Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 105 (2007) 533–539
Food Chemistry
www.elsevier.com/locate/foodchem

Preparation, identification and their antitumor activities *in vitro* of polysaccharides from *Chlorella pyrenoidosa*

Jianchun Sheng, Fang Yu, Zhihong Xin, Liyan Zhao, Xiaojun Zhu, Qiuhui Hu *

Key Laboratory of Food Processing and Quality Control, College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, PR China

Received 21 December 2006; received in revised form 29 January 2007; accepted 3 April 2007

Abstract

Ultrafiltration membranes of different pore size were applied to fractionate *Chlorella pyrenoidosa* polysaccharides (CPPS) and the main fraction could be separated by a membrane with nominal molecular weight cut-off (NMWCO) of 30 kDa. Ultrafiltration parameters of 40 °C 14.0 psi were optimized for obtaining the main fraction. The resulting sample was further purified by anion-exchange chromatography and size exclusion chromatography, and two distinctive polysaccharides, CPPS Ia and IIa were recovered. CPPS IIa had infrared spectral characteristic of polysaccharides similar to CPPS Ia, and the symmetrical stretching peak at 1408–1382 cm^{-1} was an indication of the presence of carboxyl groups. The peak molecular weights were 69658 Da and 109406 Da, for CPPS Ia and CPPS IIa, respectively. Both CPPS Ia and IIa were composed of rhamnose, mannose, glucose, galactose and an unknown monosaccharide. Galactose (relative mass 46.5%) was the predominant monosaccharide of CPPS Ia and in CPPS IIa, rhamnose (37.8%) was predominant. CPPS Ia and IIa presented significantly higher antitumor activity against A549 *in vitro* than did a blank control, in a dose-dependent manner. Both fractions might be useful for developing natural safe antitumor drugs from *C. pyrenoidosa* resources.

Keywords: *Chlorella pyrenoidosa*; Polysaccharide; Ultrafiltration; Purification; Antitumor activity

1. Introduction

Chlorella is a unicellular green alga found in both fresh and marine water and it is widely used as a food supplement (Kay, 1991). Significant attention has recently been drawn to the use of microalgae for developing functional food, as microalgae produce a great variety of nutrients that are essential for human health. *Chlorella pyrenoidosa* has been named green healthy food by FAO, rich in nutrients, such as protein (dry weight 50–65%), total lipid (5–10%), total carbohydrate (10–20%) and the antioxidants, vitamin C (200–500 mg kg^{-1}) and vitamin E (120–300 mg kg^{-1}) (Hu, Pan, Xu, Sheng, & Shi, 2007). A haemagglutinin with strong activity was identified from the freshwater unicellular *C. pyrenoidosa* (Chu, Huang, & Ling, 2006). A pilot study indicated (for some subjects with mild to moderate hypertension), that a daily dietary supplement of *C. pyrenoidosa* reduced their sitting diastolic blood pressure or kept it stable (Merchant, Andre, & Sica, 2002). Administration of *C. pyrenoidosa* could prevent dyslipidemia in rats and hamsters after a chronic high fat diet treatment (Cherng & Shih, 2005). Furthermore, the bioactive component polysaccharides from *C. pyrenoidosa* were responsible for its great potential antitumor and immunomodulatory activities (Miyazawa et al., 1988; Yang, Shi, Sheng, & Hu, 2006). The structures of the polysaccharides may not be identical as a result of differences in cultivation conditions, harvest season and extraction procedures (Zhang et al., 2005). Shi, Sheng, Yang, and Hu (2007) found two different polysaccharides in *C. pyrenoid-*

* Corresponding author. Fax: +86 25 84395618.
E-mail address: qiuhuihu@njau.edu.cn (Q. Hu).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.04.018

osa with peak molecular weights of 81,877 Da and 1749 Da, respectively, and both of them were composed mainly of mannose (78.0% and 76.5% of relative mass, respectively) with low levels of glucose (13.2% and 8.4% of relative mass, respectively) (Shi et al., 2007). An arabinogalactan was isolated from a hot water extract of freeze-dried cells of *C. pyrenoidosa*, the weight average molecular weight of which was about 47 ± 4 kDa (Suarez et al., 2005).

