Carbohydrate Polymers 185 (2018) 120–126

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Physicochemical characterization of polysaccharides from *Chlorella pyrenoidosa* and its anti-ageing effects in *Drosophila melanogaster*

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ARTICLE INFO

Keywords:
Physicochemical characterization
Chlorella pyrenoidosa polysaccharides
Anti-ageing
Drosophila melanogaster

ABSTRACT

The physicochemical characteristics and *in vitro* antioxidant and *in vivo* anti-ageing activities of partial purified *Chlorella pyrenoidosa* polysaccharides (PCPPs) were investigated. The building blocks of PCPPs were mainly composed of D-glucose, D-galactose and D-mannose. The average molecular weight of PCPPs was 9,950 Da. *In vitro* antioxidant activity assays showed that PCPPs could effectively scavenge hydroxyl, 1,1-diphenyl-2-picrylhydrazyl, and superoxide radicals, with stronger effect on hydroxyl radicals. Furthermore, the mean lifespan of the male and female *Drosophila melanogaster* was extended by 11.5% and 10.6%, respectively. This was accompanied by an increase in the total activity of the endogenous antioxidant enzymes, superoxide dismutase, glutathione peroxidase, and catalase in young or old *D. melanogaster* administered with PCPPs. Moreover, a gender-dependent difference was observed both in lifespan and antioxidant enzyme activities in *D. melanogaster*. The results indicated that *C. pyrenoidosa* polysaccharides are potential natural antioxidants in extending lifespan.

1. Introduction

Chlorella is an edible unicellular green microalgae with a diameter ranging from 2 to 10 μm, which has drawn increasing attention during the last century as a potential food and energy source (Safi et al., 2015; Yamamoto, Fujishita, Hirata, & Kawano, 2004). *Chlorella* has been developed to be one of the most widely cultivated species of microalgae due to its fast growth rate, high content of valuable components and resistance to variable inhibitory growth conditions (Zhao, Wu, Yang, Liu, & Huang, 2015). In recent years, the potential therapeutic effects of *Chlorella* have been reported in wound healing, detoxification, antitumor activity, growth stimulation, and enhancement of immunity (Hagino & Ichimura, 1975; Kotrbáček, Doubek, & Doucha, 2015; Queiroz et al., 2008; Shim et al., 2008). Many of these biological activities are mainly associated with polysaccharides and protein complexes (Kralovec et al., 2007). *C. pyrenoidosa* has been named "green healthy food" by the Food and Agriculture Organization of the United Nations (FAO) (Kotrbáček et al., 2015) because of its valuable biological activities, such as inhibition of the production of IL-5 and the immunostimulatory effects of mast cells (Kralovec et al., 2005).

Reactive oxygen species (ROS), including superoxide radicals, hydroxyl radicals and others, are normal metabolic products (Wang et al., 2015). However, over production of ROS can damage various macromolecules and can lead to the development of many diseases. Endogenous antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are defense molecules protecting the organism from the damage caused by harmful oxygen and the ageing process. However, sometimes endogenous antioxidant systems are insufficient to prevent oxidative damages (Silva et al., 2013). Thus, dietary supplementation of antioxidants is required. The literature indicates that consumed ascorbic acid, vitamin A, plant flavonoids, polysaccharides, and other antioxidants are able to scavenge ROS in cells (Peng, Chan, Li, Huang, & Chen, 2009).

Polysaccharides from natural sources are nontoxic, effective natural antioxidants, which have drawn the attention of many scientists. Numerous studies have shown that the polysaccharides extracted from natural resources have the ability to scavenge free radicals *in vitro* and enhance the activities of SOD, GPx, CAT, and GSH in the brain and

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https://doi.org/10.1016/j.carbpol.2017.12.077
Received 10 October 2017; Received in revised form 17 December 2017; Accepted 28 December 2017
Available online 05 January 2018
0144-8617/

serum of ageing mice (Ding et al., 2016; Yan, Wang, Ma, Wang, & Pei, 2016). In a previous study, the polysaccharides extracted from *C. pyrenoidosa* were considered to be a potential immune builder, improving the immunostimulatory activity of living organisms (Miyazawa et al., 1988). However, there are limited reports on the bioactivities of polysaccharides from *C. pyrenoidosa*, especially on their anti-ageing activities.

Drosophila melanogaster (Fruit fly) is one of the most commonly used models to investigate the ageing and age-related diseases, due to its short lifespan and easy maintenance, and what's more, it shares homology with more than 70% of the known disease-causing genes in humans (Minois, 2006). Furthermore, it has been reported that the lifespan of *D. melanogaster* is largely associated with oxidative stress, and it can be extended by dietary modifications (Fleming, Reveillaud, & Niedzwiecki, 1992; Piper & Bartke, 2008). Therefore, the present study mainly focused on investigating the free radicals scavenging activity of polysaccharides extracted from *C. pyrenoidosa* and its effects on longevity and endogenous antioxidant enzymes in fruit flies.

2. Materials and methods

2.1. Materials and chemicals

C. pyrenoidosa was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology (Wuhan, China). 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid, ferricchloride, L-rhamnose, D-arabinose, D-xylose, D-mannose, D-glucose, D-galactose and D-fructose were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, US). Ethanol, HCl and other chemicals were of analytical grade.

