Extraction optimization, characterization and antioxidant activity in vitro of polysaccharides from mulberry (Morus alba L.) leaves

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A R T I C L E   I N F O

Article history:
Received 10 February 2015
Received in revised form 3 April 2015
Accepted 7 April 2015
Available online 21 April 2015

Keywords:
Mulberry leaf
Polysaccharide
Extraction
Characterization
Antioxidant

A B S T R A C T

Extraction optimization, characterization and antioxidant activity in vitro of polysaccharides from mulberry leaves (MLP) were investigated in the present study. The optimal extraction conditions with an extraction yield of 10.0 ± 0.5% for MLP were determined as follows: extraction temperature 92 °C, extraction time 3.5 h and ratio (v/w, mL/g) of extraction solvent (water) to raw material 34. Two purified fractions, MLP-3a and MLP-3b with molecular weights of 80.99 and 3.64 kDa, respectively, were obtained from crude MLP by chromatography of DEAE-Cellulose 52 and Sephadex G-100. Fourier transform-infrared spectroscopy revealed that crude MLP, MLP-3a and MLP-3b were acidic polysaccharides. Furthermore, crude MLP and MLP-3a had more complicated monosaccharide compositions, while MLP-3b had a relatively higher content of uronic acid. Crude MLP, MLP-3a and MLP-3b exhibited potent Fe 2+ chelating power and scavenging activities on 1,1-diphenyl-2-picrylhydrazyl, hydroxyl, superoxide and 2,2′-azinobis-(3-ethyl-benzothiazolin-6-sulfonic acid) radicals. The results suggested that MLP could be explored as natural antioxidant.

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1. Introduction

Mulberry (Morus alba L.), a multipurpose agro-forestry plant that belongs to the family of Moraceae, is widely distributed in tropical, subtropical and temperate areas (Agarwal & Kanwar, 2007). It is commonly used as a silkworm (Bombyx mori L.) diet, alternative medicine in China and Japan and a kind of tea due to its low toxicity and good therapeutic performance (Chung, Kim, Kim, & Kwon, 2013; Jia, Tang, & Wu, 1999; Nookabkaew, Rangkadilok, & Satayavivad, 2006; Wang, Fang, Ma, & Zhang, 2014; Zhong, Furne, & Levitt, 2006). It has been reported that mulberry contains a lot of bioactive compounds including polysaccharides, 1-deoxynojirimycin, moracin, chlorogenic acid, rutin, flavonol glycosides and anthocyanins, which are associated with its biological functions such as anti-obesity, anti-diabetes, anti-oxidation, anti-inflammation and anti-atherosclerosis (Harauma et al., 2007; Hunyadi, Martins, Hsieh, Seres, & Zupkò, 2012; Katsube, Tsurunaga, Sugiyama, Furuno, & Yamashita, 2009; Kimura, Nakagawa, Kubota, Kojima, & Goto, 2007; Li et al., 2011; Peng et al., 2011; Yang, Wang, Wang, & Zhang, 2012; Yatsunami, Ichida, & Onodera, 2008; Zhang et al., 2014a).

Mulberry leaves polysaccharides (MLP), the main active components of mulberry leaves, have been attracting increasing attention as other herb polysaccharides, due to their multiple biological activities such as anti-diabetic, anti-tumor, anti-inflammatory and immunostimulatory effects (Li, Chen, Wang, Tian, & Zhang, 2010; Li et al., 2011; Wang, Li, & Jiang, 2010b; Yan, Wang, & Wu, 2014; Yang, Zhao, Yang, & Ruan, 2008; Yang et al., 2012; Zhang et al., 2010, 2014a). Besides, it has been reported that many polysaccharides including MLP have potential antioxidant activities (Scarazzini & Speroni, 2000; Wang et al., 2013). However, the reports on the antioxidant activity of MLP are relatively insufficient (Samavati & Yarmarad, 2013; Wang et al., 2010b). It is well known that the biological functions including antioxidant activity of polysaccharides are intimately related to their structure features such as chemical components, molecular weight, monosaccharide composition and glycosidic linkage (You et al., 2013; Zeng, Zhang, & Jia, 2014). Accordingly, a comprehensive study of purification, characterization and antioxidant activities of MLP will provide useful information on the relationship between antioxidant activity and structure features. Furthermore, in order to study and