Most current means of extracting polysaccharides are quite time-consuming and need large amounts of organic solvents to precipitate the polysaccharides, which will result in a problem of recovery to avoid environmental pollution. Membrane separation has traditionally been used for size-based separations with the advantages of high-throughput and being environment-friendly, and it is especially suitable for isolation of heat-sensitive substances (Van Reis & Zydney, 2001). However, most current studies on polysaccharides put an emphasis on structure activity relationships; there have been few reports on membrane separation of polysaccharides. In this paper, advanced membrane technology separation was applied for the mass separation of polysaccharides from *C. pyrenoidosa* and two purified fractions, named *C. pyrenoidosa* polysaccharides Ia and IIa (CPPS Ia and IIa), were obtained by anion exchange chromatography and size exclusion chromatography. Furthermore, their basic structures and antitumor activities *in vitro* were evaluated for developing a potential high-effective and low-toxic antitumor drug.

2. Materials and methods

2.1. Materials and reagents

Lyophilized *C. pyrenoidosa* (CP) cells were obtained from Jiangsu Academy of Agricultural Sciences (Nanjing, China). Rhamnose, arabinose, xylose, mannose, glucose, galactose, inositol, dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade and obtained locally.

2.2. Extraction of Chlorella pyrenoidosa polysaccharides

Extraction of polysaccharides was carried out by the procedure reported in our previous study (Shi et al., 2007). Briefly, *C. pyrenoidosa* cells were disrupted by ultrasound with JY98-III cell breaking apparatus prior to extraction. The resulting sample solution was incubated in a 100 °C water for 4 h and then centrifuged at 4500 rpm for 20 min to remove the pellet. Trichloroacetic acid (TCA) was added to the samples and centrifuged at 7000 rpm for 15 min. The supernatant was adjusted pH to 7 by 1 M sodium hydroxide and kept in the refrigerator for membrane separation.

2.3. Separation of Chlorella pyrenoidosa polysaccharides by ultrafiltration

The feed solution (2 l) was pretreated through a membrane with the pore size of 0.1 μm to avoid the fouling of the ultrafiltration membranes. The ultrafiltration was performed on a Millipore ultrafiltration system equipped with different membranes with the area of 0.1 m^2 (Millipore, USA). The feed solution was pumped to the membrane surface (tangential flow) and the filtrate was collected while the retentate was directed back to the recycle tank. The circulation process lasted until the feed solution was concentrated to about 200 ml, which was later reduced to one quarter of its original volume by rotary evaporation, dialyzed and lyophilized. The filtrate after each ultrafiltration process was the feed solution for the next ultrafiltration with a different membrane. Membranes with different nominal molecular weight cut-offs (100 kDa, 30 kDa, 10 kDa, 3 kDa, 1 kDa), in turn, were chosen to determine which part the main fraction of CPPS was in. After confirming the main fraction, the parameters for ultrafiltration, including temperature and pressure, were optimized, using flux as the evaluation index.

The flux (J) was expressed in terms of the permeate passing through the membrane in one unit of time and area until the ultrafiltration system reached the stable state. The formula was as follows:

$J = V/(t \times A)$, where V was sampling volume, t was sampling time and A was the area of the membrane.

2.4. Purification of Chlorella pyrenoidosa polysaccharides after ultrafiltration

10 ml of 2 mg ml^{-1} of *C. pyrenoidosa* polysaccharides dissolved in 0.1 M sodium chloride were applied to a DEAE Cellulose-52 (Whatman, England) anion-exchange chromatography column (2.6 × 20 cm), followed by stepwise elution with 0.1, 0.5 and 1 M sodium chloride solutions (200 ml each) at a flow rate of 60 ml h^{-1}. Eluate (6 ml/tube) was collected automatically and carbohydrates were determined by the phenol–sulfuric acid method, using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Finally, two fractions of polysaccharides, CPPS I and II, were obtained, dialyzed and lyophilized for further separation.

