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Research Article

Evaluation of Antioxidative Properties of Various Extracts from *Cyclina sinensis*

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Abstract: The mollusk *Cyclina sinensis* has been widely used as a food and folk medicine in China, Korea and south eastern Asia. In this study, Total Water-soluble Extracts (TWE), Crude Polysaccharides (CPS), Total Crude Proteins (TCP) were isolated from *C. sinensis* and the Deproteinized Polysaccharides (DPS) was obtained after deproteination of CPS. Their contents, such as neutral carbohydrate, protein and uronic acid, were measured. Their antioxidative properties against 1, 1'-Diphenyl-2-Picrylhydrazyl (DPPH) free radical, hydroxyl free radical and superoxide anion free radical, as well as their reducing capacity were investigated. Results showed that various fractions of *C. sinensis* can scavenge all free radicals tested above and possessed the increasing reducing capacity in a dose-dependent mode. Compared with the other three fractions, TWE represents the comprehensively best antioxidative properties. The antioxidative activities of TWE from *C. sinensis* might contribute to antioxidant-related functional food and pharmaceutical industries.

Keywords: Antioxidative activity, *Cyclina sinensis*, polysaccharide, protein, total water-soluble extracts

INTRODUCTION

The free radicals are derived from by-products of biological reactions or exogenous factors and can cause oxidative damages related to aging and many diseases, such as cancer, atherosclerosis and Alzheimer's disease **(**Duan and Kasper, 2011**)**. Almost all organisms may be protected by enzymes of Superoxide Dismutase (SOD), peroxidase and Catalase (CAT) from oxidative damages, but unbalanced mechanisms such as ageing and diseases frequently cause damages because of inadequate enzymes. The synthetic antioxidants are often used to reduce the oxidative damages and obstructing the progress of some chronic diseases **(**Turkoglu *et al*., 2007**)**. However, it has been found that the synthetic antioxidants may cause many toxic effects (Guo *et al*., 2013). Consequently, there has been increasing interest in finding potent natural antioxidants with less toxicity to substitute synthetic antioxidants **(**Podsedek, 2007; Guo and Liu, 2012**)**.

Cyclina sinesis, also known as Chinese venus, iron clam and black clam, is a bivalve mollusk belonging to Veneridae (Wang *et al*., 2006). It is widely distributed along the coastal of China, Korea and south eastern Asia and one of the commonly bivalve species in Chinese aquaculture (Liu *et al*., 2002). It has been demonstrated that it is rich in protein, polysaccharides, essential amino acids and lipid which may conducive to the bioactivity of antioxidant, anti-tumor, anti-inflammation and immune-regulation (Gu *et al*., 2006; Li *et al*., 2010). In addition, *C. sinesis* was used as an effective folk medicine in China to treat asthma, inflammation and dental ulcer (Wang *et al*., 2007). At present, there were reported that the extraction and bioactivities such as anti-tumor, antioxidant of polysaccharides from *C. sinesis* (Jiang *et al*., 2011; Guo *et al*., 2013). However, to the best of our knowledge, there were no reports of systematic studies of antioxidant fractions of *C. sinesis*.

Herein, we reported antioxidative effects of Total Water-soluble Extracts (TWE), Crude Polysaccharides (CPS), Deproteinized Polysaccharides (DPS) and Total Crude Proteins (TCP) from *C. sinensis* on the free radical scavenging activities, as well as the reducing power.

MATERIALS AND METHODS

Materials and instruments: *C. sinensis* was purchased from the local aquatic market in Lianyungang, Jiangsu Province, China.

Glucose, phenol, sulfuric acid, coomassie brilliant blue G-250, bovine serum albumin, trichloroacetic acid, potassium ferricyanide, hydrogen peroxide, ferrous sulfate, EDTA and pyrogallol etc., were purchased from National Medicine Group Shanghai Co. (Shanghai, China). DPPH were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

HH-4 thermostatic water bath boiler (Jiangnan Equipment Co., Jintan, China) for heating extraction, Synergy HT Absorbance Microplate Reader (BioTek

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Co., USA) for content and activity analysis of sample, RE-52A rotary evaporator (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) for concentration of sample and QJ32W1000A high speed disintegrator (Tianjing TST Equipment Co., Tianjing, China) for homogenate of sample. FD-1A-50 freeze drier (Beijing BYK Equipment Co., Beijing, China) for lyophilization of sample.

Preparation of TWE, CPS, DPS and TCP: *C. sinensis* were collected and washed carefully with cold water. After removing the shells and impurities, the fresh flesh was centrifuged to remove the moisture, crushed by a high speed disintegrator and stored at -20°C. Fifty gram of the wet flesh and 500 mL of distilled water were extracted in a thermostatic water bath boiler at 95°C for 4 h. After treatment, the mixture was centrifuged at 10,000 g for 20 min and filtrated, the supernatant were collected, concentrated and lyophilized to yield the Total Water-soluble Extracts (TWE).

