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Full Length Article

Purification and Characterization of Antiproliferative Peptide from Enzymatic Hydrolysates of Chinese Soft-Shelled Turtle Protein

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Abstract

Chinese soft-shelled turtle protein was hydrolyzed using neutrase to produce antiproliferative peptides. To identify the antiproliferative peptides, Chinese soft-shelled turtle protein hydrolysate was fractionated by Sephadex G-25. Antiproliferative activities of the identified peptides were evaluated using the MTT assay. Three novel antiproliferative peptides were identified as Asp-Glu-Gla-Asp-Leu-Leu-Gla (MW: 745.79 Da), Glu-Gla-Gly-Val-Asn-Asp-Trp (MW: 789.81 Da), and Gly-Ger-Ile-Ger-Ger-Gly-Gln-Val (MW: 733.78 Da). These peptides with exhibited antiproliferative activity on A549 cancer cells with IC_{50} values of 129.12±4.21, 150.89±3.75 and 142.47±5.12 μg/mL, respectively. This bioactivity was likely due to the small size of the peptides and the peptide sequences have hydrophobic amino acids. Further investigations were suggested on larger scale employing standard procedures. © 2018 Friends Science Publishers

Keywords: Antiproliferative activity; Peptide; Chinese soft-shelled turtle; Enzymatic hydrolysis; Q-TOF MS/MS

Introduction

Recently, researchers have increasingly focused on bioactive peptides from proteins of food origin, and use peptides as functional food ingredients to maintain health (Ryan *et al.*, 2011; Kim *et al.*, 2013). These functional peptides can be prepared by specific enzyme hydrolysate under controlled conditions (Jumeri and Kim, 2011). In United States, cancer is the second leading cause of death (Rebecca *et al.*, 2016). The incidence and mortality of lung cancer continues to rise, with 80% of all lung cancer patients suffering from non-small cell lung cancer (Chen *et al*., 2008). Some drugs are used to treat cancer, but their toxicity limits their usage (Cragg and Newman, 2005; Fouche *et al*., 2008). It is, therefore, imperative to research the production of more effective and low toxicity antiproliferative components. Peptides have a significant advantage in security. Discovery of small size peptides containing the Glu-Asp-Ser residues have been reported as potential cancer inhibitors (Nassar *et al*., 1995; Janin, 2003). Therefore, development of antiproliferative peptides has become an important anticancer therapy strategy. Global aquatic resources have been proved to be a rich source of new medicinally valuable compounds (Blunt *et al.*, 2005). In recent years, marine-sourced protein hydrolysates have attracted wide attention in terms of their biological activities. Specific residues in marine peptides including those in hydrolysates from oyster, Tilapia, *Arca subcrenata*, and Clam (*Ruditapes philippinarum*) have been shown to have potent antitumor activity on several human cancer cell lines *in vitro* (Wang *et al.*, 1997; Huang *et al.*, 2002; Song *et al.*, 2008; Chang *et al.*, 2011; Zheng *et al.*, 2011; Kim *et al.*, 2013). The protein hydrolysates antiproliferative activity derived from freshwater economic crops has been rarely studied. Chinese soft-shelled turtle (*Trionyx sinensis*) is known in Asian countries such as China, Korea and Japan as a health promotion food (Li *et al.*, 2008). There are few studies on the antiproliferative characteristics of peptides derived from soft-shelled turtles enzymatic hydrolyzates. The objective of this study was to identify antiproliferative peptides in soft-shelled turtle hydrolyzates and investigate the antiproliferative properties of those peptides on a human non-small-cell lung cancer A549 cell line.

Materials and Methods

Soft-shelled turtles (*Trionyx sinensis*) were obtained from the Zhejiang Zhongde Agriculture Group Co., Ltd. (Hangzhou, China). To analyze the potential of peptides generated from soft-shelled turtle as antiproliferative agents, four enzymes, i.e., neutrase, papain, acid proteinase, and trypsin were used to hydrolyse soft-shelled turtle protein.

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These enzymes were purchased from Novozyme Co. (Bagsvaerd, Denmark). Sephadex G-25 was purchased from GE Healthcare (Fairfield, Connecticut, USA). All other chemicals were of analytical and High Performance Liquid Chromatography (HPLC) grade.

Dimethyl sulfoxide (DMSO), 3-[4, 5-Dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide (MTT), trypsin, fetal bovine serum, and RPMI-1640 medium (pH 7.4) were purchased from GIBCO BRL (Grand Island, NY, USA).

