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Preparation and biological activities of chitosan from the larvae of housefly, *Musca domestica*

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Abstract

Many studies have indicated that the larvae of housefly possess excellent nutritional and pharmacological value. In the present study, the chitosan was isolated from the larvae of housefly, *Musca domestica*. Its antioxidant potencies were examined employing various established *in vitro* systems, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, reducing power and metal ion chelating activity, and inhibitory effects against human cervical carcinoma (HeLa) and mouse sarcoma-180 (S-180) tumor cells were also tested. The results showed that the chitosan had stronger scavenging activity on DPPH radical than ascorbic acid. The half effective concentration on DPPH radical was approximately 0.373 mg/mL. The chitosan showed efficient reducing power and considerable ferrous ions chelating potency in this study. Moreover, chitosan from housefly larvae also exhibited significant antitumor activity against HeLa and S-180 tumor cell lines *in vitro*. The inhibitory effects of showed a dose-dependent manner and reached 50.8%, 52.9% at 1 mg/mL, respectively. These *in vitro* results suggested that the chitosan from the larvae of housefly could be effectively used as a natural antioxidant to protect the human body from free radicals and retard the progress of many chronic diseases. Furthermore, the chitosan with antitumor activity from the larvae of housefly might provide useful information for the development of antitumor drugs.

Keywords: *Musca domestica*; Chitosan; Antioxidant; Reducing power; Chelating ability; Antitumor activity

1. Introduction

Indiscriminate application of synthetic chemicals has led to a number of ecological and medical problems due to residual toxicity, carcinogenicity, teratogenicity, hormonal imbalance and spermatotoxicity, etc. (Kumar, Mishra, Dubey, & Tripathi, 2007). Therefore, current more attention is focusing on natural active component from plants, animals and other organisms to pharmacologists, biochemists and other health professionals. Insects represent one of the most successful groups of evolution accounting for nearly one million species (Vilmos & Kurucz, 1998). A great array of insects and their products are used as drugs in the traditional medicines such as traditional Chinese medicine, but only few literatures are available. Insects are a large, unexplored and unexploited source of potentially useful compounds for modern medicine (Pemberton, 1999).

The larvae of housefly *Musca domestica* Linnaeus (Diptera: Muscidae) are an excellent source of high-quality protein, polyunsaturated fats, vitamins, minerals and other nutrients for human and animal (Ren & Shi, 2002). The housefly larvae have been used clinically to cure malnutritional stagnation, decubital necrosis, osteomyelitis, ecthyma and lip boil since the Ming/Qing Danysty (1368 Anno Domini) up to now in China, and also used to treat coma and gastric cancer when combined with other drugs (Hou, Shi, Zhai, & Le, 2007). It has also been used in medicine and functional food for centuries, and the biological effects of the active constituents from the larvae of housefly have been studied extensively (Li, 1981; Mumcuoglu et al., 1999; Sherman, 2002; Sherman, Tran, & Sullivan, 1996;

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Thomas, Jones, Shutler, & Jones, 1996). Currently, functional evaluation and identification of active component from the larvae of housefly is the emphasis of research in our laboratory.

Chitosan is a nontoxic biopolymer derived by the deacetylation of chitin (Qi, Xu, Jiang, Hu, & Zou, 2004). Previous studies are concentrated to the biological activity of chitosan from exoskeletons of crustaceans, such as shrimps, crabs and squids (Eweis, Elkholy, & Elsabee, 2006). Only few studies on preparation, property and biological activity of chitosan have been reported in the larvae of housefly (Ding, Tian, Zhang, Zhou, & Lei, 2006; Jing et al., 2007; Lai, Lei, Niu, Zhong, & Jiang, 1999; Wang, Zhou, Huang, Wang, & Lei, 2005). Extensive biological activities of the chitosan from housefly larvae need to be further investigated. These studies are significant to the exploitation and utilization of housefly larvae.

In the present study, free radical scavenging activity, reducing power and metal ion chelating ability of chitosan obtained from the larvae of housefly were examined, respectively. Inhibitory effects of the chitosan on two tumor cell lines were also evaluated *in vitro*.