Fractions CPPS I and II were further fractionated by Sephadex G-100 (Pharmacia, Sweden) column chromatography (2.6 × 70 cm). The sample was dissolved in 0.1 M sodium chloride, and eluted with the same mobile phase at 30 ml h^{-1} flow rate. Eluate (3 ml/tube) was collected automatically and carbohydrates were determined by the phenol–sulfuric acid method. Each fraction (CPPS Ia and IIa) was pooled, dialyzed against de-ionized water and lyophilized.

2.5. Spectroscopic methods

Fourier-transform infrared spectra were recorded from polysaccharide powder (2 mg) in KBr pellets on a Nicolet 5DXB FT-IR spectrometer in the range 4000–500 cm^{-1}.

2.6. Determination of molecular weight of Chlorella pyrenoidosa polysaccharides Ia and IIa

The molecular weights of CPPS Ia and IIa were determined by HPLC on Waters 600 instrument (Waters, USA), equipped with two Water Ultrahydrogel TM Linear 7.8 × 300 mm columns and eluted with 0.1 M of sodium nitrate solution at a flow rate of 0.9 ml min^{-1} at 45 °C. Elution was monitored by a refractive index detector. The column was calibrated with standard glucosan from Sigma Chemical Co. and a standard curve was then established (Yamamoto, Nunome, Yamauchi, Kato, & Sone, 1995).

2.7. Sugar analysis

CPPS Ia and IIa were hydrolyzed in 2 M HCl for 2.5 h at 105 °C in a sealed glass tube. After removing the residual acid, the hydrolyzates were converted to acetylated aldononitrile derivatives according to conventional protocols and analyzed by GC (Shimadzu, GC14A), using an OV-1701 capillary column (30 m × 0.32 mm ID) (Varma, Varma, & Nardi, 1973). Briefly, the hydrolysate was evaporated to dryness and dissolved in 0.5 ml of pyridine. After 10 mg hydroxylamine hydrochloride and 2 mg inositol (as internal reference) were added to the solution, it was allowed to react at 90 °C for 30 min. The mixture was cooled at room temperature. After 0.8 ml of acetic anhydride was added to the mixture, it was allowed to react at 90 °C for a further 30 min. Sugar identification was done by comparison with reference sugars. The relative molar proportions were calculated by the area normalization method. As references, the following neutral sugars were converted to their acetylated derivatives and analyzed: rhamnose, arabinose, xylose, mannose, glucose and galactose.

2.8. Cell lines and culture

The A549 human lung adenocarcinoma cells were provided by the Biology Preservation Center of China Pharmaceutical University and maintained with RPMI 1640 medium containing 10% FBS and 100 ng ml^{-1} each of penicillin and streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.9. Growth inhibition assay

The inhibition effects of CPPS Ia and IIa on the A549 human lung adenocarcinoma cells were evaluated *in vitro* using MTT assay (Chen et al., 2006). Briefly, the A549 human lung adenocarcinoma cells (5×10^4 ml^{-1}) were incubated in 96-well plates containing 100 μl of the culture medium at 37 °C in a humidified atmosphere with 5% CO_2. Cells were permitted to adhere for 24 h, then washed with phosphate buffered saline (PBS). One hundred microliter of different concentrations of polysaccharides (200, 600, 800, 1000 μg ml^{-1}), prepared in culture medium, is added to each well. After 48 h of exposure, the polysaccharide-containing medium was removed, washed with PBS and replaced by fresh medium. The cells in each well were then incubated in culture medium with 20 μl of 5 mg ml^{-1} of MTT for 4 h. After the media were removed, 150 μl of DMSO were added to each well. Absorbance at 570 nm of the maximum was detected by a Power Wave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT). 5-Fu was treated as positive control. The inhibition rate was calculated according to the formula below:

Growth inhibition rate (%) = (1 − Absorbance of experimental group/Absorbance of blank control group) × 100%.

2.10. Statistical analysis

The data were presented as means ± standard deviations of three determinations. Statistical analyses were performed using student's *t*-test and one way analysis of variance. Multiple comparisons of means were done by the least significance difference (LSD) test. All computations were done by employing the statistical software (SAS, version 8.0).

