Antioxidant activities potential of tea polysaccharide fractions obtained by ultra filtration

Yuanfeng Wang¹, Zhiwei Yang¹, Xinlin Wei*  
Institute of Food Engineering, College of Life & Environment Science, Shanghai Normal University, 100 Guilin Rd, Shanghai 200234, PR China

Abstract

Three polysaccharide fractions (TPS1, TPS2 and TPS3) with different molecular weights were obtained using ultra filtration membranes from crude tea polysaccharide (CTPS) extracted from abandoned lower grade tea leaves. Each fraction contained different contents of neutral sugar, uronic acid, protein, and total polyphenols. These differences provided basis for the antioxidant and free radical scavenging activity of these polysaccharide fractions. The molecular weights of TPS1, TPS2, and TPS3 were around 2.40 \times 10^4 Da, 2.14 \times 10^4 Da, and 2.46 \times 10^3 Da, respectively. In general, TPS1 and CTPS had stronger antioxidant activity, TPS2 and TPS3 had lower antioxidant activity, TPS1 had higher activity for DPPH and lipid per oxidation inhibition. But it had lower capacity for reducing power and metal chelating. This might be due to its higher content of hexuronic acid and larger molecular weight. The order of inhibition activity of lipid per oxidation of various polysaccharide fractions was the same as DPPH radical scavenging activity, as well as the order of metal chelating activity of various polysaccharide fractions similar to hydroxyl radical scavenging activity, which demonstrated that hydroxyl radical scavenging activity of polysaccharide relied heavily on the Fe^2+ metal chelating to decrease the generation of hydroxyl radical. 

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

In recent years, available data about activities of polysaccharides have shown that they can scavenge multiple reactive oxygen species (ROS) which derives from a series of physical and chemical reactions in biological organisms [1]. ROS can injure tissues and organs and induce system organ dysfunction or disease such as atherosclerosis, rheumatoid arthritis, muscular dystrophy, cancer, cataracts, nervous system disease, tumor serious diseases, etc. [2]. Antioxidant activity of polysaccharides mainly depends on several structural parameters including sugar composition, molecular weight, and type of glycosidic bond of the main chain, degree of sulfuric acid esterification [3].

Tea polysaccharide (TPS) is one of the main bioactive components of green tea, especially low-grade green tea [4]. It is reported that TPS has an active effect on scavenging ROS including hydroxyl radical, superoxide anion and DPPH radical [5,6].

In this study, different molecular weights of crude tea polysaccharides (TPS) which obtained from green tea were isolated by ultra filtration method. We attempted to investigate antioxidant activities of TPS fractions with different molecular weights, and effects of different molecular weight fractions of TPS on their antioxidant activities in vitro were evaluated.

2. Materials and methods

2.1. Materials and chemicals

Tea leaves were obtained commercially from Hebei province of China. 2-Deoxy-o-ribose, nitrotetrazolium blue chloride (NBT), phenazine methosulfate (PMS), linoleic acid, and 2, 2-diphenyl-1-picyrlyhydrazyl (DPPH) were purchased from Aladdin Reagent Int. (Shanghai, China). Nicotinamide adenine dinucleotide (NADH) and ferrozine were obtained from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Ascorbic acid, trichloracetic acid (TCA), thiobarbituric acid (TBA) and d-mannitol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other reagents and solvents were of analytical purity (AR) grade. All aqueous solutions were prepared by using newly double-distilled water.

2.2. General methods

Total polysaccharide was determined by phenol–sulfuric acid method [7]. Hexuronic acid content was determined by m-hydroxybiphenyl-H_2SO_4 method with galacturonic acid as a standard [8]. Protein content in tea polysaccharide conjugate fractions was measured according to Bradford’s method with bovine
serum albumin (BSA) as a standard [9]. Total polyphenols content in crude tea polysaccharide was determined with Folin–Ciocalteu’s reagent (FCR) according to Khokhar’s method [10].