The supernatant was prepared the same as above and concentrated to 100 mL. Precipitation by the addition of three times volume of absolute ethanol for 1 day at 4°C. CPS were collected by centrifugation at 5,000 g for 20 min and lyophilized to a constant weight.

The proteins in the 100 mL concentrated extract as above were removed by the Sevage reagent. Sevage reagent (100 mL: 80 mL of $CHCl_3$ and 20 mL of butanol) was added to the concentrated extract. The bulk was shaken for 20 min at 25°C. After centrifugation at 5,000 g for 10 min, the supernatant was collected and subjected to this step for 10 times. Precipitation by the addition of three times volume of absolute ethanol for 1 day at 4°C. DPS were collected by centrifugation at 5,000 g for 20 min and lyophilized to a constant weight.

The Total Proteins (TCP) in the 100 mL concentrated extract as above were precipitated by the addition of 80% saturation of $(NH_4)_2 SO_4$ at 4°C, added slowly and stired constantly. After standed for 12 h at 4°C, the total crude proteins was obtained by filtrated, dialysised under the blowing water and lyophilized to a constant weight.

Determination of total neutral carbohydrate contents: The carbohydrate contents were measured by phenol-sulfuric acid method (Guo *et al*., 2010). Briefly, 2.0 mL of sample solution, 1.0 mL 5% phenol and 5.0 mL of concentrated sulphuric acid were mixed and shaken. After the mixture was kept at room temperature for 30 min, the absorbance was measured at 490 nm. The total carbohydrate content was calculated with D-glucose as standard.

Determination of protein contents: The total protein content was determined by the method of Bradford (1976), using bovine serum albumin for the standard curve.

Determination of uronic acid contents: The uronic acid content was measured according to the method of Blumenkrantz and Asboe-Hansen (1973), using D-galacturonic acid as standard.

DPPH radical scavenging assay: The DPPH radical scavenging effects were estimated as described (Guo *et al*., 2013). Briefly, DPPH solution (0.2 mmol/L) in ethanol of 50 μL was added to 100 μL of sample and vibrated enough. The absorbance at 517 nm was measured after 20 min of incubation on the 96-well plate in the dark. The DPPH radical scavenging activity was calculated as follows:

$$Y = [(A_c - A_i + A_j) / A_c] * 100$$

where,

A_c = The absorbance of DPPH solution without sample

A_i = The absorbance of the test sample mixed with DPPH solution

A_j = The absorbance of the sample without DPPH solution

All tests were performed in triplicate and averaged.

Hydroxyl radical scavenging assay: The hydroxyl radical scavenging activity was measured according to the literature (Smirnof and Cumbes, 1989) with some modifications. Both 50 μL sample at different concentrations and 50 μL $FeSO_4$ (9 mmol/L) were thoroughly mixed with 50 μL H_2O_2 (8.8 mmol/L) and 50 μL salicylic acid-ethanol solution (10 mmol/L). The reaction mixture was incubated at 37°C for 20 min and the absorbance was determined at 508 nm. The activity for scavenge hydroxyl radical was calculated using the following equation:

$$Y = [(A_c - A_s + A_e) / A_c] * 100$$

where,

A_c = Absorbance of the control solution containing salicylic acid-ethanol solution, $FeSO_4$ and H_2O_2

A_s = Absorbance of the sample containing salicylic acid-ethanol solution, $FeSO_4$ and H_2O_2

A_e = Absorbance of the sample solution containing salicylic acid-ethanol solution and $FeSO_4$, without H_2O_2

Superoxide radical scavenging assay: The superoxide radical scavenging activity was determined at 25°C using the spectrophotometric monitoring of the inhibition of pyrogallol autoxidation as described according to the literature (Huang *et al*., 2009) with some modifications. The antioxidant activity was determined as the percentage of inhibiting pyrogallol

Table 1: Major chemical content of various fractions (mean±S.D., n = 3)

	TWE	CPS	DPS	TCP
Carbohydrate content (wt.%)	22.67±2.58	40.22±0.99	78.10±2.25	12.92±0.21
Protein content (wt.%)	11.41±0.92	26.74±1.13	10.84±0.27	70.22±3.18
Uronic acid content (wt.%)	1.55±0.09	3.25±0.12	2.22±0.40	1.71±0.08

autoxidation. Different concentrations of sample solution (50 μL) were mixed with 100 μL of Tris-HCl-EDTA buffer (0.1 mol/L, pH 7.3). After 20 min at room temperature, 50 μL of pyrogallol (60 mmol/L) was added and the mixture was shaken rapidly. The absorbance was measured at 319 nm each 50 sec for 5 min using Synergy HT micro-plate reader. The scavenging activity on superoxide radical was calculated as follows:

$$Y = [(\Delta A_o - \Delta A_s) / \Delta A_o] * 100$$

where,
ΔA_o = Pyrogallol autoxidation rate without sample
ΔA_s = Pyrogallol autoxidation rate with sample