2. Materials and methods

2.1. Materials

Human cervical carcinoma (HeLa) and mouse sarcoma-180 (S-180) tumor cells were obtained from Pharmacy Department of Tongji Medical University, Wuhan, Hubei, PR China. 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), potassium ferricyanide, ferrozine, ethylenediaminetetraacetic acid (EDTA) and ascorbic acid (Vc) were purchased from Sigma Chemical Co., USA. Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from GIBCO BRL, USA. All chemicals and reagents used were of analytical reagent grade.

2.2. Preparation of chitosan from larvae of housefly

The chitosan was prepared from larvae of housefly according to the method of Yen, Tseng, Li, and Mau (2007) with some modification. Fourth-instar larvae of housefly *M. domestica* were washed with 15% (W/V) aqueous sodium chloride solution and then freeze-dried. The dried larvae were ground using a mill to obtain crude power. The powder of larvae (10 g) was treated with 100 mL of 1 mol/L aqueous sodium hydroxide solution at 95 °C for 6 h to remove protein. The mixture was then filtered and washed with deionized water to obtain a neutral and crude chitin precipitate. The crude chitin was decolored with 10 mg/mL potassium permanganate aqueous solution for 4 h, and then reacted with 10 mg/mL oxalic acid aqueous solution for 3 h. Chitin was neutralized by washing with deionized water after decolorization and then freeze-dried. The crude chitin was *N*-deacetylated with 400 mg/mL sodium hydroxide solution at 70 °C for 8 h. After filtration, washing to neutral with deionized water and freeze drying, the chitosan was obtained and stored at −20 °C.

2.3. Determination of molecular weight and degree of deacetylation

The molecular weight of chitosan from housefly larvae was determined by the method of Jia, Shen, and Xu (2001). The degree of deacetylation of chitosan was determined according to the method of Qin et al. (2004).

2.4. DPPH free radical scavenging assay

The scavenging effect of chitosan on DPPH radical was examined using the modified method described earlier by Shimada, Fujikawa, Yahara, and Nakamura (1992). Briefly, 400 μM DPPH solution in methanol was prepared and 3.0 mL of this solution was added to 1.0 mL test samples at different concentrations. The reaction mixture was shaken well and incubated for 30 min at room temperature and the absorbance of the resulting solution was read at 517 nm against a blank. EC_{50} value (mg/mL) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. Ascorbic acid was used for comparison. The radical scavenging ability was calculated using the following equation:

$$\text{Scavenging effect } (\%) = (1 - \text{OD}_{\text{sample}}/\text{OD}_{\text{control}}) \times 100.$$

2.5. Reducing power assay

The reducing power of chitosan from the housefly larvae was quantified by the method described by Senevirathne et al. (2006). Ascorbic acid was used for comparison. Each test replicated three times.

2.6. Metal ion chelating ability assay

Metal ion chelating ability of chitosan from the housefly larvae was determined based on the method of Senevirathne et al. (2006). EDTA was used for comparison. Each test replicated three times.

2.7. Antitumor activity assays

The *in vitro* antitumor activity of chitosan from the housefly larvae against HeLa and S-180 tumor cells were, respectively, performed with a slight modified method from Yan et al. (2006). Chitosan was dissolved in RPMI 1640 medium to make the concentration of 2 mg/mL as a stock solution. Tumor cells in RPMI1640 medium with 10% fetal bovine serum was plated on 96-well microtiter plates (1.0×10^5 cells per well), and allowed to adhere at 37 °C

with 5% CO_2 for 4 h in a humidified incubator. Different concentrations of chitosan were added into the cells. The cells were incubated at 37 °C with 5% CO_2 for 72 h. At the end of the incubation, 10 μL of tetrazolium reagent (MTT) in RPMI 1640 medium without FBS was added into each well and incubated at 37 °C for 4 h. Then the supernatant after centrifuging at 1000*g* for 5 min was discarded and DMSO (100 μL/well) was added to allow formosan solubilization. The optical density (OD) of each well was detected by a Microplate reader (Bio-Rad, Model 680) at 570 nm. Each determination represents the average means of eight replicates. The 50% inhibitory concentration (IC_{50}) was determined by curve fitting. The inhibitory rate (IR%) was calculated as follows:

$$IR\ \% = (OD_{control} - OD_{sample})/OD_{control} \times 100.$$

3. Results

3.1. Molecular weight and degree of deacetylation

The viscosity average-molecular weight of chitosan from housefly larvae was determined to be 426 kDa. The degree of deacetylation of chitosan was 90.3%.

