

Isolation and purification of novel peptides derived from Sepia ink: Effects on apoptosis of prostate cancer cell PC-3

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Abstract. Novel prostate cancer therapeutics are in high demand. In order to identify potential therapeutic targets, protein from sepia ink was hydrolyzed by utilizing pepsin in an orthogonal array design. Pepsin hydrolysate (SH) obtained at optimal conditions exhibited the highest antitumor activity. Subsequently, a novel antitumor peptide, which was termed SHP, was isolated through ultrafiltration, gel filtration chromatography and reversed phase high-performance liquid chromatography. The amino acid sequence of SHP was identified as Leu-Lys-Glu-Glu-Asn-Arg-Arg-Arg-Arg-Asp with a molecular mass of 1371.53 Da. The results of the proliferation assay revealed that SHP significantly inhibited the proliferation of PC-3 cells in a time- and dose-dependent manner. Acridine orange/ethidium bromide staining indicated significant SHP-induced apoptosis. Furthermore, Annexin V/PI double-staining assays revealed that the percentage of early-stage apoptotic cells increased from 8.85 to 29% following PC-3 exposure to 5, 10 and 15 mg/ml SHP for 24 h. SHP-induced apoptosis was accompanied by the activation of cellular tumor antigen p53 and caspase-3, the upregulation of apoptosis regulator BAX, and the downregulation of apoptosis regulator Bcl-2. These findings suggest that SHP is a novel inducer of apoptosis *in vitro* and merits further investigation as a possible therapeutic agent for the treatment of cancer.

Introduction

Prostate cancer (Pca) is not only the most frequently diagnosed cancer in men (excluding skin cancer), but it is also the second leading cause of cancer-related deaths of men in the western world (1).

Watchful waiting, active surveillance radical prostatectomy, and radiotherapy are currently the main treatments for

non-metastatic Pca. These treatments, however, are not suitable for metastatic Pca. Further, no definitive strategy regarding the treatment choice (which drug/drug family to apply first) has been devised. Effective medical management with the highest efficacy and a lowest frequency of side-effects is the major goal of therapy (2,3).

The development of novel and potent therapeutics that selectively target the growth of tumor cells, but do not affect normal cells, during the treatment process, is an important goal. Bioactive peptides isolated from marine animals, such as the sea hare, cod, squid, plaice, tuna dark muscle, japonicas backbone, salmon, Nemipterus, and shrimp shell (4,5), exhibit anti-tumor activity via inhibition of cell proliferation *in vitro*.

Sepia is a genus of cuttlefish (in the Cephalopoda class) that utilizes a dark ink as a defensive weapon when threatened. In traditional Chinese medicine, sepia ink has been used for thousands of years to maintain hemostasis (6). Bioactive materials from sepia ink possess anti-oxidant (7,8), anti-retrovirus (9), anti-bacterial (10), anti-hypertensive (11), and most importantly, anti-tumor activities. For example, squid ink ameliorates chemotherapeutic injury induced by cyclophosphamide in mice and rats (12-15). Moreover, in the 1990s, Japanese researchers discovered that bioactive materials obtained from sepia ink have anti-tumor activity (16,17) and the glycosaminoglycan-like polysaccharides are also potential candidate compounds for the prevention of tumor metastasis (18). Furthermore, a peptidoglycan isolated from squid ink inhibits the proliferation of DU-145 and PC-3 Pca cells via apoptosis and antitumor mechanisms, resulting in an increased expression of caspase-3 mRNA, with concurrent decreases in the expression of Bcl-2 mRNA, and lower COX-2 and VEGF protein content levels (19). Enzymolysis was recently applied to obtain a tripeptide from sepia ink that was found to inhibit the proliferation of Pca cell lines and induce apoptosis through a Bcl-2/Bax-mediated mechanism (20,21).

Certain peptides improve immune responses, inhibit tumor angiogenesis and metastasis of tumor cells, directly eradicate tumor cells, induce tumor cell apoptosis, and arrest the cell cycle (22). In the present study, the preparation conditions of pepsin hydrolysates from sepia ink was optimized and subsequently its anti-tumor activity was analyzed. Furthermore, the amino acid sequence of a novel bioactive polypeptide (SHP) isolated from the pepsin enzyme extract was determined and its ability to induce apoptosis was investigated. The results

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of this study provide a basic theory for developing novel anti-tumor agents against Pca.

Materials and methods

Materials. Sepia ink (*sepia esculenta*) was purchased from Nanzhen Market (Zhoushan, China), and authenticated by Prof. Sheng-Long Zhao (Zhejiang Ocean University, Zhoushan, China).

Pepsin was purchased from Shanghai Ruji Biological Technology Co., Ltd., China. Sephadex G-25 was purchased from YTHX Biotechnology Co., Ltd. (Beijing, China). The ultrafiltration membrane was purchased from Shanghai Mosu Scientific Equipment (China). Acetonitrile was of LC grade and purchased from Oceanpak Scientific Co., Ltd. (Sweden). CCK-8 assay kit was purchased from Dojindo Laboratories (Japan). Annexin V-FITCC cell apoptosis test kit was purchased from Best Bio (Beijing, China). Rabbit polyclonal antibodies against p53, Caspase-3, Bcl-2, Bax and HRP-conjugated secondary antibodies were purchased from Celling Signaling (Beijing, China). PVDF membrane was purchased from Bio Rad (USA). All other reagents used in the experiment were of the analytica grade.