Enzymatic Hydrolyzates Preparation from Soft-shelled Turtles

Soft-shelled turtle meat was defatted, lyophilized, and pulverized into a powder. Enzymatic hydrolyzates were obtained as described previously with some modifications (Kim *et al*., 2013). Briefly, the soft-shelled turtle was rinsed and its head, bone and peel were removed. The turtle meat section was chopped and defatted with 95% ethanol, and then dried under a vacuum. Added 1000 mL phosphate buffer solution to 100 g dried sample, followed by 3 g enzyme after a 30 min pre-incubation. The enzymatic hydrolysis reactions were performed for 8 h, followed by immediate heating to 100℃ for 10 min to inactivate the enzyme. At last, the enzymatic hydrolyzates were rapidly cooled to 20–25℃, centrifuged at 4℃, and then lyophilized use the freeze-drying equipment and stored at −20℃.

Gel Filtration Chromatography to Isolate and Purify Peptides

The lyophilized powder of the soft-shelled turtle was resuspended in distilled water and centrifuged at 10,000 rpm for 10 min. The supernatant was separated by the Sephadex G-25 gel filtration column (2.6 cm×80 cm), distilled water was used as eluting solvent at 0.75 mL/min flow rate. Each 6 mL of the lotion was collected and monitored at 220 nm. Fractions with the desired peak were pooled and lyophilized to test antiproliferative activity.

Cell Culture

Human lung cancer A549 cells (ATCC, Manassas, VA, United States) were cultured in RPMI-1640 medium (pH 7.0) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, USA), 100 μg/mL streptomycin sulfate (Beyotime Institute of Biotechnology, Shanghai, China), and 100 units/mL penicillin. Cells were cultured at 37°C in an atmosphere with 5% CO_2.

Analysis of Cell Proliferation

MTT assays were performed as described previously (Perera *et al*., 2012; Wang *et al.*, 2016). Briefly, A549 cells were seeded in a 96-well plate with 1×10^5 cells/mL concentration and incubated at 37°C in a humidified 5% CO_2 for 24 h. Then 100 μL serial samples dissolved in the appropriate solvent (DMSO) were added to each well, after which cells were incubated for 72 h. At that point 20 μL 5 mg/mL MTT solution was added to each well, and then the plates were incubated for another 4 h at 37°C. Optical density at 570 nm was then measured using an ELISA plate reader (Bio-Rad, Hercules, California, USA). The anti-proliferative activity on A549 cells was expressed as the concentration of samples inhibition by 50% (IC_{50}).

HPLC

HPLC was used to analyze the molecular weight distribution of peptides. A reverse-phase HPLC (RP-HPLC) system consisting of an HPLC (2690 Waters, Milford, MA, USA) equipped with a Symmetry C18 column (4.60×150 mm, 5 μm, Waters) and a 2996 photodiode array detector (DAD). The injection volume was 10 μL. The mobile phase was 0.1% Trifluoroacetic Acid (TFA) and 10% acetonitrile in water, and the flow rate was 0.5 mL/min. The eluate was analyzed at 220 nm, and the column temperature maintained at 30℃. Molecular weight standards included cytochrome C (MW 12327 Da), aprotinin (MW 6512 Da), bacitracin (MW 1422.7 Da), thymopentin (MW 268.930 Da), and reduced glutathione (MW 307.33 Da).

Identification and Synthesis of the Purified Peptide

Build 88 M protein libraries: 88 M protein/peptide databases were obtained from NCBI. The eluted fraction with the strongest antiproliferative activity was analyzed by Waters Q-TOF MS/MS spectrometer (Micromass, Altrincham, UK). MS/MS spectra were analyzed using PLGS 3.02 software (Thermo Electron, Beverly, USA) to identify peptides. The purified peptide was synthesized using the solid phase method of GL Biochem Ltd. (Shanghai). The synthesized peptide was used for further analysis.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) of three replicate results and analyzed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Using one-way analysis of variance (ANOVA) analyze the data. IC_{50} values were calculated using GraphPad Prism 5. A $p<0.05$ was considered statistically significant.

Results

IC_{50} values revealed that all hydrolysates inhibited the growth of A549 cells (Table 1). Hydrolysate prepared using neutrase had the highest antiproliferative activity (IC_{50} 210.8 ±2.07 μg/mL; dose-dependent) thus hydrolysate prepared with neutrase was selected for further experiments. Neutrase hydrolysate was lyophilized with freeze drying equipment.

The resulting sample was then further fractionated using a Sephadex G-25 gel filtration column. Molecular weight was determined by HPLC. The molecular weight distribution of the peptides hydrolyzed by neutral protease enzyme was 229-5495 Da (Fig. 1).