2.3. Extraction of crude polysaccharide from tea leaves and fractionation

The dried group tea leaves (250 g) were extracted with 2.5 L distilled water in bath at 90 °C for 2 h. After filtered, the residues were extracted again with 2.5 L distilled water at the same temperature for another 2 h. Then the extracts were combined and centrifuged to remove the contaminants. Supernatant was concentrated to obtain 500 mL of concentrated solution which was precipitated with four volumes of anhydrous ethanol for 12 h at 4 °C. The precipitate (crude TPS) was obtained by centrifugation (4500 x g for 15 min). It was freeze-dried to yield crude tea polysaccharides (CTPS). It was ultra-filtered successively by ultra filtration membranes with molecular weight cutoff of 100 kD and 10 kD (Millipore Co., Ltd.) after it was microfiltrated under the optimized operation conditions. Three fractions (TPS1, TPS2, and TPS3) obtained from CTPS solution were concentrated to small volumes and also precipitated with four volumes of anhydrous ethanol for 12 h at 4 °C. The three precipitates were dissolved in deionized water, dialyzed, and freeze-dried respectively. The preparation process flow diagram of various TPS fractions was shown in Fig. 1.

2.4. Determination of molecular weight of TPS

Molecular weight of TPS was determined by gel permeation chromatography. Samples (10 mg) were dissolved in 1 mL of 0.02 M phosphate buffer solution and centrifuged at 16,000 x g for 10 min. 20 μL of the supernatant was injected into a Shodex SB-804 HQ GPC column (300 mm x 8 mm) with a Shodex SB-G guard column (50 mm x 6 mm) from Showa Denko K.K. (Tokyo, Japan). GPC system was maintained at 45 °C and eluted with phosphate buffer solution at a rate of 0.3 mL/min. Molecular weight was calculated by calibration curve obtained by using various standard dextran with different molecular weights (T3, T6, T10, T40, T100, T500, and T1000).

2.5. Antioxidant activity assay

2.5.1. DPPH radical scavenging activity

Antioxidant activities of various polysaccharide fractions were firstly measured on the basis of scavenging activity of DPPH free radical. Briefly, the sample solution (1 mL) with variable concentrations (25–400 μg/mL) was added to tube containing 1 mL of DPPH solution (0.2 mM in ethanol). The mixture was vortexed and set for 30 min. Then the absorbance, due to proton donating activity by TPS, was measured at 517 nm after 30 min [11]. Ascorbic acid was used for positive comparison. The DPPH radical scavenging activity was calculated using the following formula:

\[
\text{DPPH radical scavenging activity (\%) = \left(1 - \frac{A_{1}}{A_{0}}\right) \times 100,}
\]

where \(A_0\) is the absorbance of the control, \(A_1\) is the absorbance of various polysaccharides and ascorbic acid.

2.5.2. Hydroxyl radical scavenging activity

Hydroxyl radical activity was determined by the method of Haliewell et al. [12]. Briefly, the sample solution (1 mL) with variable concentrations (100–1600 μg/mL) was added to tube containing 0.7 mL of deoxyribose (2.8 mM), 1 mL of phosphate buffer (20 mM, pH 7.4) containing FeCl₃ (0.1 mM), ascorbic acid (0.1 mM), EDTA (0.1 mM) and H₂O₂ (1 mM) for 60 min at 37 °C. The extent of deoxyribose degradation was measured by TBA method. The reaction was terminated by adding 1 mL of TCA (1%, w/v) and 0.3 mL of TBA (2.8%, w/v), and then heating tubes in a boiling water bath for 20 min. After cooling to room temperature, absorbance of the mixture was measured at 532 nm against a blank. Mannitol was used as positive control. The capability of scavenging on hydroxyl radical was calculated using the following formula:

\[
\text{Hydroxyl radical scavenging activity (\%) = \left(1 - \frac{A_{1} - A_{2}}{A_{0}}\right) \times 100,}
\]

where \(A_0\) is the absorbance of the control only without tested samples, \(A_1\) is the absorbance of tested samples, and \(A_2\) is the absorbance of TPS only with addition of deoxyribose.

