) = (1 − [the optical density values for experimental group/the optical density values for control group]) × 100%.

Cellular morphology analysis

Cells in the logarithmic growth phase were plated in 24-well microtiter plates at a density of 1.5 × 10^5 cells/well and allowed to attach and grow for 24 h at 37°C. After treating with hydrolysate fraction solutions (100–500 µg/mL) and incubating for 24 h, the cellular morphology was observed using phase contrast microscopy (Model CK41, Olympus).

Microencapsulation of antitumor polypeptides

The microencapsulation of hydrolysate fraction was performed by using the complex coacervation method. Briefly,
chitosan (0.2 g) was dissolved in 200 mL of 1% v/v acetic acid solution, and CaCl₂ (6 g) was added by stirring in an Erlenmeyer flask. A hydrolysate fraction (1 g) was introduced into 200 mL of 3% sodium alginate solutions, and the well-mixed solutions were slowly dropped into the above chitosan–acetic acid solutions, the mixed solutions were subjected to thermal treatment in a water bath (45°C) for 20 min, then centrifugation at 8,000 r/min for 30 min. The resulting solids were dried at 45°C in a drying oven (DHG-9075-A, Shanghai Yiheng Instruments Company, China).

Nanoencapsulation of antitumor polypeptides

The nanoencapsulation of a hydrolysate fraction was conducted by using the ionotropic gelation method. Briefly, chitosan (0.3 g) was dissolved in 200 mL of 1% v/v acetic acid solution and stirred at 1,000 rpm. A hydrolysate fraction (0.2 g) was added, adjusting pH to 4.0, and then 70 mL of sodium tripolyphosphate solutions (1.5 mg/mL) were introduced while maintaining stirring for 30 min. The final gelation solution was subjected to centrifugation at 10,000 rpm for 30 min, and the solids were collected and dried at 45°C in an oven (DHG-9075-A, Shanghai Yiheng Instruments Company, China).

Characterization of encapsulated antitumor polypeptides

The morphology of micro/nanoencapsulated polypeptides was visualized by field emission scanning electron microscopy (FESEM, JEOL JSM-740 1F). The particle size was determined by laser Doppler anemometry. To determine the encapsulation efficiency, the freshly prepared capsules were centrifuged at 15,000 rpm at 4°C for 30 min. The amount of free polypeptides in the supernatant could be measured; hence, the actual amount of polypeptides encapsulated in capsules (w₁) = the initial input amount of polypeptides (w₂) – the free polypeptides amount. To determine the polypeptides content, the fresh capsules were washed by distilled water and freeze-dried; hence, the weight of capsules (w₃) could be obtained. Then, the encapsulation efficiency and polypeptides content could be calculated using the following equations:

Encapsulation efficiency (%) = \( \frac{w_1}{w_2} \times 100 \)

Polypeptides content (%) = \( \frac{w_1}{w_3} \times 100 \)

In vitro-controlled release of encapsulated antitumor polypeptides

The simulated gastric juice (SGI) consisted of pepsin (10 g) and hydrochloric acid (16.4 mL); the pH was 1.37 for a total volume of 1,000 mL. Simulated intestinal juice (SIJ) was prepared by dissolving KH₂PO₄ (6.8 g) and trypsin (10 g) in distilled water (600 mL), adjusting the pH to 6.8 with NaOH (0.1 mol/L), and adding water to attain a final volume of 1,000 mL.

To study the release of polypeptides from capsules in SGI, the dried capsules with entrapped polypeptides (500 mg) were added to a flask with 100 mL of SGI, incubated for 2, 4, 6, 8, 10, and 12 h at 37°C with constant agitation at 60 rpm. For the release of capsules in SIJ, SGI was replaced by SIJ while keeping other factors unchanged. The release curve was drawn based on the following equation:

Release extent (%) = the total polypeptide amount released in SGI or SIJ/(the added weight of capsules × polypeptides content)

Statistical analysis

All of the tests were conducted in triplicate. The experimental data were expressed as the mean ± standard deviation. The data were subjected to analysis of variance (ANOVA). Duncan’s new multiple range test was performed to determine the significance differences using SPSS 13.0 software (SPSS Inc., Chicago, IL).

Results

Antitumor activities of different enzymatic hydrolysates

After low-temperature high-pressure extraction, three proteases (papain, trypsin, and alcalase) were employed to hydrolyze the extracted C. pyrenoidosa proteins using each enzyme’s optimal conditions. The hydrolysates were separated via an ultrafiltration membrane to obtain four fractions (0–3, 3–5, 5–10, and >10 kDa). The inhibitory activity of each fraction on human liver cancer HepG2 cells was measured by the MTT method. The results are shown in Figure 1. For papain hydrolysates, the inhibition rates of 3–5 and 5–10 kDa polypeptides on HepG2 cells were 37 and 17%, respectively, whereas 0–3 kDa polypeptides have a small growth-stimulating effect. For the trypsin hydrolysates, the inhibition rates of 0–3, 3–5, and 5–10 kDa polypeptides on HepG2 cells were 7, 18, and 10%, respectively. For the Alacase 2.4 L hydrolysate, all the inhibition rates of 0–3 and 3–5 kDa polypeptides on HepG2 cells were 19%. In contrast, for the original undigested proteins, the inhibition rates of 0–3, 3–5, and 5–10 kDa polypeptides on HepG2 cells were 3, 10, and 6%, respectively. In general, the antitumor activities of enzymatic hydrolysates are 4–27% higher than that of the original undigested proteins, except for papain-digested 0–3 kDa and alacase-digested 5–10 kDa polypeptides. The fraction with the highest antitumor activity was the 3–5 kDa hydrolysate digested by papain, named as fraction A, which was chosen for subsequent separation experiments.

