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Antioxidant activities of polysaccharides from the fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao*

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ABSTRACT

Four water-soluble polysaccharides fractions (ZSP1b, ZSP2, ZSP3c and ZSP4b) were isolated and purified from the fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao* by DEAE-SepharoseCL-6B and SepharoseCL-6B column chromatography. Their chemical and physical characteristics were determined by chemical methods, gas chromatography (GC) and high-performance size-exclusion chromatography (HPSEC). Antioxidant activities of crude polysaccharide from the fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao* (CZSP) and its purified fractions were investigated including superoxide anion, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical-scavenging activity and reducing power. Antioxidant results showed that ZSP3c and ZSP4b containing more uronic acid had the stronger free radical scavenging activities than ZSP1b containing no uronic acid. Available data obtained with in vitro models suggested that uronic acid was an effective indicator of antioxidant activity of the samples.

1. Introduction

Oxidation is essential to many living organisms for the energy production of biological processes. However, the uncontrolled production of oxygen-derived free radicals is hostile and damaging to cells and their functions. It can also cause a chain reaction resulting to the multiplication of new free radicals. The damage they cause includes interference and manipulation of protein, tissue loosening, genetic damage and the promotion of disease and aging. In order to reduce damage to the human body, many synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, tert-butyl-hydroquinone and propyl gallate are used widely at present. However, recent research suggested that synthetic antioxidants were restricted due to potential hazards related to health, such as liver damage and carcinogenesis (Grice, 1988; Yuan, Zhang, Fan, & Yang, 2008). Thus, it is essential to develop and utilize effective natural antioxidants to protect the human body from free radicals and reduce risk of many diseases such as heart disease, cancer, arthritis and the aging process (Nandita & Rajini, 2004).

In recent years, natural polysaccharides, which are widely distributed in animals, plants and microorganisms, have been demonstrated to play an important role as free radical scavengers in the prevention of oxidative damage in living organism and can be explored as novel potential antioxidants (Ge, Duan, Fang, Zhang, & Wang, 2009; Matkowski, Tasarz, & Szypula, 2008; Yuan et al., 2008). Moreover, previous studies indicated that polysaccharides antioxidant activity might be closely related to chemical properties and structural characteristics of polysaccharides (Chen, Zhang, Qu, & Xie, 2008; Wang & Luo, 2007). Therefore, discovery and evaluation of polysaccharides extracted from plants and fungus as new safe compounds for functional foods or medicine has become a hot research spot.

Chinese jujube (*Zizyphus jujuba Miller*), which has been mainly distributed in the subtropical and tropical regions of Asia and America, is a tree of the *Rhamnaceae* family. The fruiting bodies of Chinese jujube is a kind of favorable and profitable fruit, which has been commonly used as a crude drug in traditional Chinese medicine for the purpose of analeptic, palliative, antibechic and also as food, food additives and flavors for thousands of years.

Nowadays, *Zizyphus Jujuba cv. Jinsixiaozao*, a variety of Chinese jujubes is commonly planted in China. However, up to now, no detailed investigation has been carried out on the composition and antioxidative capacity of different polysaccharides isolated from the fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao*. Therefore, this paper was concerned with the purification and properties of the major polysaccharides from the fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao* and evaluated their antioxidant activities in vitro for seeking a new functional factor used in food and pharmaceutical industry.

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2. Materials and methods

2.1. Materials

The fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao* were obtained from the Research Institute of Jujube (Shandong, China). It was dried at 60 °C in an oven for 48 h after removal of seeds, and then it was ground to pass through a 1 mm screen and stored in a refrigerator.

DEAE-SepharoseCL-6B and SepharoseCL-6B were purchased from Pharmacia Chemical Co. Dextrans with different molecular weights, standard sugars, sodium salicylate, 2,2-diphenyl-1-picrylhydrazyl(DPPH), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), and potassium ferricyanide were purchased from Sigma–Aldrich (St. Louis, USA). Ferrous sulfate, ferric chloride and trichloracetic acid were purchased from Shanghai Chemical Reagent Company (Shanghai, China). All other chemicals and solvents were analytical grade and used without further purification.