2.5.3. Non-enzymatic lipid peroxidation induced by Fe²⁺/ascorbate

Male mice (18–25 g body weight) were purchased from Sino-British Sippr/BK Lab. Animal Ltd., Co. and housed in plastic cages with free access to water and food at 25 °C. The liver of each mouse was pulled out from abdominal cavity by laparotomy after it was anesthetized by diethyl ether. The livers were cut into small pieces and washed extensively with 0.15 M NaCl. A homogenate 10% (w/v) was prepared in phosphate buffer (50 mM, pH 7.4) using a pestle in a mortar. The liver homogenate was centrifuged at 10,000 x g for 10 min. All these steps were carried out at 4 °C. Supernatant of the liver homogenate was collected. The most prominent assay currently being used as an index for lipid per oxidation in biological systems is measurement of thiobarbituric acid reactive substances (TBARS) in rat liver homogenate according to thiobarbituric acid (TBA) test [11,13]. The reaction mixture was composed of tissue homogenate (0.5 mL), phosphate buffer (50 mM, pH 7.4) 0.9 mL, FeSO₄ (0.01 mM) 0.25 mL, ascorbic acid (0.1 mM) 0.25 mL, and 0.1 mL of different concentrations of polysaccharide fractions and positive control. The reaction mixture was incubated at 37 °C for 30 min and then terminated by adding 1 mL of butyraldehydetoxyxylene (BHA, 2% (w/v) in 95% (v/v) ethanol), which was immediately added to 1 mL of TCA (20%, w/v) and centrifuged for 10 min at 3000 x g. The supernatant was again incubated with 1 mL of TBA (0.67%) at 100 °C for 15 min. Ascorbic acid was used as positive control. Malondialdehyde (MDA), a secondary product of oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid (TBA), yielding a pinkish red chromogen with an absorbance maximum at 532 nm. The percent inhibition of lipid peroxidation of samples was calculated accordingly.

Fig. 1. Scheme of extraction and fractionation of different molecular weights of crude polysaccharides from tea leaves.
2.5.4. Superoxide anion scavenging activity
Superoxide anion (•O₂⁻) scavenging activity of various polysaccharide fractions was determined by modified methods reported by Robak and Gryglewski [14]. In this experiment, •O₂⁻ was generated in 3 mL of sodium phosphate buffer (100 mM, pH 7.4) containing 1 mL of (NBTH) nitroblue tetrazolium (150 μM) solution, 1 mL of (NADH) nicotinamide adenine dinucleotide (468 μM) solution and different concentrations of polysaccharide fractions in water. The reaction started by adding 1 mL of phenazine methosulfate (PMS, 60 μM) to mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured. Galactic acid (GA), butylated hydroxytoluene (BHT) and ascorbic acid were used as positive controls. Compared to the value with no test sample added, the reduction of the absorbance was estimated as superoxide scavenging activity. The scavenging capability of various polysaccharide fractions on superoxide radical was calculated using the following equation:

Superoxide anion scavenging activity (%) = \( \left( 1 - \frac{A_1}{A_0} \right) \times 100 \)

where \( A_0 \) is the absorbance of the blank control only without and \( A_1 \) is the absorbance of TPS or positive control.

2.5.5. Total antioxidant activity
The total antioxidant activity of various polysaccharide fractions was determined using reported linoleic system with some modifications [11]. In brief, the linoleic acid emulsion was firstly prepared by homogenizing mixture containing 0.28 g of linoleic acid, 0.28 g of emulsifier Tween 20, and 50 mL of phosphate buffer (0.2 M, pH 7.0). Subsequently, 0.5 mL of different concentration of tested sample (25, 50, 100, 200, 400 μg/mL) was mixed with 2.5 mL of linoleic acid emulsion (0.2 M, pH 7.0) and 2 mL of phosphate buffer (0.2 M, pH 7.0), and then incubated in dark chamber at 37 °C for accelerating the per oxidation process. The levels of per oxidation were determined according to the thiocyanate method by adding ethanol (5 mL, 75%), ammonium thiocyanate (0.1 mL, 30%), 0.1 mL of above mixed solution, and ferrous chloride (0.1 mL, 20 mM in 3.5% HCl). The absorbance was measured at 500 nm after mixing for 3 min. Ascorbic acid was used as positive control.