2.2. Extraction and purification of polysaccharides of C. pyrenoidosa

The polysaccharides of *C. pyrenoidosa* were extracted by the ultrasonic-assisted extraction method. In brief, the raw *C. pyrenoidosa* powder was dissolved in distilled water (1:30 w/v) in a pretreated ultrasonic bath at 60 °C for 24 min with 300 W. After that, the mixture was incubated at 90 °C for 2 h and centrifuged at 4500 rpm for 15 min to separate the pellets. The supernatant was concentrated to 1/5 of the original volume by vacuum at 55 °C and then mixed with trichloroacetic acid (TCA) to remove free proteins and dialyzed with distilled water to remove small molecules. Ethanol was slowly added to the final concentration of 40% (v/v) and the sample was kept at 4 °C overnight. After centrifugation and freeze-drying, the partial purified polysaccharides of *C. pyrenoidosa* (PCPPs) were collected.

2.3. Preliminary characterization of PCPPs

2.3.1. Chemical analysis

Total sugar, protein, and phenol contents of PCPPs were determined with the phenol-sulfuric acid method using D-glucose as a standard, Coomassie brilliant blue assay using vine serum albumin as a standard, and Folin-cicoalteu's regent using gallic acid as a standard, respectively (Bradford, 1976; Dubois, Gilles, Hamilton, Rebers, & Smith, 2002; Khokhar & Magnusdottir, 2002).

2.3.2. Determination of molecular weights

The molecular weight of PCPPs was determined by the method described by Gómezordóñez, Jiménezescrig and Rupérez (2012) with some modifications. The analysis was performed in the Waters 1515 HPLC system fitted with a Refractive Index Detector (Waters 2414) and TSK-GEL G-4000PW$_{XL}$ columns (7.8 mm × 300 mm, *i.d.* 10 μm). The mobile phase was composed of 0.05% NaN_3 with flow rate 0.8 mL/min. All sample solutions were diluted to a concentration of 1 mg/mL and filtered through a 0.45 μm membrane filter before analysis. Ten microliters of each sample were injected for analysis and retention times were recorded. Different molecular weights (M_W) of Dextran standards (T-2000, T-500, T-70, T-40, and T-10) were used as reference standards respectively for calculating the M_W of each sample.

2.3.3. Monosaccharide composition analysis

The composition analysis of monosaccharides was analyzed by Agilent 7890A gas chromatography (GC, Agilent Technologies Co. Ltd., USA) as previously reported (Pu et al., 2016). L-rhamnose, D-arabinose, D-xylose, D-mannose, D-glucose, D-galactose and D-fructose were used as standards. The sample was hydrolyzed with 2 mL of 2 M triflouroacetic acid (TFA) at 110 °C in a sealed-tube for 4 h. After TFA was removed, 10 mg of hydroxylamine hydrochloride and 0.5 mL of pyridine were added and incubated at 90 °C for 30 min. Then a 0.5 mL of acetic anhydride was added and the reaction continued reacting for 30 min at 90 °C, at which point the glyc-nitrile derivatives were collected.

2.4. In vitro antioxidant activity assays

2.4.1. Hydroxyl radicals scavenging activity

Although hydroxyl radicals are very short-lived *in vivo*, they are still harmful to the organism. Therefore, the removal of hydroxyl radicals is important for preventing cell damage (Shao, Chen, & Sun, 2013). The hydroxyl radicals scavenging activity of our samples was measured according to the method described previously (Sies, 1993). Different concentrations of PCPPs solution (0.4, 0.8, 1.2, 1.6 and 2.0 mg/mL) were prepared. And, 1 mL $FeSO_4$ (9 mM), 1 mL salicylic acid–ethanol (9 mM), and 1 mL H_2O_2 (9 mM) was added to 1 mL PCPPs solution (0.4, 0.8, 1.2, 1.6 or 2.0 mg/mL). After incubation at 37 °C for 30 min, the absorbance was measured at 510 nm. The scavenging activity was calculated using the following equation:

$$\cdot OH \text{ scavenging activity } (\%) = \left[1 - \frac{(A1 - A2)}{A0}\right] \times 100$$

Where A_0 is the absorbance of the blank (containing all regents except the sample); A_1 is the absorbance of the sample, and A_2 is the background absorbance (water instead of H_2O_2). Ascorbic acid was used as positive control.

2.4.2. DPPH radicals scavenging assay

DPPH is a stable free radical and has been widely accepted to evaluate the radical scavenging abilities of antioxidants (Zhang, Zhou, Sun, Feng, & Wang, 2014). Two milliliters of sample were added to 2.0 mL of DPPH solution (0.1 μmol/L) and absorbance was measured at 517 nm after incubation in the dark, at room temperature, for 30 min. The scavenging activity was calculated according to the following formula:

$$\text{DPPH scavenging activity } (\%) = \left[1 - \frac{(A1 - A2)}{A0}\right] \times 100$$

Where A_0 is the absorbance of the blank (containing all regents except the sample), A_1 is the final absorbance of the sample, and A_2 is the background absorbance (ethanol instead of DPPH).

2.4.3. Superoxide radicals scavenging activity

The superoxide radicals scavenging activity of samples was measured according to the method described previously (Halliwell, Gutteridge, & Aruoma, 1987) with some modifications. The reaction mixture included 2.5 mL of Tris-HCl buffer (50 mM, pH 8.2, incubated at 25 °C before testing), 0.6 mL of pyrogallol solution (25 mM)and 4 mL of sample. After incubation at 25 °C for 5 min, the reaction was