Antioxidant activities of polysaccharides obtained from Chlorella pyrenoidosa via different ethanol concentrations

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An ultrasonic-assisted extraction of Chlorella pyrenoidosa polysaccharides (CPP) was carried out using different concentrations of ethanol for precipitation, and named as CPP60, CPP70 and CPP85, respectively. The monosaccharide composition of each polysaccharide (CPP) was determined using gas chromatography (GC) and the antioxidant activity of each was investigated via the reducing power and scavenging activity of hydroxyl radicals, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and superoxide anion radicals, respectively. All of the polysaccharides examined possessed antioxidant activity in vitro. CPP70 exhibited stronger scavenging activity against superoxide, DPPH and hydroxyl radicals, when compared with CPP60 and CPP85. This suggests that polysaccharides from C. pyrenoidosa precipitated by a final ethanol concentration of 70%, have the potential to be developed as natural antioxidants for use in food and pharmaceuticals.

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1. Introduction

Recently, microalgae have received quite a lot of attention as potential commercial food supplements given that they are rich in various bioactive compounds, such as carotenoids, phycobilins, fatty acids, polysaccharides, vitamins and sterols [1]. Most of these bioactive compounds exhibit anti-inflammatory, antimicrobial, immunological, and antiviral activity and can protect against toxic reactive oxygen species (ROS) [2–5].

Chlorella is a unicellular green microalga, which exists in both fresh and marine water [6]. The chlorella growth factor (CGF), extracted by water from chlorella cells, has the ability to promote tissue regeneration, cell growth and division, formation of antibody producing lymphocytes, resistance against gamma irradiation and tumors, and enhancement of immunity [7]. CGF contains free amino acids, peptides, glycoproteins, polyamines, phytotormones, vitamins, minerals, and other unknown components [8]. Thus, Chlorella has been widely used as a supplement for human nutrition and in animal feed. Water extracts from Chlorella are complicated, thus it is difficult to distinguish which compounds are the most bioactive; however recent research indicates that the bioactivity might be associated with polysaccharides [9].

The Food and Agriculture Organization of the United Nations (FAO) has named C. pyrenoidosa a green healthy food [10]. Most research into the bioactivity of polysaccharides extracted from C. pyrenoidosa has focused on antitumor and immune activities [11,12]; however a few studies have concentrated on antioxidant activity. Furthermore, to the best of our knowledge, there have been no published studies on the antioxidant bioactivities of C. pyrenoidosa polysaccharides precipitated with different ethanol concentrations. Traditionally, ethanol has been used for the initial purification of an aqueous extract, since it is simple, rapid and easily scalable [13]. Importantly, ethanol concentration is a decisive factor in the structural features and molecular size of polysaccharides, which may be related to polysaccharide bioactivity [14]. However, few studies have taken ethanol concentration into account when preparing polysaccharide samples. In the current study, the chemical composition and antioxidant activity of polysaccharides from C. pyrenoidosa were estimated after precipitation using different concentrations of ethanol. In addition, the antioxidant capabilities of three differently-precipitated polysaccharide fractions were investigated with the aim of finding a new natural antioxidant for human health.

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2. Materials

The *C. pyrenoidosa* was obtained from Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wu Han, China). Rhamnose, arabinose, xylose, mannose, glucose, galactose and fructose were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All other chemicals were of analytical grade.

3. Methods

3.1. Preparation of the polysaccharide extract

*C. pyrenoidosa* has a rigid cell wall, which is comprised mainly of cellulose and thickens during maturation [15]. Therefore, it is necessary to disrupt the cell wall prior to extraction. The *C. pyrenoidosa* sample was treated in an ultrasonic processor at 300 W, with 10-fold distilled water, for 24 min. The ratio of solid to liquid was adjusted to 1:30 (W/V). The extraction process was carried out twice with distilled water. The residue was separated by centrifugation at 4500 rpm for 15 min, and the supernatant was concentrated to one-fifth of the original volume using a rotary evaporator at 55 °C under vacuum. Appropriate volumes of ethanol were added to the concentrated solution to obtain final ethanol concentrations of 60% (CPP60), 70% (CPP70), and 85% (CPP85), respectively. The mixture was stored overnight at 4 °C, and then centrifuged at 4500 rpm for 10 min to obtain the precipitates. After precipitates were freeze dried, the CPP60, CPP70 and CPP85 were obtained (Fig. 1).