2.2. Analytical methods

Total carbohydrate was determined by the phenol–sulphuric acid colorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with D-glucose as the standard at 490 nm. The contents of uronic acid were determined according to Blumenkrantz and Asboe-Hansen's method by measuring the absorbance at 525 nm with D-galacturonic acid as the standard (Blumenkrantz & Asbose-Hansen, 1973). The protein content was determined by using the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as the standard.

The composition of neutral monosaccharide was analyzed by gas chromatography. The polysaccharides were dissolved in 2 M TFA and hydrolyzed at 121 °C for 3 h in a sealed glass tube. The solution was evaporated to dryness and then a mixture of methanol–water was added to give a clear solution, which was evaporated again to dryness. Acetylation was carried out with 10 mg hydroxylamine hydrochloride and 0.5 mL pyridine for 30 min at 90 °C. A half mL of acetic anhydride was then added with continuously heating and the alditol acetate derivative was analyzed by using a gas chromatograph.

Molecular weights of all samples were determined by high-performance gel permeation chromatography (HPGPC) with a Waters HPLC apparatus equipped with two serially linked UltrahydrogelTM Linear (Ø7.8 mm × 300 mm ID) columns, a Waters 2410 interferometric refractometer detector and UV detector connected in series with a Millennium32 workstation. The molecular weights were estimated by reference to the calibration curve made under the conditions described above from Dextran T-series standards of known molecular weights.

2.3. Extraction and purification of polysaccharides

The fruiting bodies of *Zizyphus jujuba cv. Jinsixiaozao* were refluxed with 95% ethanol at 70 °C in a water bath for 3 h. Subsequently, the dried ethanol extracted residue was extracted with distilled water at 80 °C for 3 h. The aqueous extract was concentrated in a rotary evaporator under reduced pressure at 45 °C, and then precipitated with four volumes of 95% ethanol at 4 °C for 12 h. Brown crude polysaccharides (CZSP) were obtained (Fig. 1).

CZSP was dissolved in distilled water, centrifuged, and then the supernatant was applied on a DEAE-SepharoseCL-6B column (2.6 cm × 37 cm). The column was eluted with 0.1 M NaAc-buffer (pH 5.0) and then a linear gradient from 0 to 1.5 M sodium chloride in 0.1 M NaAc-buffer (pH 5.0) were applied to the column. Four main fractions were collected and denoted as ZSP1, ZSP2, ZSP3 and ZSP4. Four fractions were further purified on a gel-filtration column (2.6 cm × 160 cm) of SepharoseCL-6B with 0.02 M sodium chloride. Four main new fractions were collected and denoted as ZSP1b, ZSP2, ZSP3c and ZSP4b (Fig. 1).

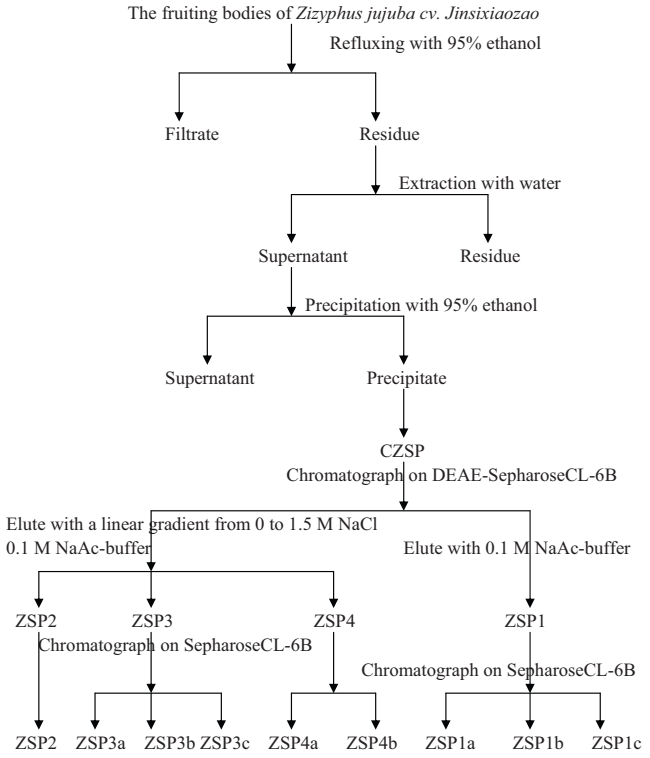

Fig. 1. Scheme for extraction and purification of polysaccharides from the fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao*.