2.5.6. Reducing power assay
The reducing power of various polysaccharide fractions was assayed by the method described by Kong et al. [15] with minor modification. Briefly, 5 mL of reaction mixture, containing 1 mL of different concentrations of tested samples (25–400 μg/mL), 2 mL of phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferri-cyanide (1%, w/v), was incubated at 50 °C for 20 min. After cooling rapidly, the reaction was terminated by adding 2 mL of TCA solution (10%, w/v) and centrifuged at 1750 × g for 10 min. The supernatant (2.5 mL) mixed with 2.5 mL of distilled water and 1 mL of 0.1% ferric chloride. The absorbance of the reaction mixture was read at 700 nm. Ascorbic acid was used as positive control. Increased absorbance of the reaction mixture indicated stronger reducing power.

2.5.7. Metal chelating assay
The metal chelating effect of various polysaccharide fractions was determined according to method reported by Carter [16] with some modifications. A reaction solution, composed of 1.0 mL of tested sample with different concentrations (0.5–5 mg/mL) and 0.1 mL of FeCl₂ (2 mM), was shaken well and stood for 30 s before it was activated by addition of 0.2 mL of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrrozine, 5 mM) and 2.7 mL of ultrapure water. After again mixed well, it was incubated at room temperature for 10 min before the absorbance of it was measured at 562 nm against a blank. In control, sample was substituted with EDTA. The ferrous ion chelating ability of different concentrations of tested samples was calculated using the following equation:

Chelating ability (%) = \( \left( 1 - \frac{A_2}{A_1} \right) \times 100 \)

where \( A_0 \) is the absorbance of the control only, \( A_1 \) is the absorbance of tested sample, and \( A_2 \) is the absorbance of the tested sample only without FeCl₂.

2.5.8. Self-oxidation of 1,2,3-phenatriol assay
The scavenging potency for self-oxidation of 1,2,3-phenatriol of all samples was evaluated according to the method of Marklund and Marklund [17] with minor modification. Briefly, 1 mL of tested sample with different concentrations (250–2000 μg/mL) was mixed with 2.8 mL of Tris–HCl buffer (0.05 M, pH 8.0) containing 1 mM EDTA and 0.2 mL of 1,2,3-phenatriol (6 mM). The mixture was then shaken rapidly at room temperature. Absorbance of it was measured at 325 nm per 30 s for 4 min against a blank, and a slope was calculated as absorbance/min. The scavenging ability for self-oxidation of 1,2,3-phenatriol of tested samples was calculated using the equation:

Scavenging (%) = \( \left( 1 - \frac{\text{slope of sample}}{\text{slope of control}} \right) \times 100 \)

3. Results and discussion
3.1. Chemical composition and molecular weight of crude tea polysaccharide

Ultra filtration is a good method for isolation and purification of TPS. Three fractions (TPS1, TPS2, and TPS3) were obtained by it. The neutral sugar, uronic acid, protein, polyphenol contents and molecular weight of various polysaccharide fractions are summarized in Table 1. Neutral sugar content of TPS3 was the highest (71.60%), but the content of uronic acid in it was lower (10.86%) than TPS1 (48.90%), TPS1 (55.70%) and TPS3 (59.60%). Each fraction contained hexuronic acid and the content of it was decreased in the order TPS1 > TPS2 > TPS3, which suggested that acid polysaccharides were the main polysaccharide in TPS. The molecular weights of TPS1, TPS2, and TPS3 were around 2.40 × 10⁴ Da, 2.14 × 10⁴ Da, and 2.46 × 10⁴ Da, respectively. The Mw/Mn value of TPS 3 was closer to 1 and less than that of TPS1 and TPS2, indicating that TPS3 had higher of homogeneity. Polyphenols compounds were important components in TPS; the occurrence of them may affect the antioxidant activity of TPS. Therefore, it would be valuable to determine the total polyphenols of various polysaccharide fractions (Table 1). The content of protein and polyphenol were relatively low in TPS1 (2.41% and 4.58%) and TPS3 (1.51% and 4.64%), and lower than those of TPS (4.00% and 8.86%) and TPS2 (3.75% and 11.83%).

