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Original Research Article

Studies on the Amino Acid Composition and Antioxidant Activities of Collagen Polypeptides from *Holothuria nobilis* Selenka

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Abstract: Studies on amino acid composition and antioxidant activities of collagen Polypeptides from the *Holothuria nobilis* Selenka were carried out in this paper. Results revealed that collagen polypeptides mainly contains glycine(Gly), alanine(Ala), glutamate (Glu), proline (Pro) and hydroxyproline (Hyp) with high medicinal values and good tasting. The rate of delicious amino acid, pharmacodynamics amino acid and essential amino acid in the total collagen polypeptide were accounted for 65.1%, 58.08% and 24.96% with IC_{50} value of 5.78 mg/ml, 4.20mg/ml and 0.27mg/ml in the antioxidant free-radical tests, respectively.

Keywords: *Holothuria nobilis* Selenka; collagen polypeptide; amino acid composition; free radicals; antioxidant activity.

INTRODUCTION

Collagen is composed of three polypeptide chains with stranded helix structures, which is key-protein in organisms and connective tissues [1, 2]. Collagen polypeptide is the product of collagen or gelatin degradation [3]. Researches show that collagen polypeptide has many physiological and pharmacological activities, such as the high protein absorption rate, protecting gastric mucosa, antiulcer, promoting the collagen metabolism of skin, inhibiting the rise of blood pressure, promoting the absorption of Ca, and decreasing the content of cholesterol in serum, etc [4-9]. In recent years, it was reported that polypeptides from sea cucumber possess various bioactivities such as antioxidant, immune regulation, influence on cell activity of tyrosinase in the cells of B16, protection of vascular endothelial cells, etc. Studies showed that whether the activity of sea cucumber collagen polypeptide is positive or not does not only depend on the molecular weight, but also on the composition and amino acid sequence [4]. *Holothuria nobilis* Selenkae whose bodies are large, wide and thick belongs to echinodermata, holothuroidea, aspidochirotida, holothuriidae. In general, the length of them is about 300 mm. The species are widely distributed in the sea from Dongshan county, Fujian province to Paracel Islands [10]. At present, the research on *Holothuria nobilis* Selenka has mainly addressed structural identification of polysaccharide, sterols, and pyrimidine [5, 7, 11, 12]. Collagen accounts for about 70% of the total body wall protein of sea cucumber [13]. However, no studies about collagen polypeptide and its compositions from *Holothuria nobilis* Selenka have been reported to date. In this paper, we reported the amino acid composition and free radicals scavenging activity in vitro, providing basic data for development and utilization of *Holothuria nobilis* Selenka.

MATERIAL AND METHODS

Preparation of collagen polypeptide

Sea Cucumber *Holothuria nobilis* Selenka were obtained from Guangxi province, China. Sea Cucumber (100g wet weight) was cut into pieces and doused in warm water for 5h. After that, bleached the sample by 10% H_2O_2 and treated with NaOH and n-butyl alcohol. The sea cucumber flesh was hydrolysed with flavourzyme. Proteases degrade protein and produce peptides with various lengths and sequences. The peptides from flavourzyme prepared Sea Cucumber *Holothuria nobilis* Selenka hydrolysate were isolated by using alcohol precipitation, ultrafiltration, gel filtration

on a Sephadex G-25 column and high performance liquid chromatography on an ODS column. *Holothuria nobilis* Selenkae collagen polypeptide (HNSCP) ultimately was obtained, named as HNSCP-F-A(MW:1kDa-10kDa).

Analysis of amino acid composition of HNSCP-F-A

By using peeling methods, 0.9mg HNSCP-F-A weighed accurately, then transferred to a hydrolysis tube, 1ml 6M hydrochloric acid added to the same hydrolysis tube, N_2 was inflated for 10 minutes. After that, the HNSCP-F-A was hydrolyzed for 24 hours in the condition of 110℃. The samples were then processed for analysis of amino acid composition by reversed phase high performance liquid chromatography [13].

The Buffer solution is composed of A and B (A: 50mM sodium acetic acid aqueous solution, B: methanol: acetonitrile: water -2:6:2). The column was balanced by 5% A liquid following by gradient eluting with B liquid from 5%-48%, 0-39 minutes, from 8%-100%，39-40 minutes. At last, the linear gradient of B liquid was stabilized at 100% for 40-45 minutes.

STUDY ON THE ANTIOXIDANT ACTIVITY OF HNSCP-F-A

1.0mg/ml, 2.0mg/ml, 3.0mg/ml, 4.0mg/ml and 5.0mg/ml *Holothuria nobilis* Selenka collagen polypeptide samples were prepared respectively. Then they were stored at -20℃.

DPPH radical scavenging activity

0.0794 grams of DPPH was transferred into a 1000ml flask and dissolved in 1000ml of ethanol. At first 5 test tubes were taken to make aliquots of 5 different concentrations level (1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL). Different concentrations of test plant samples were mixed with 2ml of DPPH. The rest of 5 test plant samples were mixed with 2ml of ethanol solution instead of DPPH. The mixture was shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517 nm. DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.

The percentage inhibition was calculated according to the formula.

The formulation of clearance rate: $E(\%)=[A_0-(A_i-A_j)]/A_0\times100\%$.

A_0: absorbance of blank control;
A_i: the absorbance of samples in the presence of DPPH,
A_j: the absorbance of samples without DPPH.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of HNSCP-F-A was assayed by the method [14]. The reaction mixture 8.0 ml contained 2.0 ml of 9 mmol salicylic acid, 2.0 ml of 9 mmol $FeSO_4$ and 2.0ml of 8.8 mmol hydrogen peroxide and varied concentrations of the HNSCP-F-A. After incubation for 30 minutes at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 510 nm. The scavenging activity of hydroxyl radical effect was calculated as follows:

Hydroxyl radical scavenging activity $(\%)=[1-(A_1-A_2)/A_0]\times100$

A_0: absorbance of the control (without HNSCP-F-A);
A_1: absorbance of the HNSCP-F-A;
A_2: absorbance without salicylic acid.