2.4. Assay for antioxidant activity

2.4.1. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was investigated by the method of Ghiselli et al. with a minor modification (Ghiselli, Nardini, Baldi, & Scaccini, 1998). Briefly, the polysaccharides sample was dissolved in 10 mL of distilled water at the concentration of 0.25, 0.5, 1, 2 and 4 mg/mL, respectively. The sample solution (0.1 mL) was mixed with 0.8 mL of 0.2 M phosphate buffer (pH 7.4), 1.75 mM deoxyribose, 0.1 mM ferrous ammonium sulfate and 0.1 mM EDTA, then 0.1 mL of 1.0 mM ascorbic acid and 0.1 mL of 10 mM H_2O_2 were added to the reaction solution. The reaction solution was incubated for 10 min at 37 °C and then 0.5 mL of 1% thiobarbituric acid and 1 mL of 2.8% trichloroacetic acid were added to the mixture. The mixture was boiled for 10 min and cooled on ice. The absorbance of the mixture was measured at 532 nm. The capability of scavenging hydroxyl radical was calculated according to the following equation:

$$\text{scavenging effect}\,(\%) = \left(1 - \frac{A_1}{A_0}\right) \times 100$$

where A_0 is the absorbance of the control (without polysaccharides) and A_1 is the absorbance of the polysaccharides.

2.4.2. Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity was investigated according to the method reported by Li, Zhou, and Han (2006). Briefly, each 1.0 ml of NBT solution (156 μmol/L of NBT in 0.1 M phosphate buffer, pH 7.4), NADH solution (468 μmol/L of NADH in 0.1 M phosphate buffer, pH 7.4) and ZSP solution

were mixed. The reaction started by adding 1.0 ml of PMS solution (60 μmol/L PMS in 0.1 M phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against a blank (water and 0.1 M phosphate buffer instead of the sample and NBT solution, respectively). The capability of scavenging to superoxide radical was calculated using the following equation:

$$\text{inhibition rate}\,(\%) = \left[1 - \left(\frac{A_1 - A_2}{A_0}\right)\right] \times 100$$

where A_0 is the absorbance of the control (water instead of ZSP solution), A_1 is the absorbance of the sample and A_2 is the absorbance of the sample under identical conditions as A_1 with 0.1 M phosphate buffer instead of NBT solution.

2.4.3. DPPH radical scavenging activity

The scavenging of DPPH radical was carried out according to the method described by Shimada, Fujikawa, Yahara, and Nakamura (1992) with some modifications. Briefly, 3.0 ml of various concentrations of CZSP and its purified fractions (0.25–1 mg/ml) was added to 1.0 ml solution of DPPH radical (0.1 mM, in 95% ethanol) and allowed to react at room temperature. The mixture was shaken vigorously and allowed to stand for 30 min, and the absorbance of the resulting solution was measured at 517 nm with a UV–Vis spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percent DPPH radical scavenging effect was calculated according to the following equation:

$$\text{DPPH scavenging effect}\,(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100$$

where A_0 is the absorbance of DPPH solution without the tested samples and A_1 is the absorbance of the tested samples with DPPH solution.

2.4.4. Reducing power

The reducing power was determined according to the method of Oyaizu (1986) with some modification. Various concentrations of the polysaccharides sample in phosphate buffer (1.25 ml, 0.2 M, pH 6.6) were added to potassium ferricyanide (1.25 ml, 1.0%) and the mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (2.5 ml, 10%) was added to the mixture, then centrifuged at 650 × *g* for 10 min. The supernatant (2.5 ml) was mixed with ferric chloride (0.25 ml, 0.1%), and then the absorbance was read spectrophotometrically at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power.

2.5. Statistical analysis

All experiments were performed at least in duplicate, and analyses of all samples were run in triplicate and averaged. Statistical analysis involved use of the Statistical Analysis Systems (SAS, version 8.1) software package. The results shown were presented as means of three determinations ± SD (standard deviation). The results obtained were analyzed using one-way analysis of variance (ANOVA) for mean differences among the samples. *P*-values of <0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Isolation, purification and physicochemical properties of different polysaccharides fractions

