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Fractionation of enzymatic hydrolysis lignin by sequential extraction for enhancing antioxidant performance

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ABSTRACT

The heterogeneity of lignin chemical structure and molecular weight results in the lignin inhomogeneous properties which also covers the antioxidant performance. In order to evaluate the effects of lignin heterogeneity on its antioxidant activity, four lignin fractions from enzymatic hydrolysis lignin were classified by sequential organic solvent extraction and further evaluated by DPPH (1,1-Diphenyl-2-Picrylhydrazyl) free radical scavenging capacity and reducing power analysis. The characterization including FTIR, ^{1}H NMR and GPC showed that the fractionation process could effectively separate lignin fractions with distinctly different molecular weight and weaken the heterogeneity of unfractionated lignin. The antioxidant performance comparison of lignin fractions indicated that the dichloromethane fraction (F1) with lowest molecular weight (4585 g/mol) and highest total phenolics content (246.13 mg GAE/g) exhibited the highest antioxidant activity whose value was close to commercial antioxidant BHT (butylated hydroxytoluene). Moreover, the relationship between the antioxidant activity and the structure of lignin was further discussed to elucidate the mechanism of antioxidant activity improvement of lignin fractionation. Consequently, this study suggested that the sequential extraction was an effective way to obtain relatively homogeneous enzymatic hydrolysis lignin fractions which showed the potential for the value-added antioxidant application.

1. Introduction

Antioxidants that prevent the objects from adverse effects of oxygen by capturing and neutralizing free radicals, are widely applied to food, medicine and chemical industry. Especially, antioxidants are important food additives, which are mainly used to prevent or delay the oxidation of oils and fats, and also avoid nutrition of food damaging, browning and fading [1]. Compared with synthetic antioxidants, such as butyl hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), which occupy a major share of the current antioxidant market, natural antioxidants because of their pure nature, non-toxicity, high security and strong antioxidant capacity, are more and more favored in the present life [2].

Lignin, as one of the three major components of lignocellulosic biomass, is the most abundant aromatic polymer in nature. Three C_6-C_3 phenyl propane units with phenolic hydroxyl groups, namely syringyl alcohol (S), guaiacyl alcohol (G) and *p*-hydroxyphenol alcohol (H), form a randomized three-dimensional network structure of lignin polymers. As a natural and abundant compound with polyphenol structure, lignin shows promising antioxidant activity and many studies have focused on the exploration of lignin antioxidant properties for the objective to use lignin as natural antioxidants [3–5]. After investigating the effects of lignin from typical sources on the irritation of eye and skin, Vinardell et al. found lignin was safe to eye and skin and suggested the lignin applications in cosmetic formulations [6]. Ugartondo et al. studied the cytotoxic effects of lignin and found that the lignin showed a high antioxidant capacity over arrange of concentrations that were not harmful to normal human cells, which further confirmed the suitability of lignin as commercial antioxidants, mainly in cosmetics and pharmaceuticals [7]. In addition to cosmetics and pharmaceuticals fields, lignin also shows promising applications in composite

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materials, such as biodegradable packing materials [8], polyolefin materials [9] and functional films [10].

However, the heterogeneity of lignin including the polydispersity of molecular weight and the diversity of functional group content, results in the inhomogeneous structures and properties of lignin which also covers the antioxidant activities. Pan et al. explored the relationships between lignin structure and antioxidant activity, and found that ethanol lignin from hybrid poplar with more phenolic hydroxyl groups and narrow polydispersity showed high antioxidant activity [11]. Dizhbite et al. found that high molecular weight with enhanced heterogeneity and polydispersity was a crucial factor that decreased the radical scavenging activity of lignin [5]. Consequently, previous studies have clearly indicated that the antioxidant activity of lignin depends highly on its molecular weight and chemical structure. Due to the structural and molecular weight heterogeneity, there are lignin fractions with high and low antioxidant activities in one lignin sample, which decreases the whole antioxidant activity of lignin sample. Therefore, using classification technology is an important way to separated lignin fractions with different antioxidant activities and obtain the fraction with higher antioxidant properties.

With the emergence of biomass refining, cellulosic ethanol has been regarded as promising bio-fuel due to its economic, environmental and social sustainability. However, during the process of ethanol production, more and more enzymatic hydrolysis residue (EHR) which mainly contains lignin is discharged. Compared with other lignin samples, the antioxidant activity of lignin from EHR was little studied. And the exploration of antioxidant activities of lignin from EHR would facilitate the value-added utilization of lignin as natural antioxidants and offer a significant opportunity for the commercial operation of a cellulosic ethanol based biorefinery.

In this study, lignin extracted from enzymatic hydrolysis residue was fractionated by sequential extraction with different organic solvents. Then all lignin samples were characterized by FTIR, GPC and ^{1}H NMR. Meanwhile, the chemical analysis including carbohydrate content and total phenolics content was also conducted. The DPPH free radical scavenging assay and reducing power assay were carried out to evaluate the antioxidant activities of all lignin samples. In addition, the relationship between the antioxidant activity and the structure of lignin was further discussed. The objective of this study is not only to investigate the effect of lignin heterogeneity on its antioxidant performance but also to obtain lignin with high antioxidant activity as efficient antioxidants from EHR.