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http://dx.doi.org/10.1016/j.carbpol.2015.04.028
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explore MLP better, an efficient technique for the extraction of MLP is necessary. For polysaccharide extraction, hot water extraction technology is still the main and classic method due to its convenience, low cost and high extraction yield (Passos & Coimbra, 2013; Wei et al., 2010). However, the existing water extraction method for MLP needs long extraction time and several extraction cycles (Samavati & Varmand, 2013). Therefore, we report here the extraction, optimization, characterization and antioxidant activity in vitro of MLP. First, response surface methodology (RSM) based on a Box–Behnken design (BBD) was applied to optimize the extraction conditions, and the resulting extraction conditions were used to prepare crude MLP through water extraction and ethanol precipitation. The crude MLP was then purified by ion-exchange chromatography of DEAE-52 cellulose and size exclusion chromatography of Sephadex G-100, and the crude MLP and its purified fractions were further characterized via chemical analysis, high performance liquid chromatography (HPLC) and Fourier transform-infrared (FT-IR) spectroscopy. Finally, the antioxidant activities in vitro of crude MLP and its purified fractions were investigated. To the best of our knowledge, it is the first report on the antioxidant activities of the purified fractions of MLP.

2. Materials and methods

2.1. Materials and chemicals

The mulberry leaves were collected from the mulberry plantation in Bozhou (Anhui, China), washed with top water, air dried at room temperature and ground into fine powder. DEAE-52 cellulose, Sephadex G-100, mannose (Man), arabinoose (Ara), galactose (Gal), galacturonic acid (GaIA), glucose (Glc), glucuronic acid (GlcA), ribose (Rib), xylose (Xyl), 3-methyl-1-phenyl-2-pyrazolin-5-one (PMP), nitroblue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), ferrozone, [2,2’-azinobis-(3-ethylbenzothiazol-6-sulfonic acid)] diazonium salt (ABTS) and 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rhamnose (Rha) and fucose (Fuc) were purchased from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals used were of analytical grade.

2.2. Extraction of polysaccharides

In order to defat and remove most of the monosaccharides, oligosaccharides, pigments and other small molecules, the powder of mulberry leaves was treated with 85% ethanol twice at room temperature for 24 h. The resulting residue was dried and used for next extraction. The dried pretreated sample was extracted with designed extraction temperature, extraction time and ratio of extraction solvent (deionized water) to raw material. The resulting solution was filtered, mixed with a triple volume of absolute ethanol and kept overnight. The precipitates were collected by centrifugation at 4000 rpm for 15 min, washed with absolute ethanol and acetone and dried, affording the crude MLP. The extraction yield was calculated according to the following formula:

\[
\text{Extraction yield (\%)} = \frac{W_1}{W_0} \times 100
\]

where \(W_1\) and \(W_0\) are the weights of crude MLP and pretreated sample, respectively.

2.3. Experimental design of RSM

Effects of extraction parameters including extraction temperature, extraction time, extraction cycles and ratio of water to raw material on the yields of MLP were investigated by single-factor tests (data not shown). Accordingly, three major factors (extraction temperature, extraction time and ratio of water to raw material) were chosen and their proper ranges were determined based on the preliminary experimental results. Furthermore, a three-level, three-variable BBD was applied to determine the optimal levels of extraction variables including the extraction temperature (\(X_1\)), extraction time (\(X_2\)) and ratio of water to raw material (\(X_3\)) for the extraction of MLP. For statistical calculation, the variables were coded according to the following equation:

\[
X_i = \frac{X_i - X_0}{\Delta X_i}
\]

where \(X_i\) is the coded value of independent variable, \(X_0\) is the actual value of the independent variable, \(X_0\) is the actual value of the independent variable at the central point, and \(\Delta X_i\) is the step change of the variable. Table 1 shows the range of independent variables and their levels.

The whole design consisted of 17 experimental runs, including 12 factorial points and 5 axial points. The 5 axial points were used to allow for estimation of a pure error sum of squares. The experiments were carried out in random order, and the experimental data (Table 1) were fitted to the following second-order polynomial mode

\[
Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=1 (j \neq i)}^{3} \beta_{ij} X_i X_j
\]

where \(Y\), extraction yield of MLP, is the predicted response; \(\beta_0\), \(\beta_i\), \(\beta_{ii}\) and \(\beta_{ij}\) are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively; \(X_i\) and \(X_j\) are the independent variables (\(i \neq j\)).

2.4. Purification of crude MLP

Crude MLP was dissolved in deionized water and loaded onto a DEAE-52 cellulose column (2.6 × 50 cm). Then, the column was stepwise eluted with 0, 0.1, 0.3 and 0.5 M sodium chloride (NaCl) solution at a flow rate of 60 mL/h. Three completely separated fractions, MLP-1, MLP-2 and MLP-3, were collected by checking the absorbance at 490 nm by using the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). As the main fraction, MLP-3 was loaded onto a column (1.6 × 100 cm) of Sephadex G-100 and the column was eluted with 0.2 M NaCl solution at a flow rate of 15 mL/h. The elution was checked as described above. As a result, two purified fractions were collected, dialyzed and lyophilized, affording MLP-3a and MLP-3b, respectively.