3.2. Chemical analysis

The chemical composition analysis of precipitates prepared above was conducted, including total sugar, protein and total phenol contents. Total sugar contents were determined by phenol-sulfuric acid method using d-glucose as standard [16], protein contents were determined by Coomasie brilliant blue method using vine serum albumin as standard [17], and total phenol contents were assayed by Folin-Ciocalteu’s regent method and using gallic acid as standard [18].

3.3. Monosaccharide composition analysis

The monosaccharide composition analysis of the precipitates (CPP60, CPP70 and CPP85) was determined by Gas Chromatography (GC) according to the method of Pu et al. [19] with some modifications. In brief, the sample was hydrolyzed with 2 mL 2M trifluoroacetic acid (TFA) at 110 °C in a sealed-tube for 4 h. The TFA was removed and cooled down to room temperature, thereafter 10 mg of hydroxylamine hydrochloride and 0.5 mL of pyridine were added and incubated at 90 °C for 30 min. A 0.5 mL of acetic anhydride was added into mixture to continue reacting for 30 min at 90 °C to achieve glyc-nitride derivatization. GC analysis was performed by an instrument (Agilent 7890A, Agilent Technologies Co. Ltd., USA) equipped with an DB – 1701 quartz capillary column (30 m x 0.32 mm, 0.25 μm) and a flame-ionization detector (FID). The initial temperature was set at 170 °C and maintained for 2 min, then increased to 250 °C at the rate of 10 °C/min and kept at 250 °C for 30 min. The detector and injector temperatures was 250 °C, i-rhamnose, d-arabinose, d-xylene, d-mannose, d-glucose, d-galactose and d-fructose were used as standards and the molar ratio of monosaccharide in the polysaccharide samples was calculated.

3.4. Antioxidant activity assays

The polysaccharides (CPP60, CPP70 and CPP85) prepared above was diluted with distilled water into different concentrations of 0.4 mg/mL, 0.8 mg/mL, 1.2 mg/mL, 1.6 mg/mL and 2 mg/mL. The antioxidant activity was investigated by reducing power, scavenging activity of hydroxyl, DPPH and superoxide anion radicals.

3.4.1. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the method of Liu et al. [20] with some modifications. Briefly, 1 mL of FeSO₄ (9 mM) and 1 mL of salicylic acid–ethanol (9 mM) were added to 1 mL of polysaccharide sample, 1 mL of H₂O₂ (9 mM) was added finally to start the reactions, then incubated at...
37 °C for 30 min. The absorbance was measured at 510 nm. The hydroxyl radical scavenging activity was calculated as follows:

\[
\text{OH}^* \text{ scavenging activity } = \left[ 1 - \left( \frac{A_1 - A_2}{A_0} \right) \right] \times 100
\]

where \(A_0\) is the absorbance of the blank (water instead of sample) and \(A_1\) is the final absorbance of sample, \(A_2\) is the background absorbance (water instead of \(\text{H}_2\text{O}_2\)).

3.4.2. DPPH radical scavenging assay

The DPPH radical scavenging activity was measured by the method of Chen et al. [21] with some modifications. 2 mL of 0.1 μmol/L/DPPH dissolved in ethanol was added to 2 mL of polysaccharide sample. Then the mixture was kept in the dark for 30 min at room temperature, and absorbance was measured at 517 nm. The DPPH radical scavenging activity was calculated by the following equation:

\[
\text{DPPH scavenging activity (\%)} = \left[ 1 - \left( \frac{A_1 - A_2}{A_0} \right) \right] \times 100
\]

where \(A_0\) is the absorbance of the blank (ethanol instead of the sample) and \(A_1\) is the absorbance of sample, \(A_2\) is the background absorbance (ethanol instead of DPPH).