Superoxide anion scavenging activity

Superoxide anion scavenging activity was evaluated by measuring the inhibition of the auto-oxidation of pyrogallol using a modified methods of Marklund and Marklund [15, 16]. 4.5 ml of Tris–HCl buffer (50 mM, pH 8.2) and 4.2 ml distilled water were added into freshly prepared 0.3 ml of 3 mM pyrogallol (dissolved in 10 mM HCl). Pyrogallol was replaced with HCl in the blank control. The absorbance control at 325 nm was measured at every 0.5 min interval for 4 min. The increment of the absorbance per minute was regarded as the auto-oxidation rate of pyrogallol (A_1). The inhibited auto-oxidation rates of pyrogallol (A_2) were gained by replacing the distilled water with the samples. The ability to scavenging superoxide anion was calculated by the following equation:

Superoxide anion scavenging activity $(\%) = [(A1-A2)/A1]\times100$

Statistical analysis

All assays were carried out in triplicates and result s are expressed as mean ± SD.

The data were analyzed by one way analysis of variance (ANOVA) and regression analysis, which were carried out using SPSS21.0 software. The P values of < 0.05 were considered significant. IC_{50} was calculated by previous method (Probit) with a reliability interval of 95%.

RESULTS AND DISCUSSIONS

Amino acid composition analysis of HNSCP-F-A

In these studies, amino acid composition of collagen polypeptide HNSCP-F-A from *Holothuria nobilis* was analyzed by HPLC and showed in Figure 1 and Table 1. The results showed that *Holothuria nobilis* Selenka mainly contains glycine(Gly), alanine (Ala), glutamate (Glu), proline (Pro), hydroxyproline (Hyp), as well as few Histidine(His) and methionine(Met). The molar percentages of Gly, Pro, and Hyp were 28.19%, 9.87% and 5.91%, respectively. High content of Gly, Pro, Hyp are characteristics distinguished from other collagen. Analysis of amino acid composition showed that the

HNSCP-F-A is collagen polypeptide, and its amino acid composition is complete, tasting good, suggesting it has high medicinal and nutritional value, high Hyp content shows that *Holothuria nobilis* Selenka has a broad prospects. Composition analysis of sea cucumber collagen polypeptide HNSCP-F-A indicats that it contains 17 kinds of common amino acids, 7 kinds of essential amino acids and 10 kinds of non-essential amino acids, which is consistent with the study of Cui Fengxia.

Study on the antioxidant activity of HNSCP-F-A

DPPH radical scavenging activity

The DPPH free-radical is a stable free-radical, which has been widely accepted as a tool for estimating the free-radical-scavenging activities of antioxidants [17]. When DPPH free-radical encounters an antioxidant, the radical would be scavenged and the absorbance at 517 nm is reduced. Based on this principle, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free-radical [18].

Figure 2 shows the DPPH radical-scavenging activity of HNSCP-F-A. The strongest scavenging activity of HNSCP-F-A was 45% at 4mg/mL concentration. The assay showed that DPPH radical scavenging activity as a whole was not dose-response relationship. But Scavenging DPPH radical-scavenging activity of HNSCP-F-A showed the dose-response relationship at concentration of 4mg/ml or less, while the scavenging effect was decreased with the increasing of concentration at concentration of more than 4mg/ml. Collagen Polypeptide HNSCP-F-A showed an IC_{50} of 5.78mg/ml.

Hydroxyl radical scavenging assay

Hydroxyl radicals have the strongest chemical activity among various reactive oxygen species, they can damage a wide range of essential biomolecules such as amino acid, protein, and DNA [19]. However, there is no specific enzyme to defense against hydroxyl radicals in human body. Therefore, it would be of great significance to discover some compounds with good hydroxyl radical scavenging activity for the oxidative stress induced diseases [20].

Hydroxyl radical scavenging assay revealed HNSCP-F-A with strong scavenging effect in Figure 3. HNSCP-F-A showed 55% scavenging effect at 5 mg/mL. When concentration was in range of 4-5mg/ml, hydroxyl radical scavenging activity was smooth. The IC_{50} values of HNSCP-F-A was 4.20mg/ml.

Superoxide anion scavenging activity

Superoxide radical can be generated by auto-oxidation and produce a coloured compound. Resulting from a colour change from purple to yellow, the absorbance at 320 nm increased when the superoxide anion was scavenged by an antioxidant, which can represent the content of superoxide radicals and indicate the antioxidant activity of the sample [21].

As depicted in Fig 4, HNSCP-F-A showed superoxide anion scavenging activity. Its scavenging effect was smoothly increased with the concentration increasing. HNSCP-F-A showed 88% scavenging effect at 4mg/ml. By means of regression analysis, the IC_{50} of HNSCP-F-A was 0.27mg/ml.

CONCLUSION

Experimental results showed that the HNSCP-F-A from *Holothuria nobilis* selenka is collagen polypeptide with high medicinal value and good tastes. IC_{50} values of HNSCP-F-A on the three free radicals (DPPH radical, Hydroxyl radical and Superoxide anionis) were 5.78mg/ml, 4.20mg/ml and 0.27mg/ml, respectively. Compared with this result, it demonstrated that superoxide scavenging effect of HNSCP-F-A is more significant that provides basic data for development and utilization of *Holothuria nobilis* Selenka.

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