Preparation of enzym hydrolysate of Sepia ink. The sepia ink was separated manually and homogenated by high-speed tissue-masher (Shanghai YouYi limited company, Shanghai, China), as described previously (23). The optimum hydrolysis condition was determined using an orthogonal array design (Table I). An additional orthogonal array design (L16(4⁵)) was applied to optimize the enzymolysis conditions, which consisted of material/distilled water, pH, enzyme dose, enzymolysis temperature, and enzymolysis time. The hydrolysates prepared with Pepsin were inactivated at 95°C for 15 min and centrifuged at 12,000 rpm/min for 10 min (Hitachi Koki Co., Ltd., Japan). Supernatants were lyophilized and stored at -20°C.

Purification of anti-cancer peptides from Sepia ink hydrolysate (SH). SH solutions were fractionated into five groups using four different molecular weight cut-off membranes of 10, 5, 3, 1 kD at 0.2 MPa, 25°C. Fractions were collected and named as SH-1, SH-2, SH-3, SH-4, and SH-5 and their MW ranges were: <1, 1-3, 3-5, 5-10 and >10 kDa, respectively. Lyophilized SH-2 (120 mg) was re-suspended in 2 ml distilled water and loaded onto a Sephadex G-25 gel filtration column (2.6x65 cm), which was pre-equilibrated and eluted with distilled water at a flow rate of 1.8 ml/min. The elution solution was collected at 2 min intervals and monitored at 220 nm; three peaks (SH-2-1, SH-2-2, SH-2-3) were pooled and lyophilized. A subfraction of SH-2-2 was further separated by reversed-phase high Performance Liquid Chromatography (RP-HPLC, Agilent 1260 HPLC, Agilent Ltd., USA) on a Xbridge BEH130 C18 column (4.6x250 mm, 5 µm particle size, Waters Scientific, USA) with a linear gradient of acetonitrile at a flow rate of 1.0 ml/min, which was equilibrated by 0.1% trifluoroacetic acid (TFA) in water. The elution (SHP) was monitored at 220 nm, collected, and lyophilized. Finally, the amino acid sequences of the purified peptides was sequenced on a Shimadzu PPSQ-31A automated gas-phase sequencer (Shimadzu, Kyoto, Japan). The purified peptides were dissolved in 10 µl of a 37% CH₃CN (v/v) solution and

then applied to TFA-treated glass fiber membranes, precycled with Polybrene (Shimadzu Corporation).

Cell culture. Prostate cancer cells line PC-3 was obtained from the China cell bank of the Institute of Biochemistry and Cell Biology and cultured in F12 medium (Gibco, USA) supplemented with 10% Fetal Calf Serum (FCS), 100 U/ml penicillin G sodium, and 100 mg/ml streptomycin sulfate in a humidified 5% CO₂ atmosphere at 37°C.

Cell proliferation assay. Cell proliferation was analyzed by Cell Counting Kit-8 (CCK-8) assay. Briefly, exponentially growing cells, at a density of 1x10⁴ cells/100 µl well in 96-well plates, were treated with different concentrations (5, 10, 15, 20 mg/ml) of SHP in complete medium or medium alone. CCK-8 was added to each well containing 200 µl of the culture medium 24, 48, and 72 h later and were then incubated at 37°C in a humidified 5% CO₂ atmosphere for 4-5 h. Absorbance was measured at 450 nm by a microplate reader (Bio-Rad, USA). All experiments were performed in triplicate and repeated three times.

The percentage inhibition of cell proliferation was calculated as follows:

$$\text{inhibition(\%)} = \left[\frac{(\text{OD}_{\text{control}} - \text{OD}_{\text{treatment}})}{(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})} \right] \times 100\%$$

Morphologic observations. The apoptotic morphologic changes were observed through the AO/EB staining (Acridine orange/Ethidium bromide) method, as described previously (24). PC-3 cells in logarithmic growth phase were plated at a final concentration of 1x10⁵ cells/well and incubated for 24 h. Adherent cells were inoculated with 2 ml/well of varying concentrations (5, 10, and 15 mg/ml) of SHP. F12 medium (10% FCS) was used as a control. The supernatant was aspirated and a AO/EB mixture [20 µl, containing 100 µg/ml AO and 100 µg/ml EB in phosphate buffered saline (PBS)] was added to the cells for 24 h and observed under a fluorescence microscope (Olympus, Japan).

Cell apoptosis analysis. PC-3 cells were seeded in six-well plates, grown for 24 h before, exposed to SHP (0, 5, 10, 15 mg/ml) and collected 24 h later, centrifuged, washed twice with cold PBS and stained with annexin V-FITC and PI at room temperature for 15 min in the dark. After incubation, 1X binding buffer was added to each tube and then the cells were immediately analyzed by FACS Calibur flow cytometry (Becton Dickinson, NY, USA).