3.2. DPPH radical scavenging activity

The DPPH radical is a stable organic free radical with absorption maximum band around 515–528 nm and thus, it is a useful reagent for investigating free radical scavenging activities of antioxidant materials. Antioxidants transfer either electrons or hydrogen atoms to DPPH and thus reduce a number of DPPH radical equal to their number of available hydroxyl groups [18]. In addition, the stable yellow-colored diphenylpicrylhydrazine (DPPH-H) is simultaneously formed, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants [19]. Various polysaccharide fractions demonstrated a concentration dependent scavenging activity by quenching DPPH radicals (Fig. 2(A)). The hydrogen donating activity, measured using DPPH test,
Table 1
Composition and molecular weight of tea polysaccharide.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Neutral sugar (wt%)</th>
<th>Uronic acid (%)</th>
<th>Protein (%)</th>
<th>Total polyphenols (%)</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>bMw bMn Mw/Mn</td>
</tr>
<tr>
<td>CTPS</td>
<td>48.90</td>
<td>28.52</td>
<td>4.00</td>
<td>8.86</td>
<td>3,597,730 3,444,710 1.04</td>
</tr>
<tr>
<td>TPS1</td>
<td>55.70</td>
<td>34.82</td>
<td>2.41</td>
<td>4.58</td>
<td>240,218 67,268 3.57</td>
</tr>
<tr>
<td>TPS2</td>
<td>71.60</td>
<td>10.86</td>
<td>3.75</td>
<td>11.83</td>
<td>21,354 13,600 1.57</td>
</tr>
<tr>
<td>TPS3</td>
<td>59.60</td>
<td>1.95</td>
<td>1.51</td>
<td>4.64</td>
<td>2462 2384 1.03</td>
</tr>
</tbody>
</table>

\(^a\) Mw: weight – average molecular weight.
\(^b\) Mn: number – average molecular weight.

Fig. 2. Antioxidant activity analysis of TPS with various methods: (A) DPPH radical scavenging activity of TPS; (B) hydroxyl radical scavenging activity of TPS; (C) inhibition by TPS on Fe\(^{3+}\)/ascorbate induced lipid per oxidation of mouse liver homogenates. Each value represents the mean ± SD (n = 3).

Table 2
EC\textsubscript{50} values of different molecular weight fractions of crude tea polysaccharide in antioxidant properties.

<table>
<thead>
<tr>
<th>Antioxidant activity</th>
<th>CTPS</th>
<th>TPS1</th>
<th>TPS2</th>
<th>TPS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH radical scavenging activity</td>
<td>119</td>
<td>36</td>
<td>96</td>
<td>64</td>
</tr>
<tr>
<td>Non-enzymatic lipid per oxidation induced by Fe(^{3+})/ascorbate</td>
<td>679</td>
<td>79</td>
<td>171</td>
<td>127</td>
</tr>
<tr>
<td>Hydroxyl radical scavenging activity</td>
<td>365</td>
<td>1040</td>
<td>632</td>
<td>322</td>
</tr>
<tr>
<td>Superoxide anion scavenging activity</td>
<td>53</td>
<td>71</td>
<td>43</td>
<td>67</td>
</tr>
<tr>
<td>Metal chelating assay</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>4000</td>
<td>1300</td>
</tr>
<tr>
<td>Reducing power assay</td>
<td>60</td>
<td>121</td>
<td>38</td>
<td>94</td>
</tr>
</tbody>
</table>

\(^a\) EC\textsubscript{50} value: the effective concentration at which the antioxidant activity was 50%.
\(^b\) RP\textsubscript{0.5AU} value: the absorbance was 0.5 for reducing power. EC\textsubscript{50} and RP\textsubscript{0.5AU} value were obtained by interpolation from linear regression analysis.
appeared that various polysaccharide fractions possessed hydrogen donating capabilities and would act as antioxidants. The scavenging effects of various polysaccharide fractions increased with increasing concentration at the dosage range of 25–400 μg/mL. The DPPH radical scavenging ability decreased in the order of TPS1 > TPS3 > TPS2 > CTPS. The EC_{50} value, defined as the effective concentration at which the DPPH radical was scavenged by 50% value of TPS1, TPS3, TPS2 and CTPS were 36 μg/mL, 61 μg/mL, 96 μg/mL and 119 μg/mL, respectively (Table 2). However, the concentration of total polyphenols in them was decreased in the order of TPS2 > CTPS > TPS3 > TPS1. Especially, when the concentration was over 100 μg/mL, the scavenging ability of TPS1 on DPPH radical was beyond 90% which was close to that of ascorbic acid, indicating that the DPPH radical scavenging activity of them may be due to the carboxy group in hexuronic acid but not to polyphenols compounds.