3. Results and discussion

3.1. Selection of ultrafiltration membrane and optimization of the process parameters

The membranes of different pore size were used successively to find the main fraction of the CPPS and the results are shown in Fig. 1. The fraction cut off by the 30 kDa

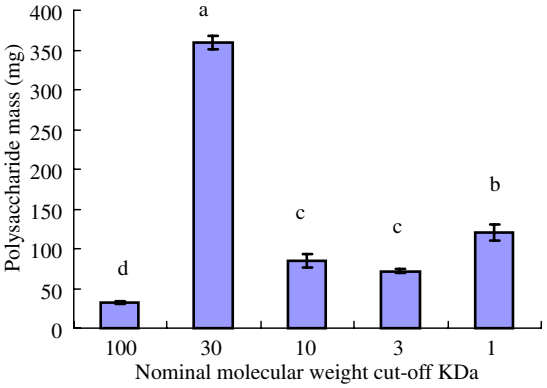

Fig. 1. Fractionation of *Chlorella pyrenoidosa* polysaccharides (CPPS) by ultrafiltration using different pore size membranes. Values were means of three determinations ± standard deviation. Columns followed by different letters were significantly different at $P < 0.05$.

membrane produced the greatest yield, 53.8% by mass percentage (significantly higher than any other fractions). It could be inferred that the molecular weight of the main fraction was about 90 kDa, based on the experience that 3-fold of nominal molecular weight cut-off (NMWCO) could be completely retained. In the paper, we focussed this fraction and its membrane separation parameters were optimized.

The effects of temperature and operation pressure on membrane flux are shown in Fig. 2. The membrane flux increased with increasing temperature (Fig. 2a), owing to the reduction of viscosity and enhancement of diffusion and mass transfer coefficient (Mulder, 1996). It reached the peak of 104.4 l m^{-2} h^{-1} at 50 °C when fixing the operation pressure at 9.0 psi. As the maximum endurable temperature of the ultrafiltration membrane was 50 °C, for comprehensive consideration, 40 °C with the membrane flux of 96 l m^{-2} h^{-1}, was optimized throughout the separation process.

The membrane flux increased linearly with operation pressure (Fig. 2b), which could be explained by the model: $J = (K_m/\mu) \times \Delta P$, where K_m represented the mass transfer coefficient and μ represented the viscosity (Mulder, 1996). As the max endurable operation pressure was 15.0 psi, in the study, operation pressure of 14.0 psi was adopted. Under the optimized conditions, with the temperature of 40 °C, the membrane flux was 128 l m^{-2} h^{-1}.

3.2. Purification of Chlorella pyrenoidosa polysaccharides

A lyophilized fraction of polysaccharides from *C. pyrenoidosa,* cut off by 30 kDa membrane, was chromatographed on a DEAE Cellulose-52 anion-exchange column to yield two peaks, CPPS I and II (Fig. 3), with CPPS I accounting for 87.9% of the total polysaccharides and CPPS II for 12.1%. Then, CPPS I and CPPS II were subjected to gel filtration on Sephadex G-100. CPPS I yielded a single fraction, CPPS Ia, and it reached the peak absorbance at tube 28 (Fig. 4a), while CPPS II a reached the peak at tube 26 (Fig. 4b). CPPS Ia and IIa were separately collected, dialyzed and lyophilized for further identification of the structure.

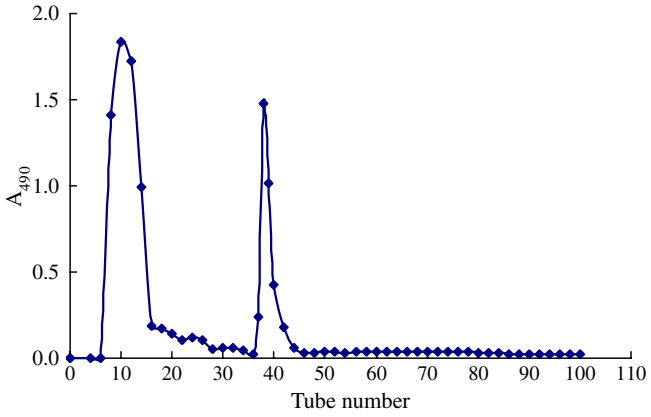

Fig. 3. Elution curve of *Chlorella pyrenoidosa* polysaccharides (CPPS) from membrane separation (30 kDa) by DEAE Cellulose-52 anion-exchange chromatography.