Western blot analysis. PC-3 cells (2x10⁵ cells/ml) were co-cultured with different concentrations of SHP and harvested after 24 h. The cells lysates were prepared with RIPA buffer (20 mmol/l (PH 7.5), 150 mmol/l NaCl, 1% Triton X-100, sodium pyrophosphate, and EDTA) and collected via centrifugation. Equal amounts of protein were subjected to electrophoresis on 10 or 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to a PVDF membrane. The membrane were incubated with primary antibody (p53, Bax, Bcl-2, Caspase3, β-actin) in 0.1% BSA at 4°C overnight after blocked 5% dried skilled milk, then rinsed, incubated with secondary antibody at room temperature for 2 h, and rinsed again. Signals were detected by enhanced chemiluminescence (ECL).

Table I. Orthogonal array design matrix L16(4⁵) and experimental results for optimization of the enzymolysis conditions (C=10 mg/ml).

Order	A	B	C (U/g)	D (C°)	E (h)	IR (%)
1	1 (1:0)	1 (2.0)	1 (300)	1 (30)	1 (2)	36.69
2	1	2 (2.5)	2 (600)	2 (35)	2 (4)	38.42
3	1	3 (3.0)	3 (900)	3 (40)	3 (6)	37.14
4	1	4 (3.5)	4 (1200)	4 (45)	4 (8)	39.32
5	2 (1:1)	1	2	3	4	32.94
6	2	2	1	4	3	37.75
7	2	3	4	1	2	27.78
8	2	4	3	2	1	34.16
9	3 (1:2)	1	3	4	2	32.75
10	3	2	4	3	1	39.43
11	3	3	1	2	4	41.38
12	3	4	2	1	3	36.95
13	4 (1:3)	1	4	2	3	29.92
14	4	2	3	1	4	30.21
15	4	3	2	4	1	34.47
16	4	4	1	3	2	29.38
K1	37.393	32.575	35.800	32.408	35.688	
K2	33.158	36.453	35.695	35.970	32.083	
K3	37.628	35.193	33.565	34.723	35.440	
K4	30.995	34.952	34.112	36.072	35.962	
Best level	A3	B2	C1	D4	E4	
R	6.632	3.878	2.235	3.665	3.879	
R order	A>E>B>D>C					

PS, A, the material water ratio; B, pH; C, the enzyme dose; D, temperature; E, time. IR, the proliferation inhibition.

Statistical analysis. One- and two-way analysis of variance (ANOVA) was used to compare the mean of each experiment. Significant differences between the means of parameters were assessed by using Tukey's test ($P<0.05$). All experimental data was analyzed using SPSS18.0 statistical software.

Results

Optimization by orthogonal experimental design. Orthogonal experimental design is a collection of mathematical and statistical techniques based on the orthogonal Latin square theory and group theory (25); it has been adopted as the primary tool for determining the best processing conditions. As such, we used an orthogonal experimental method (L16(4⁵)) to determine the best condition to hydrolyze sepia ink with pepsin (Table I). The factors affecting the cell proliferation of the hydrolysate were listed in a decreasing order as follows: A>E>B>D>C, where the material/distilled water (A) was found to be the most important factor affecting the cell proliferation inhibitory activity of the hydrolysates. The optimum extraction condition was determined to be A3B2C1D4E4, that is, material/distilled water 1:2, pH was 2.5, enzyme dose 300 U/g, enzymolysis temperature 45°C, and enzymolysis time 8 h. The material/distilled water (A) of sepia ink exhibited a significant effect ($P<0.05$) on the cell proliferation. Other factors, however, had no significant influence on the cell

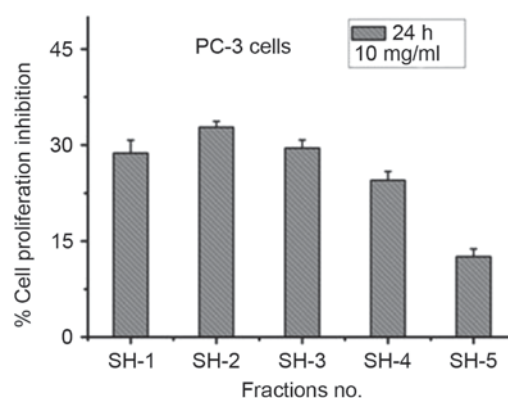


Figure 1. Inhibited proliferation of pepsin hydrolysates (SH)-treated PC-3 cells. PC-3 cells were treated with 10 mg/ml SH. Cell proliferation was measured using a CCK-8 assay at 24 h after SH treatment. SH-2 exhibited the highest activities on PC-3 cells. All results were triplicates of mean \pm SD, and each experiment performed in triplicate ($n=3$).

proliferation inhibitory effects. Purification characterization of anti-cancer peptides.