Peptide molecular weight is one of the key factors influencing the biological activity (Liu *et al*., 1997; Guo *et al*., 2009; You and Wu, 2011). Soft-shelled turtle neutrase hydrolysate was separated into five fractions (fractions I-V) by Sephadex G-25 gel filtration chromatography (Fig. 2a). Inhibitory effects exerted by the five fractions of hydrolysate on the proliferation of A549 cells were evaluated. The IC_{50} values of fractions I, II, III, IV and V were 269, 216.8, 643.1, 1035 and 185.8 μg/mL, respectively. Among the five fractions, fraction V exhibited the strongest antiproliferative activity in A549 cells (Fig. 2b), suggesting that fraction V can be a source of antiproliferative compounds. These results are consistent with earlier studies reporting that the low molecular weight peptides were particularly active in a range of biological responses (Jun *et al*., 2004; You *et al*., 2011; Zhang *et al*., 2012).

Fraction V, having the strongest antiproliferative activity, was analyzed by Waters Q-TOF MS/MS to determine its molecular weight and amino acid sequence. Peptides were identified automatically by PLGS 3.02 software that compared the experimental mass spectra with the theoretical mass spectra from the 88 M protein libraries. The MS/MS spectrum of fraction V indicated three putative amino acid sequences: Asp-Glu-Gla-Asp-Leu-Leu-Gla (DEADLLA, MW: 745.79 Da), Glu-Gla-Gly-Val-Asn-Asp-Trp (EAGVNDW, MW: 789.81 Da), and Gly-Ser-Ile-Ser-Ser-Gly-Gln-Val (GSISSGQV, MW: 733.78 Da) (Fig. 3).

Antiproliferative activities exerted by the three peptides reported above on the proliferation of A549 cells were in a dose-dependent manner (Fig. 4). IC_{50} values after 72 h incubation with peptides DEADLLA, EAGVNDW and GSISSGQV were 129.12±4.21, 150.89±3.75 and 142.47±5.12 μg/mL, respectively (Table 2). DEADLLA had the strongest effect on IC_{50}. We found no significant differences between IC_{50} values with EAGVNDW and GSISSGQV ($p > 0.05$).

Table 1: Growth inhibition of A549 cells by hydrolysates using different enzymes

Enzyme	Temperature (℃)	pH value	IC_{50} (μg/mL)
Neutrase	50	6	210.85±2.07a
Papain	55	7	278.49±3.11d
Acid Proteinase	40	3	218.52±1.56b
Trypsin	37	8	228.49±2.01c

IC_{50} was defined as the concentration of extract or compound that caused 50% inhibition of A549 cell proliferation *in vitro* after 72 h. Values are expressed as the mean ± SD of three experiments in duplicate. Different letters (a–d) for each concentration denote a significant difference between different treatments ($P < 0.05$)

Table 2: Growth inhibition of A549 cells by three peptides

Peptide	IC_{50} (μg/mL)
DEADLLA	129.12±4.21a
EAGVNDW	150.89±3.75b
GSISSGQV	142.47±5.12b

IC_{50} was defined as the concentration of peptide that caused a 50% inhibition of proliferation *in vitro* after 72 h. Values are expressed as the mean ± SD of three experiments performed in duplicate. Different letters (a–b) for each concentration denote a significant difference between different treatments ($P < 0.05$)

Discussion

Biologically active peptides obtained from aquatic animals act in diverse ways that include mineral binding, immunomodulation, antimicrobial activity, antioxidant activity, antithrombotic activity, anticancer activity, and antihypertensive actions (Kim and Wijesekara, 2010; Chen *et al*., 2012; Kim *et al*., 2013). Biological activity of peptides is remarkably dependent on molecular weight, amino acid composition and their sequences (Shahidi and Zhong, 2010; Alemán *et al*., 2011).

The molecular weight and amino acid sequences of antiproliferative peptide found in this study were analyzed by Waters Q-TOF MS/MS (Fig. 4). The molecular weights of the three peptides were 745.79 Da, 789.81 Da and 733.78 Da, and they were composed of seven and eight amino acids with the sequences Asp-Glu-Gla-Asp-Leu-Leu-Gla, Glu-Gla-Gly-Val-Asn-Asp-Trp and Gly-Ser-Ile-Ser-Ser-Gly-Gln-Val, respectively. The majority of previous studies have shown that low molecular weight peptides may have more effective antiproliferative activity and that biological active peptides usually have less than 20 amino acid residues (Pihlanto-Leppälä, 2000; Kim *et al*., 2013). Our results agree with earlier reports in this aspect. Peptides with lower molecular weight have stronger molecular mobility and dispersibility, which seems to enhance the interactions with tumor cell components and improve antiproliferative activity (Jumeri and Kim, 2011).

