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# Anticancer Activity of an Oligopeptide Isolated from Hydrolysates of *Sepia* Ink

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**[ABSTRACT] AIM:** To investigate the antitumor effect of peptides from *Sepia* ink by enzymatic hydrolysis. **METHODS:** *Sepia* ink was hydrolyzed with trypsin to prepare peptides; hydrolysates were isolated by ultrafiltration and purified using G-25 gel filtration. The purity of the *sepia* ink oligopeptides was demonstrated by HPLC and its peptide sequence was detected. Then CCK-8 method was employed to measure cellular viability, and HE staining was used to detect cell morphological change. **RESULTS:** An oligopeptide was obtained from the hydrolysates of *Sepia* ink and identified as N Gln-Pro-Lys with a molecular mass of 343.4. The yield of oligopeptides was 1.82%. The CCK-8 assay showed that N Gln-Pro-Lys significantly inhibited the proliferation of DU-145 cells and induced their death in a dose-dependent manner. The morphologic changes were observed by HE staining after treated with *sepia* ink oligopeptides. **CONCLUSION:** N Gln-Pro-Lys showed potent antitumor activity.

**[KEY WORDS]** *Sepia* ink; Enzymatic hydrolysis; Oligopeptide; Antitumor; N Gln-Pro-Lys

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## 1 Introduction

Newly discovered peptides are becoming increasingly important, not only as molecular tools for the understanding of protein-protein interactions, but also as lead compounds for the treatment of various diseases. Several studies have proved that certain products from marine sources, such as *cymbastella* sp., *Dolabella auricularia*, *mactromeris polynyma* and *halichondria okadai*, display significant anticancer activity [1-4]. Dolastatin-10 and Dolastantin-15 [5-6], peptides isolated from marine sea hare *Dolabella auricularia* have been known to have antitumor activities against several cancer cell lines. Many anticancer peptides have been extracted by hydrolysis using various enzymes [7]. The properties of functional pep- tides are highly influenced by their molecular masses and their structures, which are in turn greatly affected by their processing conditions and the enzymes employed. Enzymatic hydrolysis, an effective approach for the release of bioactive peptides from protein sources, has become a valuable tool for changing and upgrading protein nutrition and function [8]. In addition, peptide drugs have been widely employed as vaccines, antibiotics, diagnostics and as antitumor treatments because of their low dosage and superior treatment results [9]. Peptides as antitumor drugs can improve immune response, inhibit the tumor angiogenesis and metastasis of tumor cells, directly eradicate tumor cells and induce the apoptosis of tumor cells and arrest the cell cycle [10]. For example, tyroserleutide, a tripeptide and a new anticancer drug, has entered clinical trials [11].

Cuttlefish ink (*sepia esculenta*) had been used in the treatment of hemostasis for centuries in Chinese traditional medicine [12]. It has been reported that *Sepia* ink possesses antitumor activity against Meth-A fibrosarcoma in BALB/c mice and its fraction containing peptidoglycan showed higher antitumor activity than the other fractions [13]. However, no information is currently available for a tripeptide that has antitumor activity from *Sepia* ink extracted by hydrolysis. During the course of our extensive screening program on marine traditional Chinese medicines for *in vitro* antitumor activities, *Sepia* ink was selected because of its widespread

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geographic distribution in China and because there is a cheap commercial supply as it is generally discarded.

In this paper, we describe the primary structure of peptides derived from *sepia* ink hydrolysates and their anticancer activities.

## 2 Materials and Methods

### 2.1 *Materials*

*Sepia* ink was obtained from the food processing factory and the category of cuttlefish is *sepia esculenta* identified by professor Zhao Shen-Long in Zhejiang Ocean University. Trypsin, with it proteolytic activities of (2.8 × 104), (19 × 104), (15 × 104) and (1.8 × 104) $U \cdot g^{-1}$, respectively, was purchased from Shanghai Ruji Biological Technology Co., Ltd., China. The cell viability of *sepia* ink oligopeptides were measured on tumor cell lines: human prostate cancer DU-145 (Cellbank of Chinese Academy of Science, Shanghai, China). Ultrafiltrate were applied to a column saturated in Sephadex G-25 resin (Amersham Pharmacia Biotech., Shanghai, China). The *Sepia* ink oligopeptides fraction was further purified using reverse-phase HPLC on a Primesphere 10 $C_{18}$ column (Phenomenex Co., Ltd., Shanghai, China). The viability of tumor cell lines were detected by CCK-8 Kit (Hangzhou Haotian Biotechnology Co., Ltd., Hangzhou, China). Other reagents are analytical reagents made in China.

*Sepia* ink (500 g) was minced at 12 000 $r \cdot min^{-1}$ for 2 min by triturator. The homogenate was stored at −20°C before use.