3.4.3. Superoxide radical scavenging activity

The superoxide anion radical scavenging activity was measured according to the method of Lin. [22] with slight modifications. 2.5 mL of 50 mM Tris–HCl buffer (pH 8.2) was maintained at 25 °C for 20 min. 4 mL of polysaccharide sample and 0.6 mL of 25 mmol/L pyrogallol solution was added. The mixture was incubated at 25 °C for 5 min, then 1 mL of 8 mmol/L HCl solution was added into the mixture to terminate the reaction. The absorbance of the mixture was recorded at 299 nm. The scavenging activity of superoxide anion radical was calculated by the following equation:

\[
\text{O}_2^- \text{ scavenging activity (\%)} = \left[ 1 - \left( \frac{A_1 - A_2}{A_0} \right) \right] \times 100
\]

where \(A_0\) is the absorbance of the blank (Tris-HCl buffer instead of the sample), \(A_1\) is the final absorbance of sample and \(A_2\) is the background absorbance (Tris-HCl buffer instead of pyrogallol solution).

3.4.4. Determination of the reducing power

The reducing power of the samples was measured by the method of Gao et al. [23] with some modifications. 2.5 mL of phosphate buffer (pH 6.6 0.2M) was mixed with 1 mL of polysaccharide sample. 2.5 mL of K2Fe(CN)6[1% W/V] was added and the mixture was incubated at 50 °C for 20 min. The reaction was stopped by adding 2.5 mL trichloroacetic acid (TCA, 10%, W/V). The mixture was centrifuged at 5000 rpm for 10 min and 2.5 mL of the supernatant was mixed with 2.5 mL of deionised water and 0.5 mL of FeCl3 (0.1%, W/V). The mixture was kept at room temperature for 10 min, and the absorbance was measured at 700 nm.

3.4.5. Statistical analysis

Each experiment was carried out in triplicate. Statistical analysis was performed by the Statistical Analysis Systems (Originpro 8.0).

4. Results and discussion

4.1. Chemical analysis

The ethanol precipitated polysaccharides from C. pyrenoidosa (CPP60, CPP70, and CPP85) were chemically analyzed. The chemical composition, including total sugar, protein and total phenol contents, are shown in Table 1. The CPP70 contained the highest total sugar content (52.74%), far more compared to CPP60 and CPP85. In contrast, CPP70 has the least protein and total phenol content (0.75% and 0.13%, respectively). CPP85 was higher in protein (11.21% for CPP85 and 7.75% for CPP60) and slightly higher in total phenol than CPP60 (0.41% for CPP85 and 0.27% for CPP60) (Table 1).

Molecular composition is an important index for evaluating polysaccharide functionality. In this study, gas chromatography (GC) was used to evaluate monosaccharide composition and seven kinds of monosaccharides were used as standards. The results are presented in Fig. 2., and the molar ratios are shown in Table 1. The monosaccharide compositions of CPP60, CPP70 and CPP85 were identified by comparing the retention time with standards. The retention times for standard monosaccharides exhibited in increasing order are l-rhamnose, d-arabinose, d-xylene, d-glucose, d-galactose, d-mannose, and d-fructose. The results indicated that all three polysaccharide fractions are composed of l-rhamnose, d-arabinose, d-xylene, d-glucose, d-galactose and d-mannose, but none of them were found to contain d-fructose (Fig. 2B–D). The molar ratios of the above-mentioned monosaccharides for CPP60, CPP70 and CPP85 were 3.84:1.94:1:1:1.44:12.51:2.49, 1.47:3.26:1.25:46:13.91:1.06 and 1.11:1.43:1:22.05:11.33:1.71, respectively. It was also found that CPP70 contained more d-glucose, and d-xylene, as far as molar ratio, than did CPP60 and CPP85; however, the monosaccharide composition of all three polysaccharide fractions was similar.