DEADLLA, EAGVNDW and GSISSGQV exhibited strong antiproliferative activity by inhibiting the proliferation of A549 cells with IC_{50} values of 129.12±4.21, 150.89±3.75, and 142.47±5.12 μg/mL, respectively. These results were in consonance with the general findings that short peptides of 2–10 amino acids have greater biological active properties than the proteins or large peptides (Kitts and Weiler, 2003; Saito *et al*., 2003; Alileche *et al*., 2012; Nongonierma and FitzGerald, 2016). Moreover, the molecular weight of DEADLLA is less than EAGVNDW, and DEADLLA was the most active peptide among the three peptides. A novel peptide was purified from *Mytilus coruscus* that showed higher antitumor activity on prostate (IC_{50}, 940 μg/mL) cancer cells than on breast (IC_{50}, 1220 μg/mL) cancer cells. The amino acid sequence was Ala-Phe-Asn-Ile-His-Asn-Arg-Asn-Leu-Leu (Kim *et al*., 2012).

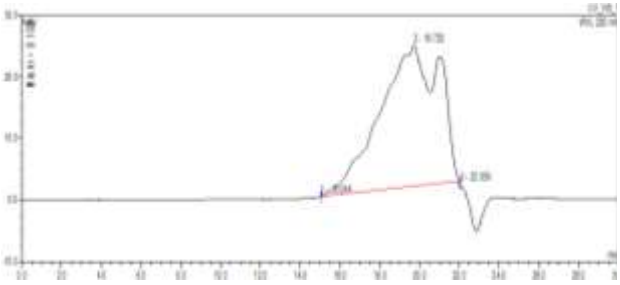

Fig. 1: Neutral protease enzyme peptide molecular weight distribution

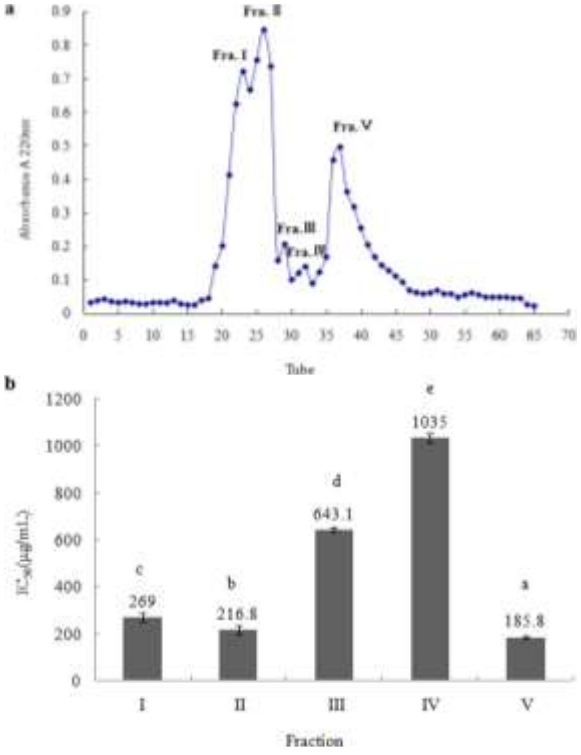

Fig. 2: Sephadex G-25 gel chromatography (a) and IC_{50} value (μg/mL) of each fraction from soft-shelled turtle neutrase hydrolysate. The column (2.6 cm × 80 cm) was equilibrated and eluted with distilled water at a flow rate of 0.75 mL/min, every 6 ml of eluted solution was collected and monitored at 220 nm. Values are expressed as mean ± SD of three experiments performed in duplicate. IC_{50} was defined as the concentration of extract or compound that caused a 50% inhibition in proliferation in vitro after 72 h in A549 cells. Letters above a column indicate significant differences between treatments ($P < 0.05$)

In addition, a novel anticancer peptide, Ala-Val-Leu-Val-Asp-Lys-Gln-Cys-Pro-Asp, was purified from *Ruditapes philippinarum* that exhibited an inhibitory activity on lung cancer cells, the IC_{50} value was 1350 μg/mL (Kim *et al*., 2013). Jumbo squid skin gelatin enzymatic hydrolyzate showed inhibitory activities on MCF-7 and U87 cells, their