### 2.2 *Enzymatic hydrolysis*

The conditions of the enzymatic hydrolysis were: temperature of 51 °C, pH 8, enzyme concentration of 1 200 $U \cdot g^{-1}$ (0.064 $g \cdot g^{-1}$), solid-liquid ratio of 1 : 1 and time of 16 h. Reactions were terminated by heating the solution to 98 °C for 15 min to inactivate the enzyme. The resulting slurry was centrifuged at 10,000 $r \cdot min^{-1}$ for 20 min.

### 2.3 *Isolation and purification of anticancer peptide*

#### 2.3.1 *Ultrafiltration*

*Sepia* ink hydrolysates were separated into a large molecular weight fraction and a low molecular weight fraction by ultrafiltration at 4 °C by PM-10 membrane (MwCO: 3 000) and kept for use in gel filtration. Prior to use, the membrane was activated by spinning 10 mL of distilled water, and the remaining liquid was carefully removed.

#### 2.3.2 *Sephadex G-25 gel chromatography*

Ultrafiltrate were again filtered through a Millipore membrane filter (0.45 μm) and applied to a column(2.6 cm × 90 cm) saturated in Sephadex G-25 resin. Sephadex G-25 column was eluted with distilled water and fractions were collected at 3-minute interval with a fraction collector. The absorbance was measured at 280 nm, according to the procedure described by Tsai. The hydrolysis fraction was fractionated into five fractions by gel filtration chromatography. Each fraction was tested for anticancer.

#### 2.3.3 *High performance liquid chromatography (HPLC)*

The fraction exhibiting the highest anticancer activity was further purified using reverse-phase HPLC on a Primesphere 10 $C_{18}$ column (10 mm × 250 mm) with a linear gradient of acetonitrile (0-50% for 20min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 $mL \cdot min^{-1}$. The absorbance of eluent was monitored at 280 nm. Active peak representing anticancer activity was pooled and lyophilized immediately. The purified peptide was analyzed its amino acid sequence.

### 2.4 *The yield of sepia ink oligopeptides*

The fraction from G-25 gel chromatography was concentrated in vacuum under 30 °C and concentrated liquid was dried under 80 °C by vacuum freeze-drying machine. Then the powder of *sepia* ink oligopeptides was collected and weighed.

### 2.5 *Peptide sequence*

Sequence of the purified anticancer peptide with the greatest activity was determined by degradation from its *N*-terminus using the Edman degradation reagent, phenylisothiocyanate. The peptide sequence was performed on automated peptide sequencer, Precise Protein Sequencing System.

### 2.6 *Cell culture and cell viability using CCK-8 assay*

DU-145, human prostate carcinoma cell was cultured and maintained in F12 containing 100 $\mu g \cdot mL^{-1}$ penicillin-streptomycin, 10% fetal bovine serum (FBS) and maintained at 37 °C under a humidified atmosphere of 5% $CO_2$. Cell viability after treatment with *sepia* ink oligopeptides was measured by the CCK-8 assay. DU-145 cells were suspended at a final concentration of $1 \times 10^4$ cells/well and cultured in a 96-well flat-bottomed microplate and allowed to adhere to the bottom of the wells for 24 h before the beginning of treatment. Cells were exposed to 3-15 $mg \cdot mL^{-1}$ doses of peptide for 24, 48 and 72 h. F12 medium (10% FCS) was used as a control for the DU-145. After different treatment of cells, CCK-8 was added to each well containing 100 μL of the culture medium and peptide mixture, and the plate was incubated for 4-5 h at 37 °C. Viable cells were counted by absorbance measurement at 450nm using auto-microplate reader. All experiments were performed in triplicate on three separate occasions. Viability percentage was calculated according to the following formula: (*A* of treated cells/*A* of corres- ponding control) × 100.

### 2.7 *Hematoxylin-Eosin staining*

DU-145 were suspended at a final concentration of $1 \times 10^5$ cells/well and cultured in a 6-well flat-bottomed microplate over a 35 mm coverslip and allowed to adhere to the bottom of the wells for 24 h before the beginning of treatment. Cells were exposed to 5, 10, 15 $mg \cdot mL^{-1}$ doses of *sepia* ink oligopeptides for 24 h. F12 medium (10% FCS) was used as a control for the DU-145. After different treat- ment, the cells were washed with PBS three times and fixed by 95% alcohol for 15 min. Then the cells were washed three times

with PBS and stained in Hematoxylin working solution for 6 min. Then the cell were washed in running tap water for 15 min and stained in working Eosin-Phloxine solution for 1 min. They were differentiated and dehydrated in 70%, 95% and 100% ethanol for 5 min, respectively. Finally, the cells were cleared in dimethylbenzene and covered coverslip with mounting medium on glass slide.