Reducing power assay: The determination of reducing capacity was performed as described (Guo *et al*., 2013). (1.0 mL) sample solutions at different concentration were mixed with 1.0 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 1.0 mL of potassium ferricyanide (1%, w/v). The mixture was incubated at 50°C for 20 min, 1.0 mL of (10%, w/v) trichloroacetic acid was added and the mixture was centrifuged at 5,000 g for 5 min. The supernatant solution (1.0 mL) was mixed with deionized water (1.0 mL) and ferric chloride (0.5 mL, 0.1%) for 10 min and then the absorbance was measured at 700 nm against a blank.

RESULTS AND DISCUSSION

Major chemical contents: As shown in Table 1, TWE, CPS, DPS and TCP from *C. sinensis* contain both neutral carbohydrates and proteins with small amount of uronic acid (<5%). After deproteinization procedures, as expected, DPS were mainly composed of neutral carbohydrates (78.10±2.25%). The strict deproteinization process through the sevage method didn't remove protein in CPS completely, implying that some proteins in CPS might be existed as polysaccharide-protein conjugates.

Antioxidant activities analysis: It is well known that evaluation of the antioxidant activities on a selected antioxidant required different test systems (Yu *et al*., 2002). Therefore, the following four assays were applied to evaluate the antioxidant capacity of the four extracts from *C. sinensis*.

DPPH radical scavenging capacity: DPPH is a well-known and stable free radical, which had been widely used to evaluate the radical scavenging capacity of antioxidants (Nagai *et al*., 2003). In the DPPH assay, DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH (Chen *et al*., 2008).

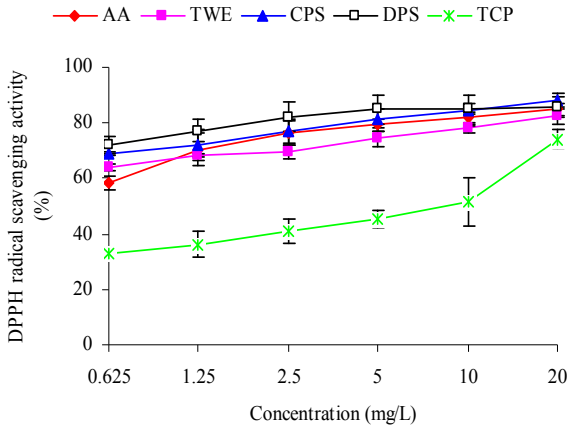

Fig. 1: Scavenging activities of sample and control standards on DPPH free radical (mean±S.D., n = 3)

The scavenging activities of TWE, CPS, DPS, TCP and Ascorbic Acid (AA) against the DPPH free radical were shown in Fig. 1. As observed, all five samples could neutralize the DPPH free radical and this activity was dose-dependent. TWE, CPS and DPS possess comparative activities of scavenging the DPPH radical compared with AA except TCP. The results suggested that TWE, CPS and DPS have significantly DPPH radical scavenging effect.

Hydroxyl radical scavenging capability: Hydroxyl radical, more likely to be produced *in vivo*, is considered to be the most reactive and poisonous free radical in living organisms. This radical can damage virtually all types of macromolecules including carbohydrates, proteins, lipids and nucleic acids (Reiter *et al*., 1995). The scavenging capability was not due to the direct scavenging but inhibition of hydroxyl free radical generation by chelating ions such as Fe^{2+} and Cu^{2+} (Qi *et al*., 2006). Hydroxyl radical can be generated by the reaction of Fe^{2+} and H_2O_2 and the antioxidants could reduce the generation of hydroxyl radical by chelating the Fe^{2+}. The hydroxyl radical in the cells can easily cross cell membranes at specific sites and cause tissue damage and cell death. Thus, it is very important of removing hydroxyl radical for the protection of living systems.