Brown crude polysaccharides (CZSP) were extracted from the fruiting bodies of *Zizyphus Jujuba cv. Jinsixiaozao* and were chromatographed on a DEAE-SepharoseCL-6B anion-exchange column to yield four peaks. ZSP1 was recovered from the NaAc-buffer eluate and ZSP2, ZSP3 and ZSP4 were recovered from the NaCl eluate. On account of molecular weight difference, ZSP1, ZSP2, ZSP3 and ZSP4 were further purified by size-exclusion chromatography on a Sepharose CL-6B column to yield four major homogeneous fractions of ZSP1b, ZSP2, ZSP3c and ZSP4b, which accounted for 73.4%, 100%, 83.1%, 56.5%, respectively. The HPGPC analysis showed that the average molecular weight (Mw) of ZSP1b, ZSP2, ZSP3c and ZSP4b were approximately 9.3×10^4 Da, 8.6×10^4 Da, 1.6×10^5 Da and 1.4×10^5 Da, respectively.

Table 1
Physicochemical property of ZSP1b, ZSP2, ZSP3c and ZSP4b.

Samples	ZSP1b	ZSP2	ZSP3c	ZSP4b
Total carbohydrate (%)	96.7	89.4	97.2	83.5
Protein (%)	nd[a]	nd[a]	nd[a]	9.7
Uronic acid (%)	nd[a]	5.9	25.7	29.0
Average molecular weight (Da)	9.3×10^4	8.6×10^4	1.6×10^5	1.4×10^5

[a] nd: not detected.

The total carbohydrate, protein content and uronic acid content of ZSP1b, ZSP2, ZSP3c and ZSP4b were given in Table 1. The content of protein of ZSG4b was determined as 9.7% while no proteins existed in the ZSP1b, ZSP2 and ZSP3c. No uronic acid existed in the ZSP1b and the contents of uronic acid evaluated in ZSP2, ZSP3c and ZSP4b increased gradually from 5.9% to 29.0%, which correlated with increasing ion exchanging ability of the eluent. The monosaccharide composition showed that ZSP1b was only composed of glucose. ZSP2 was composed of rhamnose, arabinose, glucose and galactose in molar ratios of 1:2.5:1.3:4.1. ZSP3c was composed of rhamnose, arabinose and galactose in a molar ratio of 1:2:8. ZSG4b was composed mainly of rhamnose, arabinose, mannose and galactose in a molar ratio of 13.8:4:3:8.

3.2. Antioxidant activity

3.2.1. Hydroxyl radical scavenging activities of CZSP and its purified fractions

Among the reactive oxygen species, the hydroxyl radical is the most reactive and could induce severe damage to adjacent biomolecules. Hydrogen peroxide and superoxide molecules can lead to oxidative injury in the biomolecules indirectly by producing hydroxyl radical via Fenton reaction and/or iron-catalyzed Haber–Weiss reaction, which can be prevented and/or inhibited by antioxidants (Erel, 2004). Hydroxyl radicals were generated by reaction of iron-EDTA complex with H_2O_2 in the presence of ascorbic acid, attack deoxyribose to form products upon heating with 2-thiobarbituric acid under acid conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish tint formation (Cheng, Ren, Li, Chang, & Chen, 2002). The above-mentioned model was used to evaluate the hydroxyl radicals scavenging ability of natural compounds. The results of hydroxyl radical scavenging activities of CZSP and its purified fractions (ZSP1b, ZSP2, ZSP3c and ZSP4b) were given in Fig. 2. For all the samples, the effects of scavenging hydroxyl radicals were in a concentration-dependent manner. The hydroxyl radical scavenging activities of CZSP and its purified fractions under study decreased in the order of CZSP, ZSP3C, ZSP4b, ZSP2 and ZSP1b. However, there was no significant difference on scavenging activity between ZSP2 and ZSP1b at the concentration rang of 0.25–1 mg/mL. At 2.0 mg/mL, the hydroxyl radical scavenging activity of CZSP, ZSP4b, ZSP3c, ZSP2 and ZSP1b were 77.6, 59.5, 65.5, 29.9 and 25.5%, respectively. The highest scavenging activity of CZSP may be due to the presence of some other phytochemicals such as tocopherol, pigments as well as the synergistic effects among them, which also contribute to the total scavenging