Fractionation of sepia ink hydrolysate (SH). Our results revealed that co-culture of the sepia ink hydrolysate fractions (10 mg/ml) inhibited the proliferation of PC3 (28.75 ± 2.13 , 29.54 ± 0.94 , 32.79 ± 1.27 , $12.56 \pm 1.21\%$, respectively) (Fig. 1). Based on these

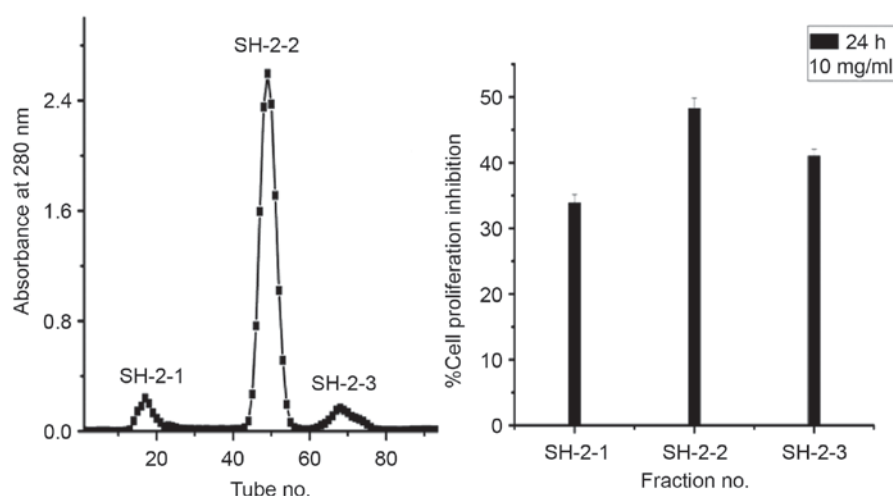


Figure 2. Gel filtration chromatography of SH-2 on a sephadex G-25 column, and right panel represented the inhibition on the proliferation of PC-3 cells. SH-2-2 demonstrated the highest anti-tumor activity. All results were triplicates of mean \pm SD, and each experiment performed in triplicate (n=3).

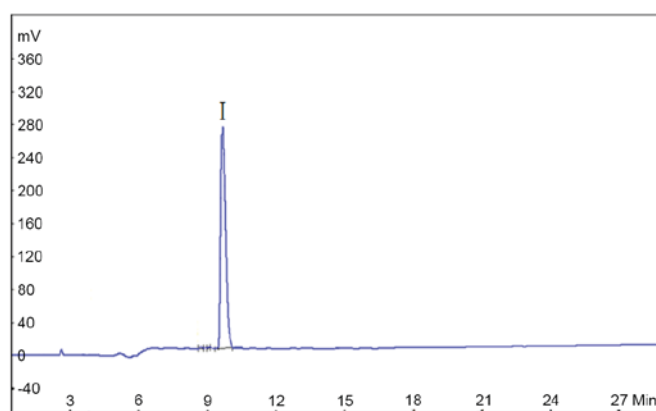


Figure 3. The high performance liquid chromatography (HPLC) chromatogram of SH-2-2.

results, we utilized the SH-2 fraction for the remaining experiments as it had the greatest effect on PC3 cells growth (Fig. 2). SH-2 was separated into three subfractions (SH-2-1, SH-2-2, and SH-2-3). Among these subfractions, SH-2-2 (10 mg/ml) exhibited the highest inhibitory activity on cell proliferation (SH-2-1, $33.89 \pm 1.53\%$; SH-2-2, $48.26 \pm 1.24\%$; SH-2-3, $40.99 \pm 2.03\%$). Isolating peptides from SH-2-2 by RP-HPLC.

SH-2-2 purification was initiated using gel chromatography, followed by separation with RP-HPLC, and then samples constituting the peak I were collected (Fig. 3). We found that peak I possessed significant anti-Pca activity, and named it SHP. Its peptide sequence was obtained by N-terminal amino acid sequencing using an Applied Biosystema 494 protein sequencer and determined to be Leu-Lys-Glu-Glu-Asn- Arg-Arg-Arg-Arg-Asp with a molecular mass of 1371.53 Da.

SHP inhibits the cell proliferation of PC-3 cell lines. We assessed the inhibitory effect of SHP on the proliferation of PC-3 cells in a CCK-8 assay (Fig. 4). SHP inhibited the growth of PC-3 cells in a time- and concentration-dependent manner and exhibited significant inhibitory effects at concentrations

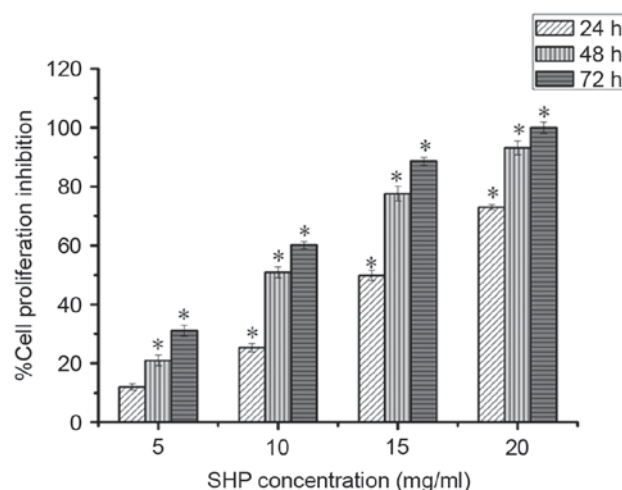


Figure 4. Inhibition proliferation of SHP-treated PC-3 cells. PC-3 cells were treated with 5, 10, 15, 20 mg/ml SHP. Cells proliferation was detected by using CCK-8 at 24, 48, and 72 h after SHP treatment. All results were expressed as mean \pm SD, each experiment performed in triplicate (n=3). *Significant difference (P<0.05) between treatments with the same concentration.

of 5, 10, 15, 20 mg/ml after SHP treatment for 24, 48, and 72 h (P<0.05).