2.8 *Data analysis*

All data were analyzed by the software of SPSS.

## 3 Results and Discussion

3.1 *Isolation and purification of peptide*

After ultrafiltration using a PM-10 membrane with molecular weight cut off of 3 000, the filtrates of trypsin digestion were loaded on a gel filtration column (Sephadex G-25). After gel filtration, five anticancer peptide fractions with the highest activity were collected and their purities were checked by HPLC. The sample hydrolyzed by trypsin was divided into five peaks and peak II had the highest anticancer activity. Peak II was further separated by reverse-phase HPLC with a linear gradient of acetonitrile (0-50% for 20 min) containing 0.1% TFA. As shown in Fig. 1, only a single peptide peak was obtained and its sequence was identified as N Gln-Pro-Lys with a molecular mass of 343.4. After freeze drying, 9.08 g *sepia* ink oligopeptide was obtained from 500 g *sepia* ink. So the yield was 1.82%.

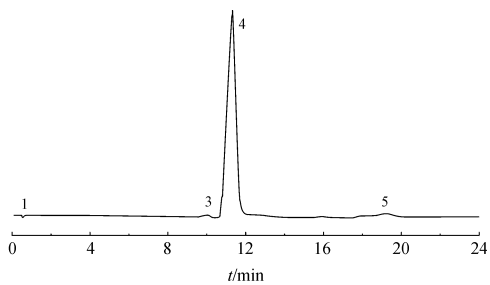


**Fig. 1 The reverse-phase HPLC chromatogram of the peak II**

3.2 *Effect of sepia* ink oligopeptides *on cell viability*

The cellular growth index of DU-145 was determined using a cell counting Kit-8, where the reduction of wst-8 to give a yellow formazan by dehydrogenases is directly proportional to the number of the living cells. DU-145 cells were treated with 3-15 mg·mL$^{-1}$ of *sepia* ink oligopeptides for 24-72 h and the cell number was estimated by the CCK-8 assay as indicated in the methods. DU-145 cells displayed dose-dependent decreases in viability, detectable as early as 24 h (Fig. 2). At 24 h, the threshold concentration which caused a decrease in DU-145 cell viability was 3 mg·mL$^{-1}$ (89.4% of control, $P > 0.05$). The *sepia* ink oligopeptides that produced the maximal effect was 15 mg/mL (25.4% of control, $P < 0.05$), and the half inhibitory concentration ($IC_{50}$) was 9.50 mg·mL$^{-1}$.

At 48 h (Fig. 2), the threshold concentration was 3 mg·mL$^{-1}$ (33.5% of control, $P < 0.05$), the maximal effect was 15 mg·mL$^{-1}$ (0.24% of control, $P < 0.05$), and the $IC_{50}$ was 1.00 mg·mL$^{-1}$. At 72 h (Fig. 2), the threshold concentration was again 3 mg·mL$^{-1}$ (42.7% of control, $P < 0.05$). The maximal effect was 15 mg·mL$^{-1}$ (0% of control, $P < 0.05$), and the $IC_{50}$ was 2.74 mg·mL$^{-1}$. The threshold concentration at 24, 48 and 72 h were the same (3 mg·mL$^{-1}$), although the level of the significance increased from day 1 to days 2 and 3. The $IC_{50}$ fell from 9.50 mg·mL$^{-1}$ at 24 h to 1.00 mg·mL$^{-1}$ at 48 h. However, there was little change at 48 h and 72 h. These results suggest that *sepia* ink oligopeptides had a dose-dependent deleterious effect on Du-145 cell viability.

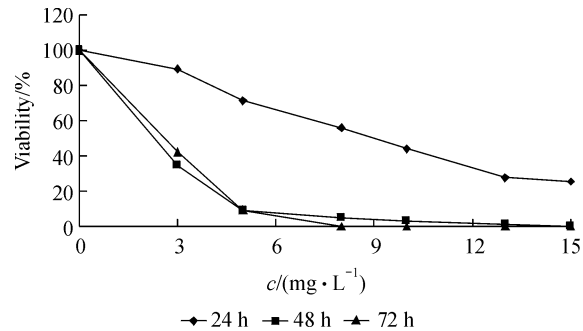


**Fig. 2 Viability curves of DU-145 cells treat with *sepia* ink oligopeptides for 24, 48 and 72 h**

3.3 *Effect of sepia ink oligopeptides on morphology of DU-145 cells detected by Hematoxylin-Eosin staining*

During the whole process, cells first showed dilated intercellular spaces with cellular shrinkage; then a few blebs were present in the middle of the cell, and the nucleus was pushed to the side; after that, the blebs were fragmented into small vesicles around nucleus zone with chromatin condensation. Finally, the vesicles were separated out, and the nucleus was fragmented. Besides, while DU-145 cells were incubated with 5, 10 and 15 mg·mL$^{-1}$ concentrations for 24 h, the morphologic changes were observed as the process as mentioned before (Fig.3 A-D). The observation of these morphologic changes was a common process during the