2. Materials and methods

2.1. Materials

EHR of corn straw produced after steam explosion pretreatment and enzymatic hydrolysis, was kindly provided by Songyuan Laihe chemical Co. Ltd, Jilin Province, China. EHR was stored at room temperature for further usage. The chemical composition of EHR was Klason lignin 55.97 ± 0.07%, acid-soluble lignin 3.18 ± 0.49%, glucosan 21.00 ± 4.85%, xylan 9.03 ± 1.70%, arabinan 1.24 ± 0.20%, protein 4.54 ± 0.14% (Kjeldah method), and ash 7.33 ± 0.32%. All chemical reagents were analytical grade.

2.2. Purification and fractionation of enzymatic hydrolysis lignin (EHL)

EHL was purified using a modified method derived from dioxane extraction process [12] and fractionated according to the scheme shown in Fig. 1. EHR was treated with 1,4-dioxane/water solution (3:1, v/v) through a solid to liquor ratio of 1:30 (g/mL), assisted with ultrasound at room temperature for 2 h. After extraction, the supernatant was collected by centrifugation and then concentrated with rotary evaporator to obtain EHL. The precipitate and 1,4-dioxane aqueous solution recovered were mixed for a second extraction. The EHL from both processes was collected and freeze-dried.

The obtained EHL was further fractionated by sequential extraction with dichloromethane, acetic ether and *n*-butyl alcohol, with a solid to liquor ratio of 1:40 (g/mL) by ultrasound at room temperature for 2 h (shown in Fig. 1). After centrifugation and rotary evaporation, the dichloromethane fraction (F1), acetic ether fraction (F2), *n*-butyl alcohol fraction (F3) and residue fraction (F4) were prepared. All of the fractions were freeze-dried and stored at room temperature before further analysis.

2.3. Lignin characterization

2.3.1. Component determination

The carbohydrate and lignin content of EHR, EHL and all fractions were determined according to the NREL standard protocol [13]. The samples were hydrolyzed by primary 72% and secondary 4% H_2SO_4. Next, the acid-insoluble lignin was quantified by difference method and the acid-soluble lignin was determined with the aid of the UV–vis (UV-2550, Shimadzu). Monosaccharides were analyzed by drawing support from High Performance Liquid Chromatography (HPLC, Agilent Technology).

2.3.2. Total phenolics content determination

Folin-Ciocalteu (F-C) was employed to determine the total phenol content of lignin [14]. The total phenolics content of sample was expressed as the gallic acid equivalent (GAE) [15]. The calibration curve of gallic acid in DMSO (with six different concentrations from 4 μg/mL–20 μg/mL) was drawn in advance. Specific steps are as follows: 0.2 mL sample (2 mg/mL in DMSO) and 0.5 mL Folin-Phenol were mixed for 5 min before addition of 1 mL 15% Na_2CO_3. Then the obtained mixture was diluted by bringing the volume to 10 mL with distilled water. The solution was incubated for 1 h in the dark before measuring the absorbance at 765 nm (UV–vis) against the blank (0.2 mL DMSO instead of sample).

2.3.3. Spectroscopic assay

Gel permeation chromatography (GPC) was used to determinate the molecular weight of lignin samples. To avoid the effect of acetylation on the molecular weight distribution, a modified method applying a hydrophile gel column (TSK G3000PWxl column) was employed [16]. Lignin solutions were diluted in tris-acetate buffer (20 mmol/L, pH 7.4) and 20 μL samples were injected. The column was operated at 25 °C and eluted with tris-acetate buffer with a flow rate of 0.5 mL/min. The FTIR spectra of the lignin fractions were obtained on FTIR spectrophotometer (FTIR-650, Gangdong Sci. & Tech. Co., Ltd, China) using potassium bromide technique in the range of 4000–400 cm^{-1}. The one-dimensional (1D) ^{1}H NMR spectra was measured using a Bruker 500 MHz spectrometer at a frequency of 250 MHz with an acquisition time of 0.011 s at 25 °C. The lignin samples for determination were dissolved in DMSO-d_6 [17].

2.4. Determination of antioxidant performance

2.4.1. DPPH free radical scavenging assay

The DPPH free radical scavenging assay of lignin fractions was evaluated on the basis of the methods represented by previous literature with modifications [18]. 6 mL lignin samples in methanol with five different concentrations from 0.01 mg/mL to 1 mg/mL were mixed with 6 mL 0.15 mmol/L DPPH-methanol solution at room temperature for 30 min in the dark. The absorbance of the mixture was measured at 517 nm with UV–vis. Commercial antioxidant BHT was used as positive control. Each test was carried out in triplicates.