Figure 2 shows the hydroxyl free radical scavenging ability of all four samples and compared with ascorbic acid as control standards. It seemed that obvious scavenging activity on hydroxyl radical in a concentration-dependent pattern and TWE represents the best activity. The values of scavenging activities

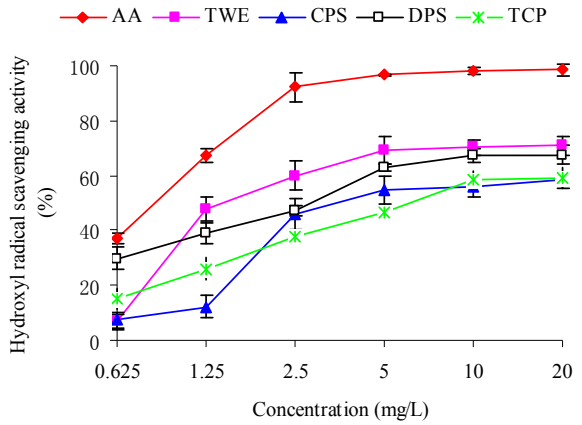

Fig. 2: Scavenging activities of sample and control standards on hydroxyl free radical (mean±S.D., n = 3)

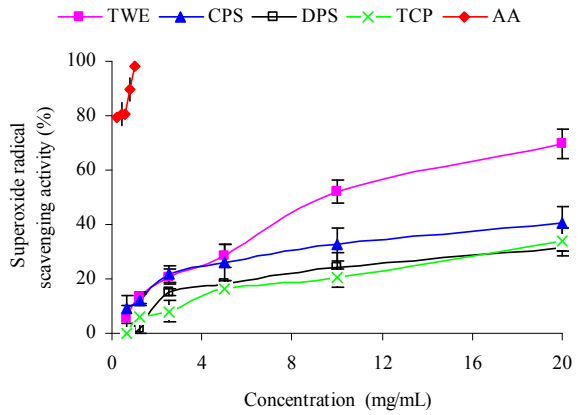

Fig. 3: Scavenging activities of sample and control standards on superoxide free radical (mean±S.D., n = 3)

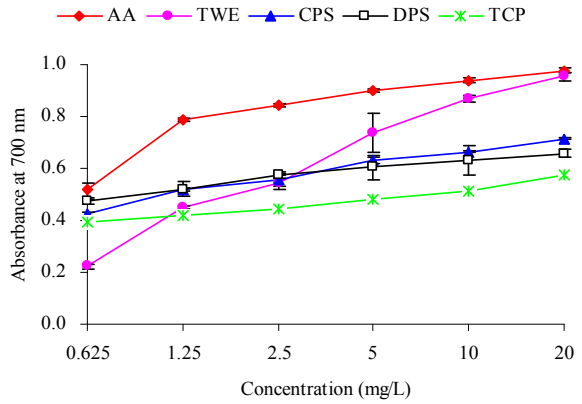

Fig. 4: Reducing power of sample and control standards (mean±S.D., n = 3)

ranged from 6.80±2.82% to 70.93±3.54% when the concentration of TWE varied from 0.625 to 20 mg/mL. TWE exhibited a certain extent of hydroxyl free radical scavenging activity, but with an efficacy lower than that of the reference ascorbic acid (98.45±2.18%) at the same concentration (20 mg/mL).

Superoxide radical scavenging activity: Superoxide radical ($O_2^{\cdot-}$), well known as another highly toxic species and to be produced *in vivo*, is related to numerous biological reactions and thus study of this radical-scavenging is also important (Kanatt *et al*., 2007). All four samples elicited the $O_2^{\cdot-}$ scavenging activity which followed a dose dependent pattern (Fig. 3). TWE presents the best ability for $O_2^{\cdot-}$ scavenging activity, whereas, the efficacy was far lower than that of the reference ascorbic acid (98.20±1.25%) at the concentration (1.0 mg/mL).

The results above from three different assays indicated that four fractions tested exhibited a certain content capabilities of scavenging DPPH radicals, hydroxyl radicals and superoxide anion. Among them, TWE represents the comprehensively best antioxidative properties.

Reducing power: During the reducing power assay, the presence of antioxidants would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Zhang *et al*., 2011). The dose-response curve for the reducing power of four samples was shown in Fig. 4 and TWE still exhibited a stronger dependence of reducing capacity on the concentration among the four samples.

CONCLUSION

In this study, four fractions, TWE, CPS, DPS and TCP were isolated from *C. sinensis*. Their chemical compositions were determined by colorimetric methods and their antioxidative properties were evaluated by free radical-scavenging activities (DPPH radical, hydroxyl radical and superoxide anion) and ferric-reducing antioxidant power assay. Results indicated that various fractions of *C. sinensis* possess multiple radical scavenging activities in a dose-dependent manner and antioxidant capacity. Compared with the other three fractions, TWE represents the comprehensively best antioxidative properties. TWE from *C. sinensis* with powerful antioxidant properties are of great potential to antioxidant-related functional food and pharmaceutical industries as confirmed by the research.

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