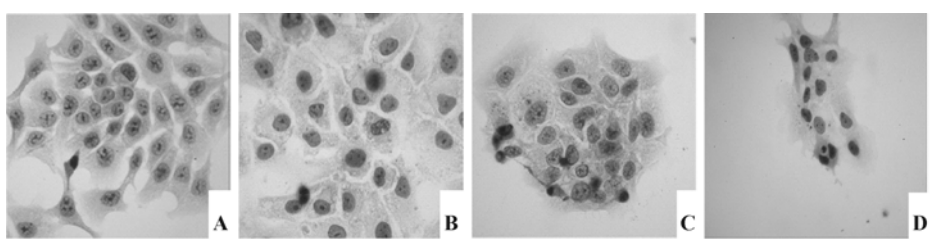


**Fig. 3 Morphologic changes of DU-145 cells. A-D: cells were incubated with *sepia* ink oligopeptide (0, 5, 10, 15 mg·mL$^{-1}$) 24 h**

cell death of DU-145 cells induced by *sepia* ink oligopeptides.

3.4 *Discussion*

In recent years, a number of researchers have focused on identifying novel natural product as anticancer drugs [6]. Anticancer peptides have characteristics of multi-function, high sensitivity, stability and so on [14]. In our studies, one peptide was purified and possessed anticancer activity.

Various peptides from marine mollusks possess anticancer activities. Xiang Xiao et al reported anticancer glycopeptides from *Meretix meretrix* and its inhibitory rate affecting the KB cell line was 69% at 200 μg·mL$^{-1}$ [15]. Relatively strong anticancer peptides were also found from *M. meretrix* with $IC_{50}$ of 10 μg·mL$^{-1}$ [16]. In addition, dalastatin-10, extracted from the sea hare *D. auricularia*, has entered into clinical trials. Dolastatin-10 is a pentapeptide with four of the residues being structurally unique. It is the most potent anti-proliferative agent known with an $ED_{50}$ of $4.6 \times 10^{-5}$ μg·mL$^{-1}$ against murine PS leukemia cells [17].

As early as 1982, it was reported that *Sepia* ink could regulate gastric juice secretion and had anti-ulceration activity [18]. Researchers in Japan found that the peptidoglycan extracted from *Sepia* ink had higher antitumor activity than the other fractions. In addition, they also found that the carbohydrate part of the peptidoglycan possessed the anticancer activity [19-20]. Interestingly, in our studies, the *sepia* ink oligopeptides prepared using the protease trypsin from *Sepia* ink also inhibited the growth of DU-145 cells. As shown in Fig. 2, in DU-145 cells, *sepia* ink oligopeptides caused a linear decrease of cell viability in a dose-dependent manner. However, the mechanism of the anticancer activity is unclear. Therefore, further studies are needed to identify the mechanism of the potent antitumor activity.

There had been a few publications on anticancer peptides from food proteins, such as fish sauce, soy protein, mollusk protein, milk protein, and beef protein [21-25]. However, no study had used *Sepia* ink protein waste as the protein source for an anticancer peptide. The results of our studies demonstrated the effect of *sepia* ink oligopeptides on growth inhibition and could be a potentially useful adjunct in the treatment of cancer. Hence, its cuttlefish ink(*sepia esculenta*) easily accessible source makes *Sepia* ink protein waste attractive as a protein source for the future industrial production of functional peptides.

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# 乌贼墨酶解肽提取方法及其抗肿瘤活性

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**【摘　要】** **目的：**用蛋白酶酶解乌贼墨, 经纯化获得寡肽, 并对其进行抗肿瘤活性研究。**方法：**通过胰蛋白酶酶解获得乌贼墨酶解物, 将酶解液用超滤、G-25 分子筛凝胶层析进行分离纯化, 高效液相进行纯度检测。分离纯化得到的寡肽进行氨基酸序列检测, 并用 CCK-8 检测寡肽对 DU-145 细胞增殖的影响和 HE 染色方法观察其对细胞形态的影响。**结果：**乌贼墨寡肽的 N 端氨基酸序列为: Gln-Pro-Lys, 得率为 1.82%。将寡肽进行抗肿瘤活性研究, 发现寡肽对 DU-145 细胞有明显的增殖抑制效应, 且有浓度依赖性。HE 染色结果显示了寡肽使细胞形态发生不规则变化, 失去原有的恶性表型。**结论：**用酶解方法制得的乌贼墨寡肽有明显的抗肿瘤活性。

**【关键词】** 乌贼墨; 酶解; 寡肽; 抗肿瘤; N Gln-Pro-Lys

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