4.2. Hydroxyl radical scavenging activity

Hydroxyl radicals can occasionally be byproducts of immune action and are consequently short-lived. These radicals are highly reactive and can damage various macromolecules, such as carbohydrates, nucleic acids, lipids and amino acids and are dangerous to human health [24–26]. Therefore, eliminating unnecessary hydroxyl radicals is vital. The hydroxyl radical scavenging activities of CPP60, CPP70 and CPP85 are shown in Fig. 3. The scavenging effects of CPP60 and CPP70 increased at concentrations ranging from 0.4–1.2 mg/mL and decreased slightly when the concentration was over 1.2 mg/mL. At a concentration of 1.2 mg/mL, the hydroxyl radical scavenging activity increased in the order of CPP85 < CPP70 < CPP60 and were 52.71%, 81.46%, and 92.71%, respectively.

4.3. DPPH radical scavenging activity

DPPH is a well-known radical that has been widely used to evaluate the radical scavenging abilities of natural compounds [27]. DPPH assumes a deep violet color in solution and has a strong absorption band centered at about 517 nm; this color fades in the presence of antioxidants [28]. Fig. 4 shows the DPPH radi-
Fig. 2. GC chromatogram of a standard mixture of monosaccharide(A) and three polysaccharide fractions of CPP60(B), CPP70(C) and CPP85(D).

Fig. 3. Scavenging activities of three different polysaccharide fractions from C. pyrenoidosa on hydroxyl radicals.

Fig. 4. Scavenging activities of three different polysaccharide fractions from C. pyrenoidosa on DPPH radicals.

Fig. 5. Scavenging activities of three different polysaccharide fractions from C. pyrenoidosa on superoxide radicals.

cal scavenging activities of CPP60, CPP70, and CPP85. The DPPH radical scavenging activities of the three polysaccharide fractions were well-correlated with the concentrations and increased as the concentrations increased. The results showed that the DPPH scavenging activities increased in the order of CPP60 < CPP70 < CPP85, and CPP70 exhibited the highest DPPH scavenging activity of 39.71% at 2 mg/mL.

4.4. Superoxide radical scavenging activity

Superoxide is quite toxic and may play a role in many diseases. The view that oxidative damage is one of several factors limiting lifespan is widely accepted, though the mechanism by which superoxide may contribute to aging is unknown [29]. The superoxide radical scavenging activities of CPP60, CPP70 and CPP85 are presented in Fig. 5. All of the polysaccharide fractions studied exhibited a concentration-dependent behavior. CPP70 had the
highest superoxide radical scavenging (76.78%) at a concentration of 2 mg/mL. The superoxide scavenging activity of CPP85 was slightly weaker than CPP70 at a concentration of 2 mg/mL, but was nearly the same as CPP60, at a concentration of 2 mg/mL, which was higher than CPP60 at lower concentrations. The order of increased superoxide radical scavenging activities was CPP60 < CPP85 < CPP70, which corresponded to the DPPH radical scavenging activities.

4.5. Reducing power

The reducing power was determined at 700 nm and the absorbance values of all samples are shown in Fig. 6. The reducing power of the three polysaccharide fractions increased with increasing concentrations. Though the reducing power of CPP70 was only 0.15 at 0.4 mg/mL, lower than the reducing powers of CPP60 and CPP85, it reached 0.328 at a concentration of 2.0 mg/mL.

5. Conclusions

Different ethanol concentrations were used for precipitation of ultrasonic-assisted extraction of three polysaccharide fractions from C. pyrenoidosa. The highest total sugar content was found in CPP70, while CPP85 had a higher in protein content. The monosaccharides d-arabinose, d-glucose, d-xyllose, d-galactose, d-mannose, and l-rhamnose were found to be constituents of all the three polysaccharide fractions with varying degrees of molar ratios. In vitro estimation of hydroxyl scavenging activity and DPPH assay were found to be sample concentration dependent and the highest activity was seen for CPP70. Similarly, CPP70 presented the highest superoxide radical scavenging activity. A positive correlation between concentration and reducing power was exhibited by CPP70. The highest CPP70 polysaccharide concentrations insti-

gated the best antioxidant activity. Ethanol concentration appears to have a marked effect on the chemical composition and antioxidant activity of precipitated polysaccharides. However, different solvents should also be tested for extraction of polysaccharides in order to compare the pure effects of the solvents.

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