Morphologic observation by acridine orange and ethidium bromide (AO/EB) staining. Acridine orange and ethidium bromide (AO/EB) staining was used to identify the morphologic changes in PC-3 cells following co-culture with SHP (5, 10, 15 mg/ml) for 24 h. The results demonstrated that SHP induced clear morphologic changes normally associated with cellular apoptosis. Fig. 5; cells stained green represent viable cells, whereas yellow staining represents early apoptotic cells and reddish or orange staining represents late apoptotic cells. PC-3 cells treated with 5 mg/ml SHP showed clear changes in cellular morphology, including membrane blebbing, chromatin condensation, and fragmented nuclei (26). Similar features were observed in PC-3 cells treated with 10 and 15 mg/ml SHP, but late-stage apoptotic activity-related apoptotic bodies were also observed. Taken together, these

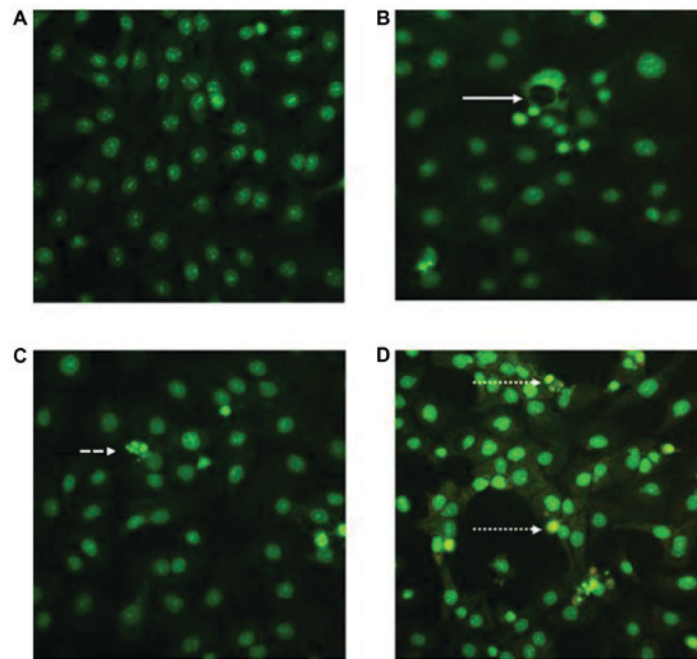


Figure 5. Morphologic observation with acridine orange/ethidium bromide (AO/EB) staining. PC-3 cells were treated without (A) and with SHP at 5 mg/ml (B), 10 mg/ml (C), 15 mg/ml (D). →, indicates viable cells; ---→, indicates early apoptotic cells; ----→, indicates late apoptotic cells. Each experiment was performed in triplicate (n=3) and generated similar morphologic features. Original magnification 400x.

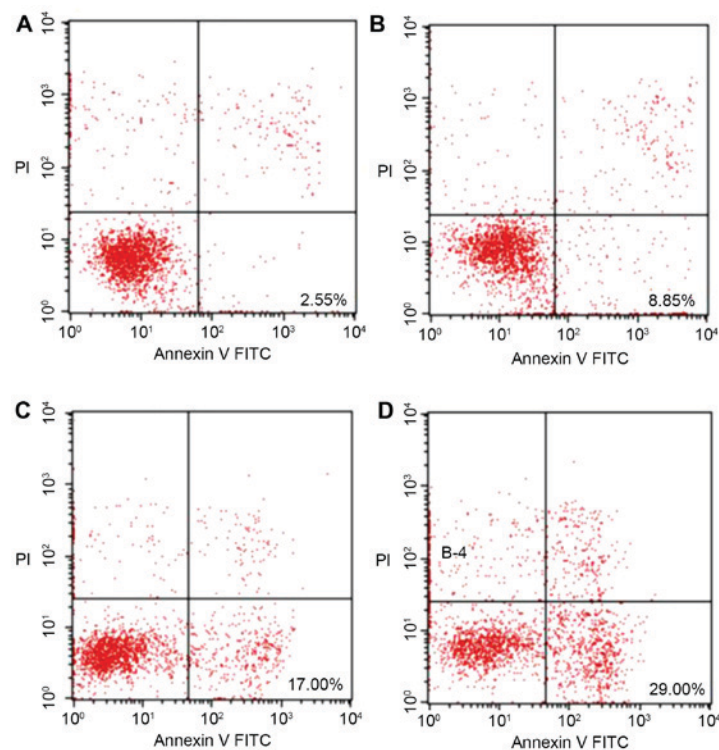


Figure 6. Flow cytometry analysis of PC-3 cells by double-labeling with Annexin-V fluorescein isothiocyanate (FITC) and PI. Quadrants: Lower left-the live cells. Lower right-the early apoptotic cells. Upper left-the necrotic cells, and Upper right-late apoptotic cells. Percentages of early apoptotic cells were: (A) control cells: $2.55 \pm 1.53\%$; (B) SHP (5 mg/ml): $8.85 \pm 1.24\%$; (C) SHP (10 mg/ml): $17.00 \pm 1.85\%$; (D) SHP (15 mg/ml): $29 \pm 1.96\%$. One representative FACS assay of three independent experiments was presented.

results show the concentration-dependency of SHP for cancer cell apoptosis.