2.5. Characterization of MLP

2.5.1. Determination of contents of carbohydrate, protein, uronic acid, sulfuric radical and total polyphenols

The contents of carbohydrate in crude MLP and its purified fractions were determined by the phenol-sulphuric acid method (Dubois et al., 1956) using glucose as the standard. The content of protein was determined according to the reported method (Bradford, 1976) using bovine serum albumin as the standard. The content of uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen (1973) using galacturonic acid as the standard. The content of sulfate radical was determined according to the reported method (Doigson & Price, 1962). The content of total polyphenols was estimated by the Folin–Ciocalteu colorimetric method (Li, Nie, Xie, & Li, 2014) using gallic acid (GA) as the standard, and it was expressed as mg GA equivalent (GAE) per 100 mg dry sample.
### Table 1
Box–Behnken design matrix and the response values for the yield of MLP.

<table>
<thead>
<tr>
<th></th>
<th>Temperature (°C)</th>
<th>Time (h)</th>
<th>Ratio of water to raw material (mL/g)</th>
<th>Polysaccharide yield (%)</th>
</tr>
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<tr>
<td><strong>Code X1</strong></td>
<td><strong>Code X2</strong></td>
<td><strong>Code X3</strong></td>
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<tr>
<td>1</td>
<td>95</td>
<td>1</td>
<td>30</td>
<td>8.5</td>
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<tr>
<td>2</td>
<td>90</td>
<td>0</td>
<td>20</td>
<td>8.2</td>
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<td>0</td>
<td>30</td>
<td>9.8</td>
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<td>5</td>
<td>95</td>
<td>1</td>
<td>40</td>
<td>9.3</td>
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<td>85</td>
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<td>6.8</td>
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<td>15</td>
<td>85</td>
<td>1</td>
<td>30</td>
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</table>

2.5.3. Analysis of monosaccharide composition

The monosaccharide composition of MLP was analyzed according to the reported method (Dai et al., 2010) with some modifications. The polysaccharide solution (100 μL, 5 mg/mL) was hydrolyzed with 100 μL 4 M trifluoroacetic acid (TFA) at 120 °C for 2 h. After hydrolysis, the excess TFA was removed by the addition of methanol and evaporated at reduced pressure. The hydrolysate was dissolved with deionized water (100 μL) and mixed with 100 μL 0.6 M NaOH. Then, 100 μL of the resulting mixture was labeled with PMP by adding 100 μL of 0.5 M methanol solution of PMP and incubating at 70 °C for 10 min. After being cooled to room temperature, the resultant solution was neutralized by adding 50 μL of 0.3 M HCl and evaporated to dryness. The residue was dissolved in 1.0 mL deionized water, and the excess PMP was leached with chloroform for three times. The aqueous layer was filtered through a 0.45 μm membrane and analyzed by an Agilent 1100 HPLC system equipped with photodiode array detector. The chromatographic conditions were as follows: column, Eclipse Plus C18 (4.6 × 250 mm, 5 μm, Agilent); column temperature, 30 °C; mobile phase, a mixture of phosphate buffered saline (PBS, 0.1 M, pH 6.7) and acetonitrile in a ratio of 83:17 (v/v); flow rate, 1.0 mL/min; detector wavelength, 245 nm. The injection volume was 20 μL. In similar manner, the monosaccharide standards were PMP-labeled and analyzed by HPLC.

2.5.4. FT-IR spectrometric analysis

The dried crude MLP and its purified fractions were mixed with spectroscopic-grade potassium bromide powder, ground and pressed into pellets for FT-IR measurement. FT-IR spectra were recorded with a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at the frequency range of 4000–400 cm⁻¹.

2.6. Assay of antioxidant activity in vitro of MLP

2.6.1. Assay of DPPH radical scavenging activity

The DPPH radical scavenging activity was measured by the method of previous report (Shimada, Fujikawa, Yahara, & Nakamura, 1992) with slight modifications. Briefly, MLP was dissolved in deionized water to afford a series of concentrations (0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/mL). Then, 50 μL of sample solution, 25 μL of DPPH-ethanol solution (0.4 mM) and 100 μL of deionized water were mixed in a 96-well plate. The mixture was kept at room temperature in the dark for 30 min, and the absorbance (Abs) at 517 nm was measured by a microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Ascorbic acid was used as positive control. DPPH radical scavenging activity was calculated by the following formula:

\[
\text{DPPH radical scavenging activity (} \% \text{)} = \left[ 1 - \frac{\text{Abs}0 - \text{Abs2}}{\text{Abs}0} \right] \times 100
\]

where Abs0 is the Abs of the control (deionized water instead of sample), Abs1 is the Abs of the sample, and Abs2 is the Abs of the sample under identical conditions as Abs1 with ethanol instead of DPPH-ethanol solution.