3.3. Molecular weight of Chlorella pyrenoidosa polysaccharides Ia and IIa

The molecular weight of the polysaccharide was determined by high performance liquid chromatography. The equation of the standard curve was: Log $M_p = 13.6–0.511\ t$ (where M_p represented the peak molecular weight, while t represented retention time). The peak molecular weights of the purified polysaccharides were estimated to be 69658 Da and 109406 Da for CPPS Ia and CPPS IIa, respectively.

3.4. Chemical composition of Chlorella pyrenoidosa polysaccharides Ia and IIa

The infrared spectra of the purified CPPS Ia and CPPS IIa fractions were shown in Fig. 5. CPPS Ia displayed a broad stretching intense characteristic peak at around 3425 cm^{-1} for the hydroxyl group (Santhiya, Subramanian, & Natarajan, 2002), and a weak C–H stretching band at 2927 cm^{-1}. The peak at around 897 cm^{-1} is characteristic of β-D-mannose (Coimbra, Goncalves, Barros, & Delgadillo, 2002). CPPS IIa revealed similar spectral characteristics to CPPS Ia, and the relative symmetrical stretching peak at 1408–1382 $cm^{-1}$was an indication of the presence of carboxyl groups (Mao, Li, Gu, Fang, & Xing, 2004). The result was

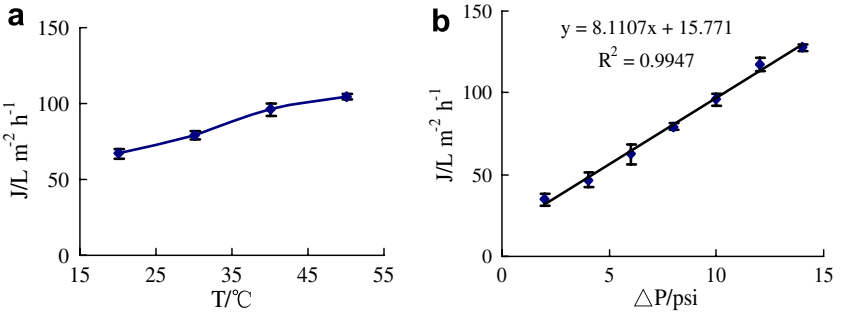

Fig. 2. Effect of ultrafiltration temperature and pressure on membrane flux. (a) Temperature (operation pressure: 9.0 psi); (b) pressure (operation temperature: 40 °C).

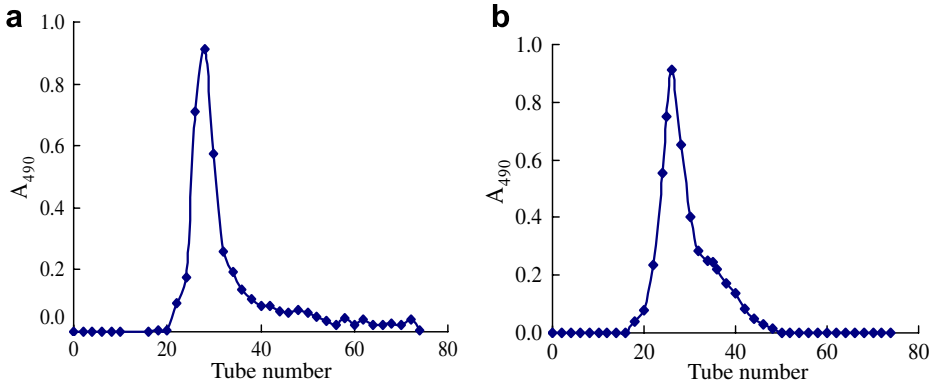

Fig. 4. Elution curves of *Chlorella pyrenoidosa* polysaccharides I and II (CPPS I and II) by Sephadex G-100 column chromatography. (a) CPPS I; (b) CPPS II.