Cellular apoptosis analysis by flow cytometry. To confirm the observed induction of cell apoptosis by SHP, we performed

flow cytometry using the Annexin V-FITC/PI assay (Fig. 6, lower right quadrants represent the early apoptotic cells [Annexin V binding and propidium iodide (PI) negative]). As shown in Fig. 6A, the majority of normal cells appeared in the Annexin V-negative section (lower left quadrants) and PC-3

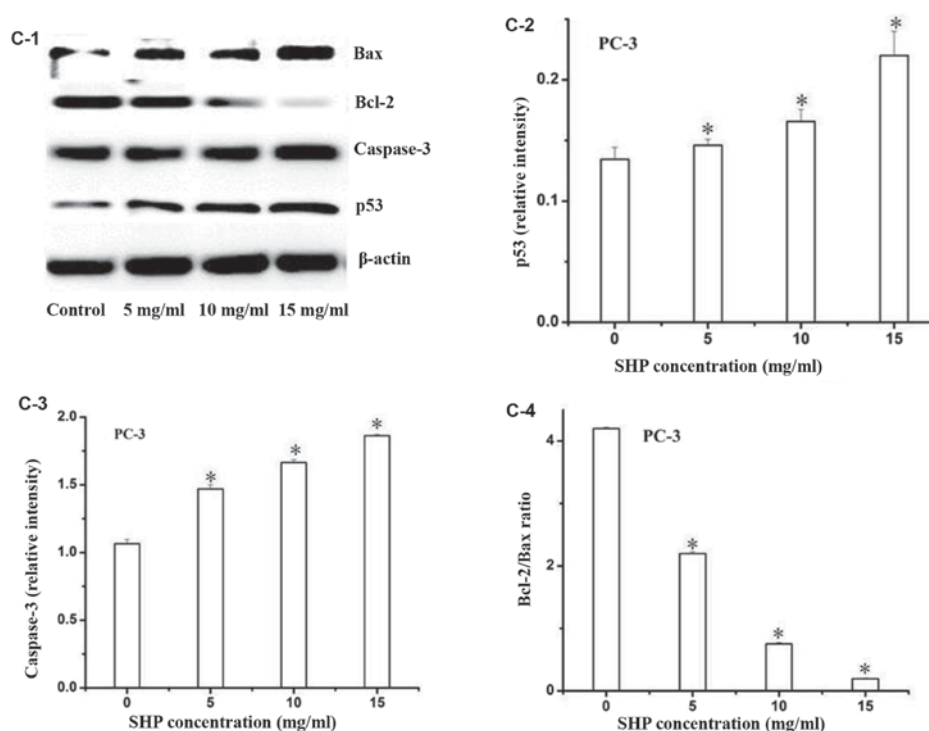


Figure 7. PC-3 cells were treated with the indicated concentrations (5, 10, 15 mg/ml) of SHP, after 24 h, the cells were harvested, the lysates were subjected to SDS-PAGE followed by immunoblotting for-actin, p53, Caspase-3, Bcl-2, Bax. The-actin was as the control (C-1). For the relative changes of proteins in p53, Caspase-3 in the (C-2), (C-3), and for the changes in the ratio of Bcl-2, Bax in the (C-4). *Significant difference ($P < 0.05$) vs. control.

cells treated with SHP (5, 10, and 15 mg/ml) increased the Annexin V-positive cell population from 8.85 to 29% (lower right quadrants).

Effects of SHP on apoptosis-related proteins in PC-3 cells. To understand the mechanisms by which SHP induced apoptosis we examined the expression of proteins typically associated with apoptosis, such as p53, caspase-3, Bcl-2, and Bax (Fig. 7). The results indicated that SHP induced an increase in p53 expression and caspase-3 protein levels ($P < 0.05$). Moreover, Bcl-2 levels were significantly downregulated ($P < 0.05$), whereas Bax expression was enhanced and the ratio of Bcl-2/Bax protein levels was decreased. These findings clearly indicate that SHP induces apoptosis in PC-3 cells via the mitochondrial apoptotic pathway.

Discussion

The uniqueness of the marine environment, the diversity of marine creatures, and the novelty of the structures of active substances utilized by marine organisms has become the foundation for novel research and development of marine biologic active substances. In recent years, more and more extracts from marine creatures have been confirmed to suppress tumor growth. Pettit reported that one of seven peptides from the sponge *Phakellia* sp., suppressed mouselymphoma P388 cells, with an ED_{50} of 5 mg/ml (23). Ma reported that a peptide isolated from *Bullacta exarata*, possessed anti-Pca activity (26). Yong *et al* and Wang *et al* reported that anticancer peptides from the protein hydrolysate of *Mytilus coruscus* (27,28).