2.6.2. Assay of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of MLP was determined according to the reported method (Liu, Luo, Ye, & Zeng, 2012). Briefly, the reaction mixture contained 50 μL ferrosin (0.75 mM), 75 μL phosphate buffer (0.15 M, pH 7.4), 50 μL FeSO4 (0.75 mM), 50 μL MLP sample solution and 50 μL H2O2 (0.01%, w/v). After incubation at 37 °C for 30 min, the Abs at 536 nm was measured. Ascorbic acid was used as positive control. The scavenging activity was calculated using the following equation:

\[
\text{Hydroxyl radical scavenging activity (} \% \text{)} = \left[ 1 - \frac{(\text{Abs}0 - \text{Abs1})}{(\text{Abs}1 - \text{Abs2})} \right] \times 100
\]

where Abs0 is the Abs of the control (deionized water instead of sample), Abs1 is the Abs of the deionized water instead of H2O2 and sample, and Abs2 is the Abs of the sample.

2.6.3. Assay of superoxide radical scavenging activity

The scavenging ability on superoxide radical was measured by the previously described method (Bi et al., 2013; Robak &
with minor modifications. The reaction mixture contained 50 μL of sample solution, 50 μL of NBT solution (156 μM), 50 μL of NADH solution (156 μM) and 50 μL of PMS solution (60 μM). The mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was determined. Ascorbic acid was used as the positive control. The superoxide radical scavenging activity was calculated according to the following formula:

Superoxide radical scavenging activity (%) = \frac{1 - \frac{(Abs_1 - Abs_2)}{Abs_0}} \times 100

where Abs₀ is the Abs of the control (deionized water instead of sample), Abs₁ is the Abs of the sample, and Abs₂ is the Abs of the sample under identical conditions as Abs₁ with 0.1 M phosphate buffer instead of NBT solution.

2.6.4. Assay of ABTS radical scavenging activity

The ABTS radical scavenging activity of MLP was measured by ABTS radical cation decolorization assay (Re et al., 1999; Xie, Hu, Wang, & Zeng, 2014). Briefly, ABTS solution (7 mM) was oxidized with 4.95 mM of potassium persulphate for 12 h in the dark at room temperature. The ABTS⁺ solution was then diluted with PBS (0.2 M, pH 7.4) to an Abs of 0.70 ± 0.02 at 734 nm, and 200 μL of the resulting ABTS⁺ solution was mixed with 20 μL of sample solution. The mixture was kept for 6 min at room temperature, and the Abs at 734 nm was then measured. The ABTS radical scavenging effect was calculated by the following equation:

ABTS radical scavenging activity (%) = \frac{1 - \frac{(Abs_1 - Abs_2)}{Abs_0}} \times 100

where Abs₀ is the Abs of the control (deionized water instead of sample), Abs₁ is the Abs of the sample, and Abs₂ is the Abs of the sample only (PBS instead of ABTS⁺ solution).

2.6.5. Assay of Fe²⁺ chelating activity

The Fe²⁺ chelating ability of MLP was measured according to the reported method (Decker & Welch, 1990). Briefly, 50 μL of sample solution was mixed with 2.5 μL of ferrous chloride (FeCl₂) solution (5.0 mM), 10 μL of ferrozine solution (5 mM) and 137 μL of deionized water. The mixture was incubated for 10 min at room temperature, and then the absorbance at 562 nm was measured. EDTA was used as positive control. The Fe²⁺ chelating activity was calculated by the following equation:

Fe²⁺ chelating activity (%) = \frac{1 - \frac{(Abs_1 - Abs_2)}{Abs_0}} \times 100

where Abs₀ is the Abs of the control (deionized water instead of sample), Abs₁ is the Abs of the sample, and Abs₂ is the Abs of the sample only (deionized water instead of FeCl₂ solution).

2.7. Statistical analysis

The Design-Expert software version 8.0.6 was used for the experimental design and data analysis of RSM. The results of the antioxidant assay are reported as mean ± SD of three replicates. The data were statistically analyzed by one-way analysis of variance (ANOVA) procedure with SPSS software version 19.0 (Chicago, IL, USA), followed by the Duncan test. P-value of less than 0.05 was regarded as significant.

3. Results and discussion

3.1. Optimization of extraction parameters

3.1.1. Predicted model and statistical analysis

The experimental design along with the extraction yields is shown in Table 1, and the data were analyzed by Design-Expert software. As a result, a second-order polynomial equation describing the correlation between the MLP yield and the test variables was obtained by the following equation:

\[Y = -454.60135 + 10.05870X_1 + 4.80828X_2 - 0.17728X_3 - 0.02612X_1X_2 + 0.00683X_1X_3 + 0.01416X_2X_3 - 0.55583X_1^2 - 0.43506X_2^2 - 0.00718X_3^2\]

where Y represents the extraction yield of MLP, X₁, X₂ and X₃ represent extraction temperature (℃), extraction time (h) and ratio (v/w, mL/g) of water to raw material, respectively.