Ion exchange and gel filtration chromatography of active fraction

The fraction A, i.e. the 3–5 kDa hydrolysate digested by papain, was further separated by DEAE-52 column

![Figure 1. Inhibitory activities of different molecular weight hydrolysates of three enzymes (papain, trypsin, and alacase) on HepG2 cells.](image-url)
After gradient elutions, four fractions A1, A2, A3, and A4 corresponding to peaks 1, 2, 3, and 4 were obtained (Figure 2a). As determined by the antitumor activity assay, the fraction A2 exhibited the strongest inhibitory effect on HepG2 cells (48.7% at 0.8 mg/mL), A4 exhibited the weakest activity, and A1 exhibited no activity (Table 1). Hence, the fraction A2 was used for further separation.

As shown in Figure 2b, it is clear that the fraction A2 was separated by gel filtration chromatography into two fractions A2-1 and A2-2. Based on the antitumor activity assay, the fraction A2-1 possessed greater inhibitory effect on HepG2 cells than the fraction A2-2 (Table 2), at 0.4 mg/mL with inhibition rates of 49.1 and 11.9%, respectively. Thus, the active fraction A2-1 was collected and freeze-dried to be the CPAP.

### Cytotoxicity and morphology analysis of antitumor polypeptides acting on cancer cells

To determine the half inhibition concentration (IC$_{50}$) of CPAP, the cytotoxicity of CPAP under different doses (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL) was evaluated, and the common anticancer drug 5-FU was used as a positive control. Figure 3 shows that CPAP and 5-FU displayed dose-dependent inhibition effects on HepG2 cells, at 0.5 mg/mL, with the highest inhibition rates of 60.9 and 80%, respectively. The IC$_{50}$ values of CPAP and 5-FU could be estimated to be 426 and 314 µg/mL, respectively.

Additionally, phase contrast microscopy was used to examine the morphological changes of CPAP with different concentrations (0.1–0.2 mg/mL), round and detached cells started to appear and the cell density was gradually reduced (Figure 4a). Compared with the negative control group, the experimental groups were significantly different. In low-dose group (0.1–0.2 mg/mL), round and detached cells started to appear and the cell density was gradually reduced (Figure 4a). In high-dose group (0.3–0.5 mg/mL), the morphology of HepG2 cells became irregular and sparse, with larger intercellular gaps and fewer intercellular junctions. Additionally, some cells exhibited the characteristics of apoptosis such as cell membrane shrinkage, condensation, and fragmentation of nuclear chromatin as well as the formation of black condensation.
apoptotic bodies (Figure 4d–f). Together, these results suggested that CPAP could induce apoptosis and necrotic death of HepG2 cells.

Figure 4. Morphological changes in HepG2 cells induced by fraction A2-1. (a) control, (b) 0.1 mg/mL, (c) 0.2 mg/mL, (d) 0.3 mg/mL, (e) 0.4 mg/mL, and (f) 0.5 mg/mL.

Micro- and nanoencapsulation of antitumor polypeptides

The microencapsulation and nanoencapsulation of CPAP were carried out by complex coacervation and ionotropic
gelation methods, respectively. The surface structures were examined by SEM. Figure 5a shows that the microencapsulated polypeptides microcapsules are uniformly spherical structures without breakage; the average diameter is within the range of 200–300 μm. Figure 5b shows that the nanoencapsulated polypeptides nanoparticles are round or oval in shape, with the average particle size of 129.2 nm (Figure 5c). The encapsulation efficiency and polypeptide contents for microencapsulation are 74.5 and 12.7%, respectively. For nanoencapsulation, the encapsulation efficiency and polypeptides contents are 30.1 and 12.3%, respectively. That is, the encapsulation efficiency of microencapsulation is 2.5-fold higher than that of nanoencapsulation, but their polypeptides contents are similar.

In vitro-controlled release and antitumor activity

The release properties of microencapsulated and nanoencapsulated CPAP were investigated in SGI and SIJ. Figure 6 shows that during 2–12 h the release extents of microencapsulated CPAP in SGI and SIJ were 10.5–26.9 and 18.92–73.3%, respectively; and the release extents of nanoencapsulated CPAP in SGI and SIJ were 14.9–34.1 and 25.2–78.2%, respectively. That is to say, the nanoencapsulated CPAP released more rapidly than the microencapsulated CPAP did whether in SGI and SIJ, and both microencapsulated and nanoencapsulated CPAP were more slowly released in SGI than in SIJ.