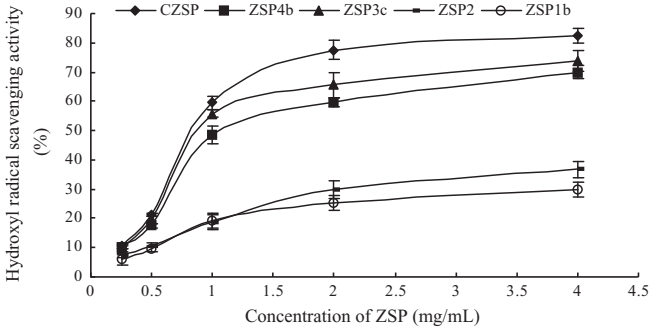

Fig. 2. Scavenging effects of CZSP and its purified fractions on hydroxyl radical.

activity. ZSP3c and ZSP4b containing more uronic acid had the stronger scavenging activities than ZSP1b containing no uronic acid, which suggested that uronic acid was an effective indicator of antioxidant activity of the samples.

3.2.2. Superoxide anion scavenging activities of CZSP and its purified fractions

Among different reactive oxygen species, superoxide anion is generated first. The superoxide radical is a highly toxic species that could be generated by numerous biological and photochemical reactions. Although the superoxide anion is a relatively weak oxidant, it may decompose to form stronger reactive oxygen species, such as singlet oxygen and hydroxyl radical, local oxidative stress and initiate cellular damage or lipid peroxidation and pathological incidents such as arthritis and Alzheimer's disease (Yuan et al., 2005). Superoxide anion is also known to initiate indirectly the lipid peroxidation as a result of the formation of H_2O_2, creating precursors of hydroxyl radical (Meyer & Isaksen, 1995). Therefore, superoxide anion scavenging is extremely important to antioxidant work. Scavenging activities of CZSP and its purified fractions on superoxide anion were presented in Fig. 3. The superoxide scavenging activities of CZSP, ZSP1b, ZSP2, ZSP3c and ZSP4b increased significantly ($P < 0.05$) with the increase of polysaccharide concentration ranging from 0.25 to 1 mg/ml. The scavenging activity increased slowly with the increase of sample concentration when the concentration was above 1 mg/ml. The scavenging effect of crude ZSP and its purified fractions on superoxide anion followed the order: CZSP, ZSP4b, ZSP3C, ZSP2, ZSP1b and were 82.3, 52.5, 49.5, 29.6 and 6.7% at the concentration of 2 mg/mL, respectively. Among four purified fractions, ZSP4b had the strongest scavenging ability and the highest content of proteinous substances and uronic acid in the polysaccharide. Higher scavenging activities were found when the content of proteinous substances and uronic acid increased. This result correlated to the findings of Liu, Ooi, & Chang (1997) that the content of proteinous substances in the

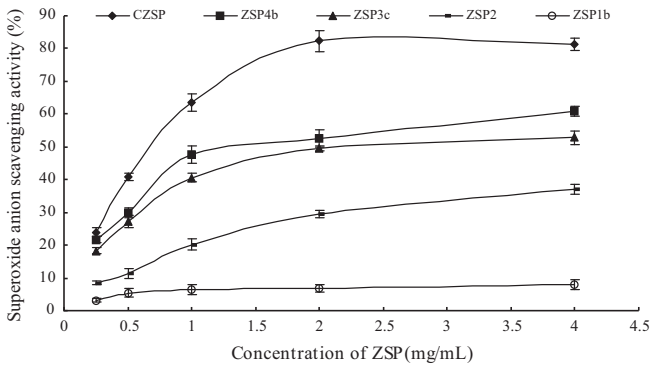

Fig. 3. Scavenging effects of CZSP and its purified fractions on superoxide anion.

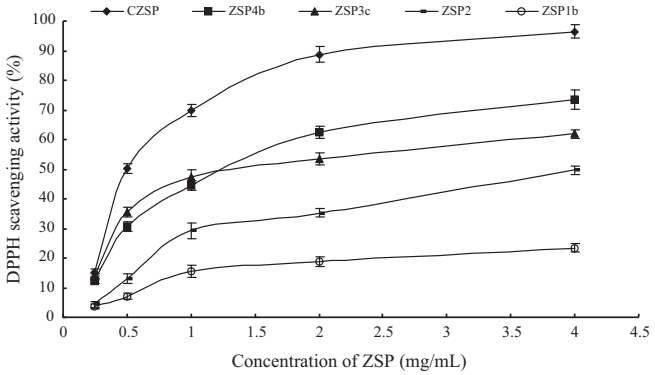

Fig. 4. Scavenging effects of CZSP and its purified fractions on the DPPH.

polysaccharide molecules potentiate their free radical scavenging activity.