In the present study, we isolated polypeptides via the hydrolysis of sepia ink and demonstrated that one of these polypeptides,

SHP, exhibited anti-Pca proliferation and apoptosis-inducing effects in human Pca PC-3 cells, *in vitro*. Although a number of reports indicate that extracts from sepia ink have anti-tumor properties (19-21). This is the first report to demonstrate that polypeptides extracted from sepia ink induced apoptosis in cancer cells. Our results clearly demonstrated that PC-3 cells treated with SHP presented with the typical cellular morphologic changes associated with apoptosis, such as membrane blebbing, chromatin condensation, fragmented nuclei, and apoptotic bodies. Moreover, Annexin V/PI double-staining assays revealed that the percentage of early apoptotic PC-3 cells increased from 8.85 to 29.00% following treating with SHP. These results suggest that SHP inhibits the proliferation of PC-3 cells indirectly via mechanisms related the induction of apoptosis. Therefore, we subsequently investigated the mechanisms of SHP-induced apoptosis in PC-3 cells.

Our data showed an upregulation of the Bcl-2/Bax ratio. Moreover, we found that p53 expression and caspase-3 protein levels were increased. Bcl-2 family proteins play an important role in the cellular apoptosis pathway. Bcl-2 and Bax, representative pro-apoptotic and anti-apoptotic genes, respectively, in the Bcl-2 family are strongly correlated with the induction of apoptosis (29). Moreover, the Bcl-2/Bax ratio appears to be the most critical factor for inducing apoptosis (30). Interestingly, p53, a typical tumor suppressor gene, might affect the BCL-2/Bax ratio, thus regulating cell apoptosis indirectly (31). Studies have shown a correlation between p53 levels and apoptosis-related gene (anti- and pro-) proteins expressed during cell apoptosis (32,33) and that p53 played a key role in the activation of apoptosis through the mitochondrial apoptosis pathway (34). Caspases are also involved in the induction of apoptosis as either an initiator (caspase-3) or an

effector (caspase-9) (35,36). Taken together, our results suggest that SHP induced apoptosis in PC-3 cells. How SHP could be used to specifically inhibit tumor growth without causing a systemic imbalance in the normal cellular environment, however, remains unknown.

In summary, our study revealed the optimized preparation conditions for pepsin hydrolysate from sepia ink and its ability to induce the apoptosis of PC3 cells. Our results clearly demonstrate that the bioactive peptide SHP, whose peptide sequence is LKEENRRRRD has a significant inhibitory effects on the proliferation of PC-3 cells in a concentration- and time-dependent manner, and can induces PC-3 cells apoptosis *in vitro*.