The ANOVA, lack-of-fit and the adequacy of the model are indicated in Table 2. The model F-value of 82.47 implied that the model was significant, indicating that there was only a 0.01% chance that the model F-value could occur due to noise. The determination coefficient (R²) and the adjusted determination coefficient (adj-R²) were 0.9907 and 0.9786, respectively, which showed a good agreement between the experimental and the predicted values of the MLP yield with goodness-of-fit of the regression equation.

The P-value is used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller the P-value is, the more significant the corresponding coefficient is. Table 2 shows that the linear coefficients (X₁, X₂ and X₃), quadratic term coefficients (X₁², X₂² and X₃²) and cross product coefficients (X₁X₂) were significant on extraction yield of MLP due to P-value <0.05. The results also suggested that, among the independent variables, the extraction temperature was the most significant parameter affecting the yield of MLP followed by ratio of water to raw material and extraction time.
temperature and longer extraction time can promote the dissolution of polysaccharides from plant tissues. However, too high extraction temperature and too long extraction time may result in degradation of polysaccharides and hence decrease the polysaccharides yield (Samavati & Yarmand, 2013). Fig. 1C and D shows the quadratic effects of $X_1$ and $X_3$ on the extraction yield when $X_2$ was fixed at level 0. The elliptical contour plot as shown in Fig. 1D indicated that the mutual interactions between the extraction temperature and ratio of water to raw material were significant. The extraction yield of MLP increased with the increases of extraction temperature and ratio of water to raw material from 85 to 93.27°C and 20 to 39.56 mL/g, but it did not further increase when extraction temperature and ratio of water to raw material were over 93.27°C and 39.56 mL/g. The result is in agreement with that
of previous reported (Chen, Li, Li, Jin, & Lu, 2015). Fig. 1E and F shows the effects of \( X_2 \) and \( X_3 \) and their reciprocal interaction on the extraction yield when \( X_1 \) was fixed at level 0. The maximum yield of MLP could be achieved when extraction time and ratio of water to raw material were in the range of 2.78–3.95 h and 29.27–38.36 mL/g, respectively. However, the extraction yield of MLP decreased when extraction time and ratio of water to raw material were over 3.95 h and 38.36 mL/g. This phenomenon could be explained that the polysaccharides will be completely dissolved in the extraction solvent and partial of polysaccharides may be hydrolyzed due to longer extraction time. The result is in agreement with that reported by Qiao et al. (2009).

3.1.3. Optimization of extracting parameters and validation of the model

By employing the Design-Expert software, the optimal conditions for MLP extraction were extraction temperature 91.52 °C, extracting time 3.53 h, and ratio (v/w, mL/g) of water to raw material 34.09. In the optimal conditions, the maximum predicted yield of MLP was 10.1%. For operation convenience, the optimal parameters were determined as follows: extraction temperature 92 °C, extracting time 3.5 h, and ratio (v/w, mL/g) of water to raw material 34. To ensure that the predicted result was not biased toward the practical value, experimental rechecking was performed using the deduced optimal conditions. A mean value of 10.0 ± 0.5% (n = 3) was obtained from actual experiments, which demonstrated the validation of the RSM model and indicating that the model was adequate for the extraction of MLP. The optimal extraction conditions with high yield were more efficient than that reported (Samavati & Yarmard, 2013).

3.2. Purification and characterization of MLP

Crude MLP was prepared using the optimal extraction conditions. Then, the crude MLP was separated by a DEAE-52 cellulose chromatography column, affording three independent elution peaks of MLP-1, MLP-2 and MLP-3 (Fig. 2A). The third fraction of MLP-3, the main fraction of crude MLP, was further purified with a Sephadex G-100 gel permeation chromatography column, resulting in two fractions of MLP-3a and MLP-3b (Fig. 2B).

3.2.2. Contents of carbohydrate, protein, uronic acid, sulfuric radical and total polyphenols in MLP

Table 3 shows the contents of carbohydrate, protein, uronic acid, sulfuric radical and total polyphenols in MLP. MLP-3a and MLP-3b. The carbohydrate contents in crude MLP, MLP-3a and MLP-3b were 52.09%, 89.74% and 37.20%, respectively. The contents of protein in crude MLP, MLP-3a and MLP-3b were 2.16%, 0.83% and 0.22%, respectively. Among all the polysaccharides tested, MLP-3b contained the highest contents of uronic acid. The contents of total polyphenols for MLP-3a and MLP-3b (0.17 and 0.16 mg GAE/100 mg, respectively) were much lower than that (1.93 mg GAE/100 mg) for crude MLP, indicating that most of the polyphenols in crude MLP was removed during the purification.