3.3. Hydroxyl radical scavenging activity

The 2-deoxy-d-ribose is degraded on exposure to hydroxyl radicals generated by Fenton reaction. A pink chromogen was formed when it was heated with TBA at low pH. The inhibitory action of tested samples on deoxyribose degradation gives an indication of hydroxyl radical scavenging capability and iron chelating activity [11]. Added TPS competed with deoxyribose for hydroxyl radicals, and then analysis of the results in terms of a simple competition between TPS and deoxyribose, that could calculate the rate constant for reaction of TPS with hydroxyl radicals (Fig. 2(B)). As seen in Fig. 2(B), various polysaccharide fractions scavenged hydroxyl radical that induced deoxyribose cleavage in a concentration-dependent manner. The scavenging effect of them increased with increasing sample concentration, with EC_{50} value of 322 μg/mL (TPS3), 365 μg/mL (CTPS), 632 μg/mL (TPS2), 1040 μg/mL (TPS1), respectively (Table 2). The scavenging activity of TPS3 was close to manitol at the concentration of 400–1600 μg/mL. In general, the order of them on scavenging activities was TPS3 > CTPS > TPS2 > TPS1. Hydroxyl radical scavengers could suppress against hydroxyl radical generation or clean the hydroxyl radical generated [20]. Various polysaccharide fractions might release hydrogen proton to react with hydroxyl radicals, causing decreased of the rate of hydroxyl radicals attack on deoxyribose. Many factors including monosaccharide constituent, molecular weight, carboxyl and sulfate group, and protein content may affect the antioxidant activity of polysaccharides. The lower molecular weight of TPS3 as well as lower content of hexuronic acid and total polyphenols exhibited that molecular weight of polysaccharide played important role in hydroxyl radical scavenging activity.

3.4. Non-enzymatic lipid peroxidation induced by Fe^{2+}/ascorbate

Lipid peroxidation (LPO), induced by free radical, is associated with various pathological events, such as inflammation, postischemic reperfusion injury, atherosclerosis, ethanol toxicity, and cancer [21]. These pathological phenomena may be due to the crosslink of MDA with biological macromolecules [22]. As shown in Table 2, the EC_{50} value of CTPS, TPS1, TPS2 and TPS3 of 679 μg/mL, 79 μg/mL, 171 μg/mL and 127 μg/mL, respectively. Fig. 2(C) showed that the inhibiting effects of the tested samples on the lipid peroxidation were concentration-dependent. The inhibiting effects rose from 10.91% to 50.97% for TPS1, 29.85% to 64.52% for TPS2, 12.58% to 54.89% for TPS3 and 19.29% to 57.89% for TPS4 with the concentration increasing from 25 to 800 μg/mL. In the mouse liver microsomal lipid system, Fe^{2+} catalyzed lipid hydroperoxides (LOOH) breakdown and the accumulation of lipid peroxidation end products (e.g., MDA) [23].