3.2. DPPH radical scavenging activity

Total DPPH scavenging potential of chitosan from the larvae of housefly at various concentrations was measured and the results were shown in Fig. 1. Chitosan had stronger scavenging activity on DPPH radical than ascorbic acid. The scavenging effect increased with increasing their concentrations. Scavenging effect of chitosan on DPPH radical was 57.1% at 0.5 mg/mL, whereas only 54.2% at 2 mg/mL for ascorbic acid. The half effective concentrations of chitosan and ascorbic acid were approximately 0.373 and 1.92 mg/mL, respectively.

3.3. Reducing power

The reducing power of chitosan from the larvae of housefly exhibited a concentration-dependent manner but value remained lower than ascorbic acid (Fig. 2). The reducing power of the chitosan and ascorbic acid were 0.307 and 0.783 at 1 mg/mL, respectively.

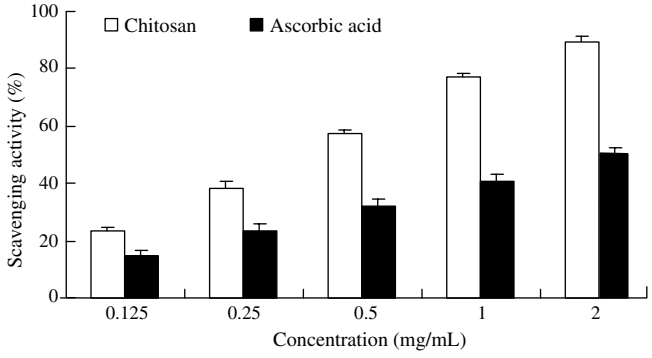

Fig. 1. Scavenging activities of the chitosan from larvae of housefly and ascorbic acid on DPPH radical. Each test replicated three times. The bar represented +SD.

3.4. Metal ion chelating ability

The ferrous ions chelating ability of chitosan from the larvae of housefly increased with increasing concentrations as shown in Fig. 3. The chitosan showed high ferrous ions chelating ability which was similar to that of EDTA. At 1 mg/mL, chelating ability of chitosan and EDTA on ferrous ions were 78.1% and 90.2%, respectively.

3.5. Antitumor activities

The *in-vitro* inhibitory effects of chitosan from the larvae of housefly on HeLa and S-180 tumor cells were investigated by the standard MTT colorimetric method. As shown in Fig. 4, chitosan exhibited similar inhibitory effects on the growth of HeLa and S-180 tumor cells. Inhibitory effects of chitosan on HeLa and S-180 tumor cells reached 50.8% and 52.9%, respectively, at 1 mg/mL. A dose-dependant manner was also observed in this antitumor activities assay.

4. Discussion

Chitosan has been widely used as a natural resource in various fields such as medicine, food and chemical engi-

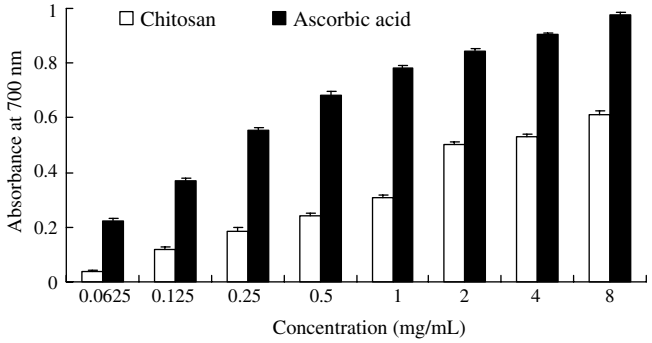

Fig. 2. Reducing power of the chitosan from larvae of housefly and ascorbic acid. The bar represented +SD.

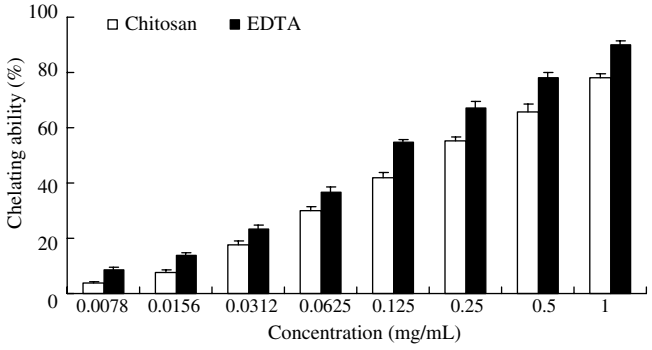

Fig. 3. Ferrous ions chelating ability of the chitosan from larvae of housefly and EDTA. The bar represented +SD.