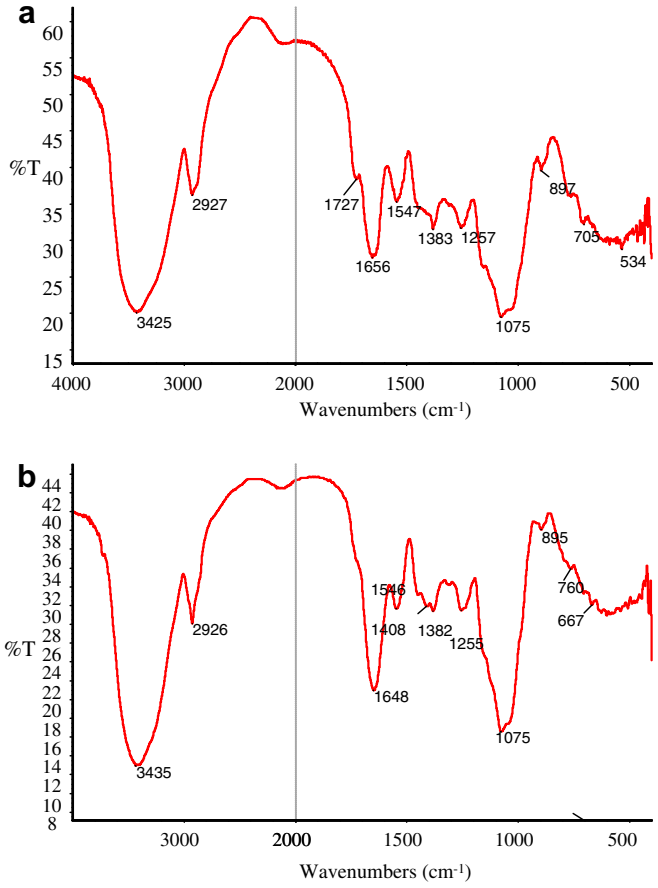

Fig. 5. FTIR spectra of *Chlorella pyrenoidosa* polysaccharides Ia and IIa (CPPS Ia and IIa). (a) CPPS Ia; (b) CPPS IIa.

in accordance with the elution curve on a DEAE Cellulose-52 anion-exchange column showing that CPPS II was a negatively charged polysaccharide.

Compared with gas chromatography of standard monosaccharides (rhamnose, arabinose, xylose, mannose, glucose and galactose), the monosaccharide compositions were shown in Table 1. Both CPPS Ia and CPPS IIa were composed of rhamnose, mannose, glucose, galactose and an unknown monosaccharide. Galactose (relative mass 46.5%) was the predominant monosaccharide of CPPS Ia, and in CPPS IIa rhamnose was predominant (37.8%). Except for the unknown monosaccharide, CPPS Ia was composed of rhamnose:mannose:glucose:galactose in molar proportion of 2.21:1:2.13:5.48 and these were 3.27:1.20:1:2.11 for CPPS IIa.

Table 2
Growth inhibition of *Chlorella pyrenoidosa* polysaccharides Ia and IIa (CPPS Ia and IIa) at different concentrations against A549 *in vitro*

Fraction	Concentration (μg ml^{-1})	A_{570}[a]	Inhibition rate (%)
Control		0.862 ± 0.042	
CPPS Ia	200	0.580 ± 0.091[b]	33.1
	600	0.542 ± 0.042[b]	37.6
	800	0.534 ± 0.099[b]	38.6
	1000	0.277 ± 0.034[b]	68.7
CPPS IIa	200	0.724 ± 0.028[b]	16.2
	600	0.550 ± 0.048[b]	36.7
	800	0.487 ± 0.023[b]	44.0
	1000	0.441 ± 0.084[b]	49.5
5-Fu	200	0.262 ± 0.023[b]	70.5
	600	0.259 ± 0.036[b]	70.8
	800	0.280 ± 0.018[b]	68.3
	1000	0.279 ± 0.035[b]	68.5

[a] Data are expressed as means of three determinations ± standard deviation.
[b] Meant significantly different from control group at $P < 0.01$ using students *t*-test.