3.5. Superoxide anion radical scavenging activity

In the PMS/NADH–NBT system, the reduction of NBT with NADH mediated by PMS under aerobic conditions was inhibited upon addition of antioxidants. The addition of PMS to this system provoked the reduction, and the reduction of NBT by O_{2}^{−} occurring in the reoxidation of reduced PMS with O_{2} [24]. Antioxidants would therefore lower the steady-state concentration of NBTH* (i.e. NBT + H^{+} + O_{2}^{−} → O_{2} + NBTH*.) by scavenging O_{2}^{−} and therefore decrease the rate of production of the formazan by reaction (i.e. 2NBTH* → NBT + NBTH_{2}). The decrease of absorbance at 560 nm after addition of various polysaccharide fractions indicated the consumption of the generated superoxide anion in the reaction mixture which was positively correlated to the superoxide anion scavenging activity. It showed the inhibitory effect of tested samples on superoxide radical. The scavenging activity of all polysaccharide fractions increased with increasing concentration in Fig. 3. The EC_{50} value of CTPS, TPS1, TPS2 and TPS3 was about 25 μg/mL, 71 μg/mL, 43 μg/mL, and 67 μg/mL, respectively (Table 2). On concentrations ranging from 25 to 400 μg/mL, the scavenging rates of TPS2 on superoxide anion radical were from 37.42% to 87.21%. TPS3 exhibited a strong scavenging activity (94.13%) at 400 μg/mL, which was close to GA. There was no significant difference in superoxide anion scavenging ability among TPS2, CTPS and control (GA). In this assay, GA exhibited potent superoxide anion scavenging activity, however, BHT and ascorbic acid exhibited the bottom level of scavenging activity in this system (Fig. 3). BHT and ascorbic acid seemed to generate more formazan dye at high concentrations, and showed similar changing trends. It also may be ascribed to the stronger reducing power of them which reacts with NBT and reduces it directly. The bottom level of the reduction of absorbance at 560 nm in this assay might reflect the balance of superoxide anion scavenging and NBT reduction. These results were in accordance with data reported by Yamaguchi et al. [25] and Lai et al. [26].

3.6. Total antioxidant activity

Total antioxidant capacity is an easily and increasingly broadly used parameter employed in clinical studies and in food science,
useful in comparison of the antioxidant content of body fluids, cell and tissue homogenates, food, and beverages [27]. The amount of peroxide was determined using ferric thiocyanate test. Lower absorbance indicates higher level of antioxidant activity. It showed the absorbance changes induced by four fractions TPS (200 μg/ml) at 37 °C compared to ascorbic acid during 96 h in Fig. 4(A). Total antioxidant activities of all polysaccharide fractions exhibited a gradually decreasing tendency with prolongation of exposure time. The total antioxidant activity of the samples decreased in the order of TPS2 > TPS1 > TPS3 > CTPS. TPS1, TPS2 and TPS3 exhibited definite antioxidant activity compared to the control. Moreover, the activities of them were higher than that of ascorbic acid. However, the scavenging ability of CTPS was lower than that of ascorbic acid, suggesting that the polysaccharide parts play important roles in antioxidant capability although total polyphenols involved in it would affect its corresponding activity. The antioxidant activities of the polysaccharides can be attributed to the mechanism that polysaccharides neutralize the linoleate free radical and other free radicals formed in this model system.

3.7 Reducing power assay

For the measurements of the reducing ability, the Fe³⁺–Fe²⁺ transformation was investigated in the presence of various polysaccharide fractions. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [28]. The presence of polysaccharide fractions may cause the Fe³⁺ to be converted to Fe²⁺. The equation was given as follows: K₃Fe(CN)₆ + TPS → K₄Fe(CN)₆ + TPSₙoxide; K₄Fe(CN)₆ + Fe³⁺ → K₄[Fe(CN)₆]₃ (Prussian blue). Thus, the Fe²⁺ concentration can be monitored recording absorbance at 700 nm which was positively correlated to the formation of Prussian blue. A higher absorbance at 700 nm indicates a higher reducing power. It depicted the reductive effects of various TPS fractions compared with ascorbic acid. Similar to the total antioxidant activity in Fig. 4(B), the reducing power of various TPS fractions increased with increasing dosage. Among the fractions tested, the TPS2 exhibited greater reducing power although other fractions showed lower activities than that of ascorbic acid. TPS2 and CTPS exhibited greater reducing powers than TPS1 and TPS3. Reducing power of all tested samples exhibited the following order: ascorbic acid > TPS2 > CTPS > TPS3 > TPS1.

The reducing properties were generally associated with the presence of electron-donating groups or hydrogen atoms. Accordingly, all TPS fractions might contain higher amounts of reductone, which could react with free radicals to stabilize and block radical chain reactions [29]. Therefore, the data presented here indicate that the marked antioxidant activity of TPS seemed to be the result of their reducing power.