3.2.3. DPPH Scavenging activities of CZSP and its purified fractions

The DPPH free radical is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant. So it has been widely accepted as a tool for evaluating the free radical scavenging activities of natural compounds (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004; Leong & Shui, 2002). Alcoholic solutions of DPPH have a characteristic absorption maximum at 517 nm. When an electron- of hydrogen-atom-donating antioxidant is added to DPPH, the absorbance at 517 nm disappears because the DPPH radical is scavenged (Marinova & Yanishlieva, 1997). On the basis of this principle, the scavenging effects of CZSP and its purified fractions on the DPPH radical were measured and shown in Fig. 4. The scavenging activity of polysaccharides on inhibition of the DPPH radical was related to the concentration of the samples. Furthermore, the DPPH scavenging activities of CZSP significantly increased with the increasing concentrations and were stronger than that of its purified fractions at every concentration point. Interestingly, ZSP3c exhibited higher radical scavenging activity than ZSP4b at the lower doses (0.25–1.0 mg/mL), whereas the radical scavenging activity of ZSP3c was lower than that of ZSP4b at the higher doses (2.0–4.0 mg/mL). At the concentration of 2 mg/mL, the scavenging effects of CZSP, ZSP4b, ZSP3C, ZSP2 and ZSP1b on the DPPH radical were 88.7, 62.4, 53.6, 35.3 and 18.9%, respectively. The results mentioned above implied that CZSP and its purified fractions might act as electron or hydrogen donator to scavenge DPPH.

3.2.4. Reducing power of CZSP and its purified fractions

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities (Makris, Psarra, Kallithraka, & Kefalas, 2003; Meir, Kanner, Akiri, & Hadas, 1995). Reducing power of CZSP and its purified fractions were investigated and showed in Fig. 5. The reducing capacities of CZSP and its purified fractions increased with the increase of sample concentration. At the concentration of 3 mg/ml, the reducing capacity of CZSP, ZSP1b, ZSP2, ZSP3C and ZSP4b was 0.732, 0.316, 0.287, 0.413 and 0.379, respectively. The results indicated that reducing power of CZSP and its purified fractions decrease in the order CZSP > ZSP3C > ZSP4b > ZSP1b > ZSP2. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with

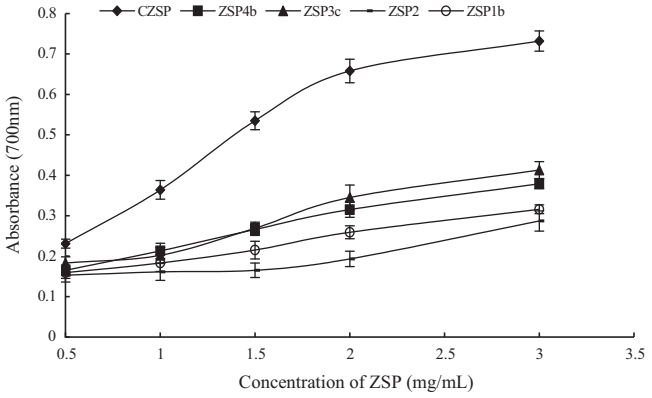

Fig. 5. Reducing power of CZSP and its purified fractions.

certain precursors of peroxide, thus preventing peroxide formation. The antioxidant activity was concomitant with the reducing power (Amarowicz et al., 2004; Pin-Der-Duh, Pin-Chan-Du, & Gow-Chin-Yen, 1999). Our data on reducing power of CZSP and its purified fractions indicates that it may likely play a role in the antioxidation observed.

4. Conclusions

According to the results stated above, it could be concluded that CZSP predominantly contained four polysaccharide fractions (ZSP1b, ZSP2, ZSP3c and ZSP4b) purified by DEAE-SepharoseCL-6B and SepharoseCL-6B column chromatography. In vitro antioxidant activities studies indicated both ZSP3c and ZSP4b exhibit greater capacity in scavenging free radicals, which may be related to the content of uronic acid. Besides, the antioxidant activity of polysaccharide is usually influenced by various factors combined rather than one single factor. Therefore, further research is needed to elucidate the complete structure, conformation and mechanism of antioxidant activity.

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