Table 1
Monosaccharide compositions and their relative mass of *Chlorella pyrenoidosa* polysaccharides Ia and IIa (CPPS Ia and IIa) determined by GC

Fraction	Rhamnose (%)	Mannose (%)	Glucose (%)	Galactose (%)	Unkown (%)
CPPS Ia	17.1	8.49	18.1	46.5	9.78
CPPS IIa	37.8	15.2	12.7	26.7	7.64

3.5. Growth inhibition of Chlorella pyrenoidosa polysaccharides Ia and IIa on A549 Cell

Both fractions of CPPS Ia and IIa presented significantly higher antitumor activity against A549 *in vitro* than did blank control groups, and the inhibition ability was dose-dependent (Table 2). At 1000 μg ml^{-1}, the inhibition rate of CPPS Ia was 68.7%, comparable to that of 5-Fu (68.5%), but significantly higher than CPPS IIa (49.5%). The difference in the antitumor activity may be attributed to their different molecular weights, monosaccharide distributions and charge characteristics (Zhang, Cui, Cheung, & Wang, 2007). Both fractions may be potential substitutes, as antitumor drugs, for 5-Fu, which was immunosuppressive as it killed many human body-friendly cells while killing tumor cells and had negative side-effects (Takiguchi et al., 2001).

4. Conclusions

Compared with proteins and nucleic acids, polysaccharides provide the highest capacity for carrying biological information because they have the greatest potential for structural variability, and, as a result, study of this kind of biopolymer has lagged far behind others (Ohno, 2005). In the paper, the main polysaccharide fraction in *C. pyrenoidosa* could be cut off by a 30 kDa membrane and the process parameters with temperature of 40 °C and operation pressure of 14.0 psi were optimized for further development into large-scale industrial production. Two distinctive fractions, CPPS Ia and CPPS IIa, were obtained by anion-exchange chromatography and size exclusion chromatography. The peak molecular weights were 69658 Da and 109406 Da for CPPS Ia and CPPS IIa, respectively. Both CPPS Ia and IIa were composed of rhamnose, mannose, glucose, galactose and an unknown monosaccharide. CPPS Ia and IIa presented significantly higher antitumor activity against A549 *in vitro* than did a blank control, in a dose-dependent manner. At 1000 μg ml^{-1}, the inhibition rate of CPPS Ia was 68.7%, comparable to that of 5-Fu (68.5%), but significantly higher than that of CPPS IIa (49.5%). A further challenge is to define the complete structure of the polysaccharides, including configuration of glycosidic linkages, position of glycosidic linkages, sequence of monosaccharides, position of branching points and the structure–function relationship, which will certainly present a good opportunity to elucidate the biological roles of polysaccharides and develop high potential antitumor drugs based on the three-dimension structures.

Acknowledgements

This research was supported by Jiangsu Science and Technology Department (N0. BE2006327) and by the 111 Project of the Ministry of Education of The People's Republic of China (N0. B07030).

References

Chen, Y. L., Lin, S. Z., Chang, J. Y., Cheng, Y. L., Tsai, N. M., Chen, S. P., et al. (2006). *In vitro* and *in vivo* studies of a novel potential anticancer agent of isochaihulactone on human lung cancer A549 cells. *Biochemical Pharmacology, 72*(3), 308–319.

Cherng, J. Y., & Shih, M. F. (2005). Preventing dyslipidemia by *Chlorella pyrenoidosa* in rats and hamsters after chronic high fat diet treatment. *Life Science, 76*(26), 3001–3013.

Chu, C. Y., Huang, R., & Ling, L. P. (2006). Purification and characterization of a novel haemagglutinin from *Chlorella pyrenoidosa*. *Journal of Industrial Microbiology and Biotechnology, 33*(11), 967–973.

Coimbra, M. A., Goncalves, F., Barros, A. S., & Delgadillo, I. (2002). Fourier transform infrared spectroscopy and chemometric analysis of white wine polysaccharide extracts. *Journal of Agricultural and Food Chemistry, 50*(12), 3405–3411.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.