3.8 Metal chelating assay

Ferrozine is a sensitive reagent. Colored species (iron (II)-ferrozine complex) was formed immediately when it was mixed with the ferrous ion. Iron (II)-ferrozine complex has maximal absorbance at 562 nm. When antioxidants were involved in this
system, they will compete with ferrozine for Fe²⁺ and result in the decrease of absorbance at 562 nm. This was often used for the quantitative determination of Fe²⁺ [30]. The binding properties of Fe²⁺ by tested samples were estimated by the decrease in the maximal absorbance of the Fe(II)–ferrozine complex. The possible reaction equation was given as follows: Fe(II) + 3ferrozine → Fe (ferrozine)₃²⁺. As shown in Fig. 4(C), the Fe²⁺ chelating effects of all polysaccharide fractions also demonstrated a concentration dependent tendency.

The chelating effect of TPS3 with Fe²⁺ (72.18%) was higher than that of the other fractions although all of them were lower than that of EDTA in concentration range of 0.5–5 mg/mL. Chelating capability of them were decreased in the order of TPS3 > TPS2 > CTPS > TPS1. The highest Fe²⁺ chelating effect of TPS3 may be due to the higher content of total polyphenols, lower content of hexuronic acid, and lower molecular weight. But for the lowest Fe²⁺ chelating capability of TPS1, the higher content of hexuronic acid, lower content of total polyphenols, and much bigger molecular weight may influence factor. Thus, the antioxidant and free radical scavenging activities of the polysaccharide were not a function of a single factor but a combination of several factors. The exact mechanism underlying the radical scavenging activity and antioxidant activity exerted by polysaccharides needs to be further investigated.

Ferrous iron can initiate lipid per oxidation as well as accelerating per oxidation [31]. Furthermore, chelating agents are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion [32]. Various polysaccharide fractions with different molecular weights exhibited remarkable binding capacity for Fe²⁺, further demonstrated they had stronger antioxidant capability.

4. Conclusions

Three polysaccharide fractions (TPS1, TPS2 and TPS3) with different weights were successively isolated by ultrafiltration method from crude polysaccharide extracted from abandoned lower grade tea leaves, which suggested that ultra filtration can be used to purified polysaccharide. Each fraction contained different contents of neutral sugar, uronic acid, protein, total polyphenols, and molecular weight. These differences provided basis for the antioxidant and free radical scavenging activity of these polysaccharide fractions. In general, TPS1 and CTPS had stronger antioxidant activity, TPS2 and TPS3 had lower antioxidant activity. CTPS and TPS1 did not exhibited prominent antioxidant and free radical scavenging activity although they contained much total polyphenols, indicating polysaccharide parts in them played key role in their activity. TPS1 had higher activity for DPPH and lipid per oxidation inhibition. But it had lower capacity for reducing power and metal chelating. This may be due to its higher content of hexuronic acid, lower content of total polyphenols, and larger molecular weight. TPS3 showed moderate antioxidant and free radical scavenging activity although it contained lower content of hexuronic acid and total polyphenols, suggesting the activity of it may be ascribed to the lower molecular weight. The order of inhibition activity of lipid per oxidation of various polysaccharide fractions was the same as DPPH radical scavenging activity. The order of metal chelating activity of various polysaccharide fractions was similar to hydroxyl radical scavenging activity, which again demonstrated that hydroxyl radical scavenging activity of polysaccharide relied heavily on the Fe²⁺ metal chelating to decrease the generation of hydroxyl radical. Further work is under way to confirm the ant oxidative effect of purified polysaccharide fractions and to characterize their structure–activity relationship, and their possible interactive antioxidant effects together with other antioxidants.

Acknowledgments

We are grateful to MS Fan Yang, Xuan Cai and Chunxi Liu for their assistance in the experiments. The authors are grateful for financial sponsored by Shanghai Rising-Star Tracking Program (11QH1401800), Innovation Program of Shanghai Municipal Education Creative Commission (11ZZ121, 12ZZ125), Produce–learn–Research Project of Shanghai Normal University (No. DCL201002, No. DCL201207), National High Technology Research and Development (863) Program of China (2008AA10Z232), National Natural Science Foundation of China (No.81072308), Shanghai Biomedicine Key Program (No.10391901700, No.083919111100), Shanghai Basic Research Key Program (No. 09JC1411500) and Shanghai Yangtze River Delta Science Joint Efforts Program (11495810500).

References