3.2.2. Molecular weight of MLP

The homogeneity and molecular weights of MLP-3a and MLP-3b were analyzed by HPLC. Both MLP-3a and MLP-3b gave a single and symmetrical peak, indicating that they were homogeneous.

Table 3

<table>
<thead>
<tr>
<th>Item</th>
<th>Crude MLP</th>
<th>MLP-3a</th>
<th>MLP-3b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate (%)</td>
<td>52.09 ± 1.10</td>
<td>89.74 ± 0.31</td>
<td>37.20 ± 0.59</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>2.16 ± 0.02</td>
<td>0.83 ± 0.03</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Uronic acid (%)</td>
<td>32.45 ± 0.92</td>
<td>6.53 ± 0.13</td>
<td>65.29 ± 1.53</td>
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<tr>
<td>Sulfuric radical (%)</td>
<td>1.73 ± 0.10</td>
<td>1.40 ± 0.02</td>
<td>1.22 ± 0.02</td>
</tr>
<tr>
<td>Total polyphenols (mg GAE/100 mg)</td>
<td>1.93 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
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<tr>
<td>Man</td>
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<td>4.53</td>
<td>1.27</td>
</tr>
<tr>
<td>GlcA</td>
<td>2.23</td>
<td>0.81</td>
<td>0.20</td>
</tr>
<tr>
<td>GalA</td>
<td>3.02</td>
<td>1.21</td>
<td>6.10</td>
</tr>
<tr>
<td>Glc</td>
<td>2.13</td>
<td>3.47</td>
<td></td>
</tr>
<tr>
<td>Gal</td>
<td>2.95</td>
<td>12.55</td>
<td></td>
</tr>
<tr>
<td>Ara</td>
<td>2.55</td>
<td>11.14</td>
<td>0.89</td>
</tr>
</tbody>
</table>

←: Not detected.
3.2.3. Monosaccharide composition of MLP

The results of monosaccharide compositions of crude MLP and its purified fractions (MLP-3a and MLP-3b) determined by HPLC are shown in Table 3. It was found that both crude MLP and MLP-3a were composed of Man, Rha, GlcA, GalA, Glc, Gal, and Ara, but the molar ration was quite different. The molar ratio of Man, Rha, GlcA, GalA, Glc, Gal, and Ara for crude MLP was 0.51: 5.13: 2.23: 3.02: 2.13: 2.95: 2.55, while it was 0.77: 4.53: 0.81: 1.21: 3.47: 12.55: 11.14 for MLP-3a. For MLP-3b, the molar ratio of Rha, GlcA, GalA, Glc, and Ara was 1.57: 0.20: 6.10: 1.27: 0.89, and Man and Glc were not detected. These results indicated that the crude MLP and its purified fractions were acidic polysaccharide. In addition, the monosaccharide composition and molar ratio of MLP were different from those of the earlier reports for mulberry leaf polysaccharides (Katayama et al., 2008; Xie et al., 2008). As reported, a pectic polysaccharide from mulberry leaves was composed of Rha, Ara, Xyl, Glc, Gal and GaLA in a molar ratio of 5: 4: 1: 2: 6: 38 (Xia et al., 2008). Katayama et al. (2008) separated and characterized a main polysaccharide from mulberry leaves, which was composed of Rha, Gal, Glc, GaLA and GlcA in a molar ratio of 1: 0.2: 0.5: 2.3: 1.5. The difference might be related to the extraction, purification and the raw material (mulberry leaf). It has been reported that different results for compositional components of tea polysaccharides were given in various reports due to different tea raw material or purification process (Nie & Xie, 2011).

3.2.4. FT-IR spectrum

The FT-IR spectra of crude MLP and its purified fractions are shown in Fig. 3. Broad and strong absorption bands around 3400 cm⁻¹ for C–H stretching vibrations and 2939 cm⁻¹ for C–H stretching vibrations were observed in the FT-IR spectra of crude MLP and its purified fractions. The absorption peaks at 1604.48 cm⁻¹ and 1421.3 cm⁻¹ for crude MLP, 1734.8 cm⁻¹ and 1641.1 cm⁻¹ for MLP-3a, 1738.6 cm⁻¹ and 1610.6 cm⁻¹ for MLP-3a were attributed to the stretching vibrations of ester carbonyl groups (C=O) and carboxylic groups (COO−) (Li et al., 2013; Santhiya, Subramanian, & Natarajan, 2002; Zhao, Yang, Yang, Jiang, & Zhang, 2007), respectively, which indicated that crude MLP, MLP-3a and MLP-3b were acidic polysaccharides. The results are consistent with the analytical results of monosaccharide compositions for crude MLP, MLP-3a and MLP-3b as mentioned above. The absorbance peaks at 1414.91 cm⁻¹ for MLP-3a and 1415.46 cm⁻¹ for MLP-3b were associated with the stretching of the pectin methyl ester group (−OCH₃), which suggested that some of the uronic acids in polysaccharides were esterified (Al-Sheraji et al., 2012). A characteristic peak at around 894 cm⁻¹ was found in crude MLP, MLP-3a and MLP-3b, indicating the existence of β-glycosidic bonds in the three polysaccharides (Coimbra, Gonçalves, Barros, & Delgadillo, 2002; Yang et al., 2006).