Hu, Q. H., Pan, B. S., Xu, J., Sheng, J. C., & Shi, Y. (2007). Effects of supercritical carbon dioxide extraction conditions on yields and antioxidant activity of *Chlorella pyrenoidosa* extracts. *Journal of Food Engineering, 80*, 997–1001.

Kay, R. A. (1991). Microalgae as food and supplement. *Critical Reviews In Food Science And Nutrition, 30*(6), 555–573.

Mao, W. J., Li, B. F., Gu, Q. Q., Fang, Y. C., & Xing, H. T. (2004). Preliminary studies on the chemical characterization and antihyperlipidemic activity of polysaccharide from the brown alga Sargassum fusiforme. *Hydrobiologia, 512*(1), 263–266.

Merchant, R. E., Andre, C. A., & Sica, D. A. (2002). Nutritional supplementation with *Chlorella pyrenoidosa* for mild to moderate hypertension. *Journal of Medicinal Food, 5*(3), 141–152.

Miyazawa, Y., Murayama, T., Ooya, N., Wang, L. F., Tung, Y. C., & Yamaguchi, N. (1988). Immunomodulation by a unicellular green algae (*Chlorella pyrenoidosa*) in tumor-bearing mice. *Journal of Ethnopharmacology, 24*(2-3), 135–146.

Mulder, M. (1996). *Basic principles of membrane technology* (2nd ed.). Dordrecht: Kluwer Academic Publishers.

Ohno, N. (2005). Structural diversity and physiological functions of β-glucans. *International Journal of Medicinal Mushrooms, 7*, 167–188.

Santhiya, D., Subramanian, S., & Natarajan, K. A. (2002). Surface chemical studies on sphalerite and galena using extracellular polysaccharides isolated from bacillus polymyxa. *Journal of Colloid and Interface Science, 256*(2), 237–248.

Shi, Y., Sheng, J. C., Yang, F. M., & Hu, Q. H. (2007). Purification and identification of polysaccharide derived from *Chlorella pyrenoidosa*. *Food Chemistry, 103*, 101–105.

Suarez, E. R., Kralovec, J. A., Noseda, M. D., Ewart, H. S., Barrow, C. J., Lumsden, M. D., et al. (2005). Isolation, characterization and structural determination of a unique type of arabinogalactan from an immunostimulatory extract of *Chlorella pyrenoidosa*. *Carbohydrate Research, 340*(8), 1489–1498.

Takiguchi, N., Saito, N., Nunomura, M., Kouda, K., Oda, K., Furuyama, N., et al. (2001). Use of 5-FU plus hyperbaric oxygen for treating malignant tumors: Evaluation of antitumor effect and measurement of 5-FU in individual organs. *Cancer Chemotherapy and Pharmacology, 47*, 11–14.

Van Reis, R., & Zydney, A. (2001). Membrane separations in biotechnology. *Current Opinion in Biotechnology, 12*(2), 208–211.

Varma, R., Varma, R. S., & Nardi, A. H. (1973). Separation of aldononitrile acetates of neutral sugars by gas–liquid chromatography and its application to polysaccharides. *Journal of Chromatography, 77*, 223–227.

Yamamoto, Y., Nunome, T., Yamauchi, R., Kato, K., & Sone, Y. (1995). Structure of an exocellular polysaccharide of *Lactobacillus helveticus* TN-4, a spontaneous mutant strain of *Lactobacillus helveticus* TY1-2. *Carbohydrate Research, 275*(2), 319–332.

Yang, F. M., Shi, Y., Sheng, J. C., & Hu, Q. H. (2006). In vivo immunomodulatory activity of polysaccharides derived from *Chlorella pyrenoidosa*. *European Food Research and Technology, 224*(2), 225–228.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: A review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science and Technology, 18*(1), 4–19.

Zhang, Q., Qi, H., Zhao, T., Deslandes, E., Ismaeli, N. M., Molloy, F., et al. (2005). Chemical characteristics of a polysaccharide from Porphyra capensis (Rhodophyta). *Carbohydrate Research, 340*(15), 2447–2450.