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References

- Jemal A, Siegel R, Xu J and Ward E: Cancer statistics, 2010. *CA Cancer J Clin* 60: 277-300, 2010.
- Heidenreich A, Bastian PJ, Bellmunt J, Bolla M, Joniau S, van der Kwast T, Mason M, Matveev V, Wiegel T, Zattoni F, *et al*: EAU guidelines on prostate cancer. Part II: Treatment of advanced, relapsing, and castration-resistant prostate cancer. *Eur Urol* 65: 467-479, 2014.
- Heidenreich A, Bastian PJ, Bellmunt J, Bolla M, Joniau S, van der Kwast T, Mason M, Matveev V, Wiegel T, Zattoni F, *et al*: EAU guidelines on prostate cancer. part I: Screening, diagnosis, and local treatment with curative intent-update 2013. *Eur Urol* 65: 124-137, 2014.
- Zheng LH, Wang YJ, Sheng J, Wang F, Zheng Y, Lin XK and Sun M: Antitumor peptides from marine organisms. *Mar Drugs* 9: 1840-1859, 2011.
- Ngo DH, Vo TS, Ngo DN, Wijesekara I and Kim SK: Biological activities and potential health benefits of bioactive peptides derived from marine organisms. *Int J Biol Macromol* 51: 378-383, 2012.
- Wang R, Sun DY and Meng ZH: Squid ink - a novel systemic hemostatic. *Chin J Zool* 1: 20-21, 1975.
- Chen SG, Xue CH, Xue Y, Li ZJ, Gao X and Ma Q: Studies on the free radical scavenging activities of melanin from squid ink. *Chin J Mar Drugs* 26: 24-27, 2007.
- Guo X, Chen S, Hua Y, Li G, Liao N, Ye X, Liu D and Xue C: Preparation of water-soluble melanin from squid ink using ultrasound-assisted degradation and its antioxidant activity. *J Food Sci Technol* 51: 3680-3690, 2014.
- Rajaganapathi J, Thyagarajan SP and Edward JK: Study on cephalopod's ink for anti-retroviral activity. *Indian J Exp Biol* 38: 519-520, 2000.
- Smiline Girijia AS, Vijayshree Priyadharshini J, Pandi Suba K, Hariprasad P and Raguraman R: Antibacterial effect of squid ink on ESBP producing strains of *Escherichia coli* and *Klebsiella pneumoniae*. *Indian J Mar Sci* 41: 338-343, 2012.
- Kim SY, Kim SH and Song KB: Partial purification and characterization of an angiotensin converting enzyme inhibitor from squid ink. *Agric Chem Biotechnol* 46: 122-123, 2003.
- Wang G, Guo YZ, Guan SB, Zhong JP, Pan JQ, Huang Y and Liu HZ: Protective effect of sepia ink extract on cyclophosphamide-induced pulmonary fibrosis in mice. *Chin J Mar Drugs* 28: 36-40, 2009.
- Wang G, Liu HZ, Wu JL, Zeng QW, Chen YP, Yang CL and Zhong JP: Study of sepia ink extract on protection from oxidative damage of cardiac muscle and brain tissue in mice. *Chin JMAP* 27: 95-98, 2010.
- Liu HZ, Wang G, Guo YZ, *et al*: Protective effect of squid ink on cyclophosphamide-induced oxidative injury of kidney in mice. *Chin J Nephrol* 25: 804-805, 2009.
- Zhong JP, Wang G, Shang JH, Pan JQ, Li K, Huang Y and Liu HZ: Protective effects of squid ink extract towards hemopoietic injuries induced by cyclophosphamine. *Mar Drugs* 7: 9-18, 2009.
- Sasaki J, Ishita K, Takaya Y, Uchisawa H and Matsue H: Anti-tumor activity of squid ink. *J Nutr Sci Vitaminol (Tokyo)* 43: 455-461, 1997.
- Takaya Y, Uchisawa H, Matsue H, Okuzaki B, Narumi F, Sasaki J and Ishida K: An investigation of the antitumor peptidoglycan fraction from squid ink. *Biol Pharm Bull* 17: 846-849, 1994.
- Chen SG, Wang JF, Xue CG, Li H, Sun B, Xue Y and Chai W: Sulfation of a squid ink polysaccharide and its inhibitory effect on tumor cell metastasis. *Carbohydrate Polymers* 81: 560-566, 2010.
- Zheng YY, Yang ZS, Yan HQ, Huang FF, Li R and Ding GF: Apoptosis induction and underlying mechanism of peptidoglycan from cuttlefish ink on prostate cancer PC-3 and DU-145 cells. *Food Sci* 34: 251-256, 2013.
- Ding GF, Huang FF and Yang ZS: Anticancer activity of an oligopeptide isolated from hydrolysates of Sepia ink. *Chin J Nat Med* 9: 151-155, 2011.
- Huang FF, Yang Z, Yu D, Wang J, Li R and Ding GF: Sepia ink oligopeptide induces apoptosis in prostate cancer cell lines via caspase-3 activation and elevation of Bax/Bcl-2 ratio. *Mar Drugs* 10: 2153-2165, 2012.
- Zeng MJ, Zhang DM and Chen JH: Research advances of anti-tumor peptides. *Chin J Bio Chem Pharm* 28: 139-141, 2007.
- Pettit GR: Isolation and structure of phakellistatin14 from the Western Pacific marine sponge *Phakellia* sp. *J Nat Prod* 68: 60-63, 2005.
- Liu YJ, Liang ZH, Li ZZ, Yao JH and Huang HL: Cellular uptake, cytotoxicity, apoptosis, antioxidant activity and DNA binding of polypyridyl ruthenium(II) complexes. *J Organomet Chem* 696: 2728-2735, 2011.
- Liu RJ, Zhang YW, Wen CW and Jian T: Study on the design and analysis methods of orthogonal experiment. *Exper Technol Manage* 27: 52-55, 2010.
- Ma J, Huang F, Lin H and Wang X: Isolation and purification of a peptide from *Bullacta exarata* and its impact on apoptosis on cancer cell. *Mar Drugs* 11: 266-273, 2013.
- Wang B, Li L, Chi CF, Ma JH, Luo HY and Xu YF: Purification and characterisation of a novel antioxidant peptide derived from blue mussel (*Mytilus edulis*) protein hydrolysate. *Food Chem* 138: 1713-1719, 2013.
- Yang YF, Yan HQ, Ding GF and Huan FF: Isolation and purification of an anticancer activity peptide from protein hydrolysate of *Mytilus coruscus*. *J China Pharm U* 42: 272-275, 2011.
- Ren G, Zhao YP, Yang L and Fu CX: Anti-proliferative effect of cliticine from the mushroom *Leucopaxillus giganteus* on human cervical cancer HeLa cells by inducing apoptosis. *Cancer Lett* 262: 190-200, 2008.
- Ghobrial IM, Witzig TE and Adjei AA: Targeting apoptosis pathways in cancer therapy. *CA Cancer J Clin* 55: 178-194, 2005.
- Fridman JS and Lowe SW: Control of apoptosis by p53. *Oncogene* 22: 9030-9040, 2003.
- Milczarek GJ, Martinez J and Bowden GT: p53 phosphorylation: Biochemical and functional consequences. *Life Sci* 60: 1-11, 1997.
- Zhou J, Luo RH, Tang CF, *et al*: Effect of Bcl-2 protein family and p53 gene on regulating and controlling cell apoptosis abstract. *J Clin Rehab Tissue Engin Res* 11: 1950-1952, 2007.
- Keim A, Rossler OG, Rothhaar TL and Thiel G: Arsenite-induced apoptosis of human neuroblastoma cells requires p53 but occurs independently of c-Jun. *Neuroscience* 206: 25-38, 2012.
- Xu HL, Yu XF, Qu SC, Zhang R, Qu XR, Chen YP, Ma XY and Sui D: Anti-proliferative effect of Juglone from *Juglans mandshurica* Maxim on human leukemia cell HL-60 by inducing apoptosis through the mitochondria-dependent pathway. *Eur J Pharmacol* 645: 14-22, 2010.
- Olsson M and Zhivotovsky B: Caspases and cancer. *Cell Death Differ* 18: 1441-1449, 2011.