3.3. Antioxidant activity in vitro of MLP

3.3.1. DPPH radical scavenging activity

DPPH free radical is a stable free radical with a characteristic absorption at 517 nm, which will decrease significantly on exposure to proton-donating substance (Yamaguchi, Takamura, Matoba, & Terao, 1998). Accordingly, it has been widely used to evaluate the antioxidant activity of natural antioxidants. In the present study, the scavenging ability of MLP on DPPH free radicals was examined and the results are shown in Fig. 4A. The scavenging rates of MLP and Vc increased with the increase of sample concentration. At a concentration of 4.0 mg/mL, the DPPH free radical scavenging activities for crude MLP, MLP-3a, MLP-3b and Vc were 68.21%, 44.96%, 60.17% and 91.16%, respectively. Compared with pure fractions, the crude MLP exhibited relative higher (P < 0.05) DPPH radical scavenging activity, which might be partly attributed to its relative higher content of polyphenols (Table 3). The scavenging abilities of MLP-3a and MLP-3b might mainly come from the polysaccharides due to their relatively low contents of total polyphenols. It has been reported that the antioxidant activity of polysaccharides was related to their molecular weight, uronic acid content, degree of sulfation, type of monosaccharide and glycosidic linkage (Liu et al., 2010; Melo, Feitosab, Freitas, & de Paula, 2002; Sun, Wang, Li, & Liu, 2014; Wang, Chang, Stephen Inbaraj, & Chen, 2010a; Zeng et al., 2014). MLP-3b displayed higher antioxidant activity than MLP-3a, which might be due to the lower molecular weight and higher content of uronic acid of MLP-3b (Asker, Mahmoud, & Ibrahim, 2007; Chen, Zhang, Qu, & Xie, 2008). But the exact mechanism is unclear.

3.3.2. Hydroxyl radical scavenging activity

Hydroxyl radical, which is well known as one of the most reactive free radicals, can react with almost the biomacromolecules in living cells and induce severe damage to the adjacent biomolecules (Spencer et al., 1994). Therefore, the hydroxyl radical scavenging activities of MLP and its purified fractions were investigated. As shown in Fig. 4B, all MLP samples and Vc exhibited scavenging activity on hydroxyl radicals in a dose-dependent manner. At a concentration of 4.0 mg/mL, the scavenging abilities of crude MLP, MLP-3a, MLP-3b and Vc were 88.85%, 57.89%, 68.12% and 92.44%, respectively. Notably, crude MLP showed similar (P > 0.05) scavenging activity as Vc. The results indicated that the crude MLP exhibited strong scavenging activity and its purified fractions had moderate scavenging activities. It has been reported that the hydroxyl scavenging ability is related to the number of hydroxyl or amino groups in polysaccharide (Guo et al., 2005). This might be explained by the factor that crude MLP would provide more active hydroxyl groups than its purified fractions as crude MLP contained much higher content of polyphenols.

3.3.3. Superoxide radical scavenging activity

Superoxide radical is a long lifetime radical that is generated by numerous biological and photochemical reactions (Banerjee,
Dasgupta, & De, 2005; Dahl & Richardson, 1978). Although superoxide radical is a relatively weak oxidant, it can further interact with other molecules to generate more strong and reactive oxidative species such as singlet oxygen and hydroxyl radicals, and cause oxidative tissue damage and various diseases. Therefore, the superoxide radical scavenging activity of MLP was measured by the PMS/NADH-NBT system in the present study. The results demonstrated that MLP exhibited dose-dependent superoxide radical scavenging capacity at concentrations ranging from 0.0625 to 2.0 mg/mL (Fig. 4C). At the concentration of 2.0 mg/mL, the
superoxide radical scavenging activities for crude MLP, MLP-3a, MLP-3b and Vc were 84.47%, 71.91%, 75.25% and 99.53%, respectively. Notably, all the MLP samples had strong superoxide radical scavenging activity. The possible mechanism of polysaccharide for scavenging superoxide anion is reported to be associated with the dissociation energy of O–H bond, that is, the more electron withdrawing groups such as carboxylic groups and aldehyde groups attached to polysaccharide, the weaker the dissociation energy of O–H bond (Lin, Wang, Chang, Inbaraj, & Chen, 2009; Jin et al., 2012). The present result showed that MLP-3b displayed higher scavenging activity than MLP-3a, which might be partly due to that of MLP-3b with higher content of carboxylic groups (Sun, Liu, & Kennedy, 2010), which is in agreement with the result of uronic acid content. The crude MLP with highest scavenging ability might be partly due to its reduction activity, which might be related to the higher polyphenols content (Wang et al., 2010a,b).