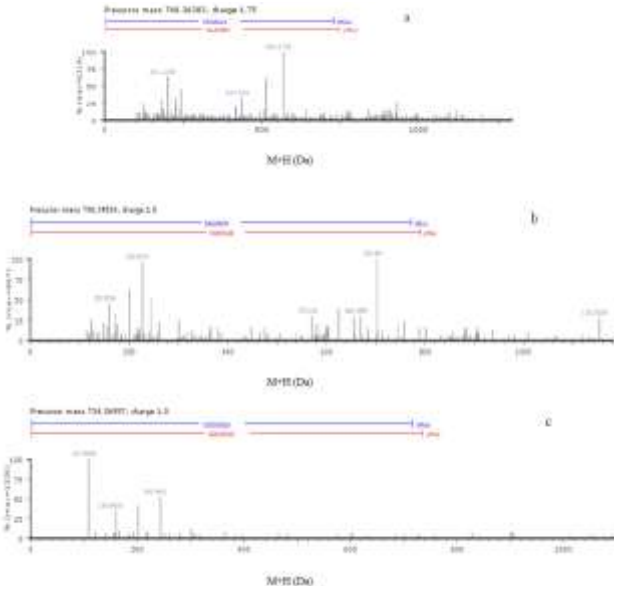

Fig. 3: Identification of the molecular mass and amino acid sequence of three isolated peptides. (a) MS/MS spectrum of a single charged ion (746.36 Da) of DEADLLA, (b) MS/MS spectrum of a single charged ion (790.35 Da) of EAGVNDW, (c) MS/MS spectrum of a single charged ion (734.37 Da) of GSISSGQV. MS/MS experiments were performed on a Q-TOF-MS spectrometer. The three peptides were sequenced by de novo sequencing algorithm using the PLGS 3.02 software

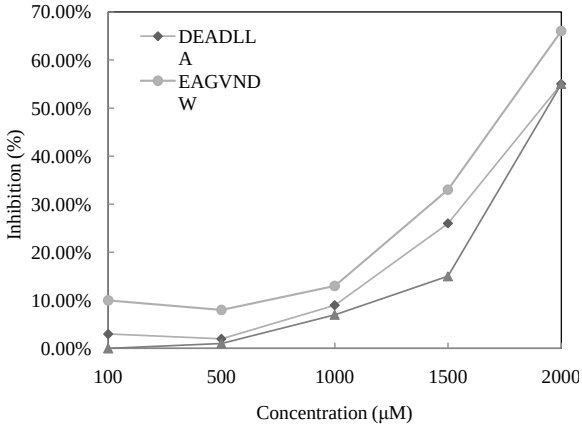

Fig. 4: Dose-dependent inhibitory effect of peptides on the proliferation of A549 cells

IC_{50} values were 130 and 100 μg/mL, respectively (Aleman *et al*., 2011a).

It has been reported that hydrophobic amino acids have a significant effect on antioxidant activity (Rajapakse *et al*., 2005; Ranathunga *et al*., 2006; Ren *et al*., 2008; Zhang *et al*., 2012). The hydrophobicity of peptides plays a crucial role in modulating effects on cancer cells (Huang *et al*., 2011). Hymenochirin-1Pa is a peptide isolated from frog *Pseudhymenochirus merlini* (Pipidae) skin secretions. It has

been reported that the main sequences of hymenochirin-1Pa were characterized by an alternating pattern of two hydrophobic and two hydrophilic amino acid residues, and the peptide showed high cytotoxic effects on human cancer A549 cells, breast cancer MDA-MB-231 cells, and colorectal cancer HT-29 cells (Serraa *et al*., 2014). In the present study, DEADLLA and EAGVNDW have three hydrophobic amino acids, respectively, while GSISSGQV has two. This raises the possibility that anticancer activity can be improved through a modification of amino acid sequence.

Conclusion

In this research, an effective method for bioactive-oriented preparative isolation was used to find antiproliferative peptides in soft-shelled turtle hydrolyzates. We obtained three peptides and determined the sequences to be: Asp-Glu-Gla-Asp-Leu-Leu-Gla (DEADLLA, MW: 745.79 Da), Glu-Gla-Gly-Val-Asn-Asp-Trp (EAGVNDW, MW: 789.81 Da), and Gly-Ser-Ile-Ser-Ser-Gly-Gln-Val (GSISSGQV, MW: 733.78 Da), respectively. In addition, these results indicated that the three novel antiproliferative peptides have effective cytotoxicity on A549 cells. The structure of antiproliferative peptides and their biological activity *in vivo* need to be further investigated. The results of this work will help to better understand the application of the bioactive food and medicine of turtle peptides.

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