Furthermore, the antitumor activity of micro- and nanoencapsulated CPAP was compared with the unencapsulated CPAP. Based on the above polypeptide contents (~12% for the two kinds of encapsulations), the micro/nanoencapsulated and unencapsulated CPAP were all prepared in solutions with the same polypeptides concentrations of 0.4 mg/mL. The MTT assay was conducted to estimate their inhibitory activity on HepG2 cells. The results showed that the inhibition rates of unencapsulated, microencapsulated, and nanoencapsulated CPAP on HepG2 cells were 47.9, 38, and 31%, respectively (Figure 7).

Discussion

Accumulating data indicate that peptides from enzymatic hydrolysis of various food proteins have been reported to exert anticancer or anti proliferative activities. For example, Bowman–Birk protease inhibitor and lunasin, two peptides isolated from soybeans, are now being intensively studied as cancer chemopreventive agents. The two anti-proliferative peptides isolated from papain and Protease XXIII hydrolyses of tuna dark muscle byproduct showed a dose-dependent inhibition effect on MCF-7 cells. Very recently, a novel polypeptide (Mere15), purified from *M. meretrix* Linnaeus, was shown to significantly suppress the growth of human lung adenocarcinoma A549 xenograft in nude mice. In particular, a peptide was isolated from pepsin hydrolysate of *C. vulgaris* protein waste, which exhibited dose-dependent antiproliferation and induced a post-G1 cell-cycle arrest in gastric cancer cells. However, no reports are available on potential antitumor peptides derived from the microalgae *C. pyrenoidosa*. In this study, using low-temperature high-pressure extraction, enzymatic hydrolysis, ion exchange, and gel filtration chromatography, we separated a polypeptide, which is a papain-digested hydrolysate of *C. pyrenoidosa* proteins that exhibits inhibitory activity on human liver cancer HepG2 cells. The IC50 value of such a polypeptide was estimated to be 426 μg/mL, and close to that of common anticancer agent 5-FU (314 μg/mL), suggesting that this polypeptide may be useful ingredients in food, nutraceutical, and pharmaceutical applications.

However, there are some limitations for the practical application of bioactive proteins or polypeptides. For example, they are easily degraded in the stomach owing to the existence of complex enzymes and gastric acids. One effective solution to this issue is the microencapsulation or nanoencapsulation of bioactive proteins or peptides so that they can be protected against degradation in the stomach and can be released in the intestinal environment for enteric cell absorption into the blood stream. Currently, carbohydrates such as starches, alginate, and chitosan are often used as encapsulating materials owing to their biodegradability, low toxicity,
low cost, and food-grade availability. In this study, we encapsulated CPAP by using two methods: complex coacervation (edible alginate, CaCl₂, and chitosan involved) and ionotropic gelation (edible chitosan and sodium tripolyphosphate involved). The results demonstrated that the encapsulation efficiency of microencapsulation (74.5%) is much higher than that of nanoencapsulation (30.1%), but their polypeptides contents are similar (12.7 vs. 12.3%). This means that the larger microcapsules (200–300 μm) do increase the encapsulation efficiency, but do not improve the polypeptide contents. Hence, their detailed encapsulation mechanisms deserve further investigation.

The in vitro release tests revealed that CPAP was well preserved against gastric enzymatic degradation after micro/nanoencapsulations and the slowly controlled release in the intestine could be achieved. However, the MTT assay results indicated that the antitumor activity of the encapsulated CPAP was partly lost in comparison with the unencapsulated CPAP, probably owing to the local encapsulation of some active sites in the CPAP. In consideration of the fact that the unencapsulated polypeptides are basically degraded in the stomach under the action of complex enzymes and gastric acids, we can assume that 80% of unencapsulated polypeptides are degraded in the gastric environment and only 20% enter into intestinal tract. On the other hand, the above release experiments indicate that ~30% of the encapsulated polypeptides are released/degraded in the stomach and ~70% enter the intestine. Hence, for a fixed polypeptide content such as 1 mg/mL, the unencapsulated CPAP will be diluted to 0.2 mg/mL in the intestine. In contrast, the encapsulated CPAP will be diluted to 0.7 mg/mL and to a final value of 0.49 mg/mL after considering that ~30% of the activity is lost by encapsulation. Thus, the final content of the encapsulated CPAP for intestinal absorption (0.49 mg/mL) is still much higher than that of the unencapsulated CPAP (0.2 mg/mL). This indicates that the encapsulation of CPAP is still beneficial for improving the bioavailability in intestinal tract although encapsulation probably caused some loss in activity. Although further in vivo studies are required to verify these findings, this study provides a good example for obtaining bioactive polypeptides from microalgae by activity-driven isolation, and it could also serve as a basis for the further development of functional foods through the addition of the encapsulated polypeptides.

Acknowledgments

This study was supported by the Scientific and Technological Research and Development Program of Guangdong Province (2009B090300271, 2011B090400537), and the Scientific and Technological Research Program of Guangzhou City (2012J4100123).

Literature Cited