3.3.4. ABTS radical scavenging activity

The ABTS radical cation has often been used in the evaluation of total antioxidant activity of single compounds and complex mixtures of various origins (body fluids, foods, beverages, plant extracts). In the present study, the scavenging ability of MLP on ABTS free radical was determined and the results are shown in Fig. 4D. The scavenging activities of all the samples increased in a concentration-dependent manner, and all the samples exhibited strong radical scavenging activities at higher doses. At the dose of 4.0 mg/mL, the scavenging abilities of crude MLP, MLP-3a, MLP-3b and Vc were 99.33%, 79.81%, 90.47% and 100.00%, respectively. Therefore, MLP had an appreciable scavenging activity on ABTS free radical. It has been reported that the antioxidant activity obtained by ABTS assay was positively correlated to that obtained by DPPH radical assay (Floegel, Kim, Chung, Koo, & Chun, 2011; Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Byrne, 2006). In our present study, however, the scavenging ability of MLP by ABTS assay was higher than that by DPPH assay. The reason might be due to that ABTS assay is more suitable for evaluating hydrophilic antioxidant while DPPH assay is more applicable for evaluating hydrophobic antioxidant (Floegel et al., 2011).

3.3.5. Fe2+ chelating activity

Iron is essential in the human body for respiration and the activity of a large range of enzymes, oxygen transport and redox reactions (Hsu, Coupar, & Ng, 2006). However, iron is an extremely reactive metal, which can accelerate the reaction of oxidation of important biological molecules (Liu, Wang, Xu, & Wang, 2007). Therefore, the ferrous ion chelating activity of MLP was investigated and the results are shown in Fig. 4E. The Fe2+ chelating activity of MLP was correlated well with increase of concentration. At a concentration of 4.0 mg/mL, the Fe2+ chelating activities of crude MLP, MLP-3a, MLP-3b and Vc were 92.86, 51.33, 87.76 and 99.91%, respectively, indicating that crude MLP and MLP-3b had potent Fe2+ chelating activity. It has been reported that compounds with metal ion chelating activity often own functional groups such as –OH, –SH, –COOH, –PO4H2, –CO, –NR2, –S– and –O– (Jiang et al., 2014; Liu et al., 2010). The strong Fe2+ chelating activity of crude MLP and MLP-3b might be partially due to the high contents of –COOH and C=O groups in their structures (Table 3). However, the exact mechanisms should be investigated further.

4. Conclusion

In the present study, the optimal extraction parameters for MLP with a extraction yield of 10.0 ± 0.5% were determined as follows: extraction temperature of 92 °C, extraction time of 3.5h, ratio of water to raw material of 34 mL/g and extraction cycles of two times. Two fractions of polysaccharides with the average molecular weights of 80.99 and 3.64 kDa (MLP-3a and MLP-3b, respectively) were obtained from crude MLP by chromatography of DEAE-Cellulose 52 and Sephadex G-100. The crude MLP and MLP-3a were composed of Man, Rha, GluA, GalA, Glu, Gal, and Ara in the molar ratio of 0.51: 5.13: 2.23: 3.02: 2.13: 2.95: 2.55 and 0.77: 4.53: 0.81: 1.21: 3.47: 12.55: 11.14, respectively, while MLP-3b was composed of Rha, GluA, GalA, Gal and Ara in a molar ratio of 1.57: 0.20: 6.10: 1.27: 0.89. The results along with the FT-IR spectra demonstrated that the crude MLP, MLP-3a and MLP-3b were acidic polysaccharides. Furthermore, the crude MLP and its purified fractions exhibited potent antioxidant activity in vitro, specifically superoxide scavenging activity, ABTS radical scavenging activity and Fe2+ chelating power. The results suggested that the mulberry leaves can be utilized much better by using the optimal extraction parameters obtained in the present study and MLP could be explored as a natural antioxidant for use in medicine or functional foods. Further studies on the mechanism of antioxidant activity, precise chemical structures of the purified fractions and other biological functions of MLP are in progress.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No.31201454), the Fundamental Research Funds for the Central Universities (KYZZ201218) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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