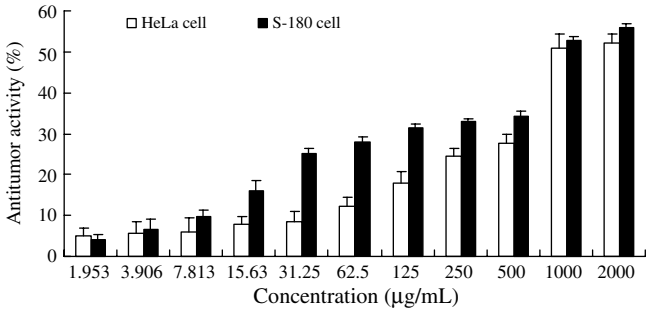

Fig. 4. Inhibitory effects of the chitosan from larvae of housefly on HeLa and S-180 tumor cells *in vitro*. The bar represented +SD.

neering, pharmaceuticals, nutrition and agriculture (Avadi et al., 2004; Fujimoto, Tsuchiya, Terao, Nakamura, & Yamamoto, 2006; New & Stevens, 2002; Qin et al., 2006; Sun, Xie, & Xu, 2004). Previous studies in our laboratory indicated that chitosan from larvae of housefly possessed hypolipidemic, fresh-keeping and antibacterial effects (Ding et al., 2006; Lai et al., 1999; Wang et al., 2005). The present results showed that chitosan from the housefly larvae exhibited excellent scavenging activity on DPPH radical and the reducing power. Many studies have demonstrated that compounds with great reducing power may serve as significant indicators of potential antioxidant activity (Duh, Du, & Yen, 1999; Gordon, 1990; Shimon, Joseph, Bezalel, & Sonia, 1995). Our data on the reducing power of chitosan demonstrated that it was likely to contribute significantly toward the observed antioxidant effect. These results suggested that chitosan from the housefly larvae could be used as a natural antioxidant to protect the human body from free radicals and retard the progress of many chronic diseases. Furthermore, it is reported that synthetic antioxidants may cause carcinogenic effects in food, health experts pay more attention to natural antioxidative ingredients instead of synthetic antioxidants (Shahidi, 2000). The use of natural chitosan from the housefly larvae has the advantage that the consumer readily accepts them, considered to be safe because of natural source and no chemical contamination.

In addition, the present results indicated that chitosan from the housefly larvae possessed excellent chelating ability on ferrous ions, and effectively inhibited the *in-vitro* growth of HeLa and S-180 tumor cells in a dose-dependent manner. Ferrous ions are considered to be the most effective pro-oxidants present in food systems (Yamauchi, Tatsumi, Asano, Kato, & Ueno, 1988). The high chelating effect of chitosan also would be beneficial if it was formulated into foods. And the metal-chelating property of this chitosan showed that it might be applied in adsorption, metal ions separation or waste water treatment. Besides, the chitosan from housefly larvae exhibited the impressive inhibitory effects on the growth of two tumor cell lines *in vitro*. It is earlier reported that the extract of housefly larvae possess excellent inhibitory effect against human colon cancer cell line CT26 (Hou et al., 2007). These studies suggested that the chitosan might be an available component existed in the extract of housefly larvae, for providing a therapeutic potential of drug against cancer. Extensive antitumor screening and intensive mechanism investigation are demanded in this area. Furthermore, the inhibitory effects of the chitosan in *vivo* tumor models also need to be further determined.

In conclusion, all results from this study provide strong evidence that the larvae of housefly could be used in medicine, described in many traditional Chinese pharmacopoeia (Li, 1981). This kind of chitosan has the potential as natural antioxidative ingredients for functional food products or antitumor therapeutic drug. These studies have important value in the utilization and exploitation of housefly larvae. The results encourage us to continue the study of this kind of chitosan. Further studies in this area are in progress and will be reported in due courses.

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