Process Biochemistry 51 (2016) 444–451

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Enzymolysis-based ultrasound extraction and antioxidant activities of polyprenol lipids from *Ginkgo biloba* leaves

Chang-Wei Zhang[a,b,c,d], Cheng-Zhang Wang[a,b,c,d,e,*], Ran Tao[a,b,c,d], Chuanjie Fang[a,b,c,d]

[a] Institute of Chemical Industry of Forest Products, CAF, Nanjing 210042, Jiangsu, China
[b] National Engineering Laboratory for Biomass Chemical Utilization, Nanjing 210042, Jiangsu, China
[c] Key and Open Laboratory on Forest Chemical Engineering, SFA, Nanjing 210042, Jiangsu, China
[d] Key Laboratory of Biomass Energy and Material, Nanjing 210042, Jiangsu, China
[e] Institute of New Technology of Forestry, CAF, Beijing 100091, China

ARTICLE INFO

Article history:
Received 7 September 2015
Received in revised form 2 December 2015
Accepted 13 December 2015
Available online 17 December 2015

Keywords:
Ginkgo biloba leaves
Polyprenols
Lipids
Enzymolysis
Ultrasound
Antioxidant activity

ABSTRACT

Polyprenols are one of the most important lipid components in *Ginkgo biloba* leaves (GBLs). In this study, an enzymolysis-based ultrasound extraction procedure of GBL polyprenol (GBP) lipids was established, and the antioxidant activities of polyprenols and six kinds of polar fractions separated from GBL lipids via an optimal extraction procedure were studied. The results showed that the optimum conditions were an enzyme quantity of 0.5 g (the mass ratio of cellulase and pectinase was 1:2, and the enzyme activity was 60 U/mg), enzymolysis pH of 4.5, and temperature of ultrasound of 45 °C. Under these conditions, the yield of polyprenols was 0.80% ± 0.22%, which is 69.70% higher than that obtained by direct petroleum ether extraction. In addition, concentration c (content of polyprenols was 91.53%), fraction 3 (content of polyprenols was 43.28%), polyprenols (content of polyprenols >99%), and fraction 3 showed the strongest scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)), superoxide anion, and hydroxyl radicals with the corresponding IC_{50} values of 236.9, 76.84, 80.23, and 113.9 μg/mL. It is inferred that GBL lipids with different concentrations of polyprenols could play different roles in the research and development of cosmetics and functional food.

1. Introduction

Ginkgo biloba is the only living species in the division Ginkgophyta, having survived for >180 million years. It is a traditional Chinese medicinal herb; according to many famous Chinese herbal medicine treatises, such as Shen Nong Ben Cao Jing (2800 BC) and Pen Ts'ao Kang Mu (1596) [1], it is commonly used in medicine, health-care food, and cosmetics [2,3]. *Ginkgo biloba* leaves (GBLs) consist of many bioactive components including flavonoids, terpene lactones, sterols, polyprenols, alkylphenols, and carboxylic acids [4]. Thus, *Ginkgo* flavonoids and terpene lactones have already been widely studied in the clinical treatment and prevention of cardiovascular as well as cerebrovascular disease; however, a limited number of studies have reported on lipids from GBL.

GBL lipids are oily non-saponifiable fractions separated from lipophilic GBL extracts. It is reported that GBL lipids contain volatile oil, long-chain alkanes, carotenoids, polyprenols, sterols, and compounds with oxygen-containing functional groups. GBL polyprenols (GBPs) are new effective lipids with similar structure and biological activities such as antiviral activity, immune enhancement, and cancer cell metastasis inhibition, for example, dolichols (DH) in humans [5,6].

Traditionally, organic solvent extraction has been used as the main extraction method of plant lipids, but its extraction efficiency is low. In order to increase the dissolution ratio of plant lipids, several cell wall disruption technologies including ultrasound, microwave, bead beating, and enzymolysis have been developed in recent years. These methods are particularly important, as the yield of the extracted lipids is determined by the effect of cell wall disruption. For example, microwave instruments generate high-frequency waves that disrupt cells and have been proven to be

Abbreviation: GBL, *Ginkgo biloba* leaves; BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate); GBP, polyprenols from *Ginkgo biloba* leaves; DH, dolichols; BBD, Box–Behnken design.

* Corresponding author at: Institute of Chemical Industry of Forest Products, CAF, Nanjing 210042, China. Fax: +86 025 8548 2471.
E-mail addresses: 13770752951@163.com (C.-W. Zhang), wangczlhs@sina.com (C.-Z. Wang), trmoon1949@126.com (R. Tao), 1249943205@qq.com (C. Fang).

http://dx.doi.org/10.1016/j.procbio.2015.12.008
1359-5113/

useful for essential oil extraction [7,8], whereas ultrasonic cavitation leads to disruption of the microbial cell wall and membrane, causing more ingredients to dissolve out [9,10]. Enzymolysis can effectively catalyze the degradation of cell walls, thus favoring the release of the inner cell components [11–13]. Bead beating involves high-speed spinning with fine beads, which causes direct mechanical damage to cells and has been used on both a laboratory and an industrial scale [14]. However, it is reported that the exclusive use of these cell wall disruption methods is less effective than in combination [15,16].

Enzymolysis-based ultrasound extraction is a relatively new technology [17], and its mechanism is as follows: Bubbles generated via cavitation collapse due to crushing, instantaneously producing enormous mechanical shearing forces. Moreover, this method is considered to be advantageous due to mild extraction conditions, lower investment cost and energy requirement, and simplified manipulation [18]. It is reported that this technique has high extraction efficiency and can enhance the antioxidant activities of the corresponding extract obtained from the plant [19–21]. However, studies on enzymolysis-based ultrasound extraction of GBL lipids have rarely been reported.

In this study, first, the enzyme with a high efficiency of cell wall disruption was screened from 10 different enzymes for GBL enzymolysis. Then, the Plackett–Burman and Box–Behnken design (BBD) tests were designed to determine the optimal conditions for obtaining a high yield of polyprenols. Scale-up experiments were conducted at the optimal conditions, and six different kinds of polar fractions were separated by freezing and silica gel separation from GBL lipids; then, the antioxidant activities of these lipids factions were evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate), superoxide anion, and hydroxyl radical scavenging assays. The aim of this work was to obtain an effective extraction method for GBPs with higher yield and lipid fractions with strong antioxidant activity.

2. Materials and methods

2.1. Materials

The dried GBLs were purchased from Pizhou Xinyuan Biological Products Co., Ltd. (Jiangsu, China). The standard polyprenols (C_{70}, C_{75}–C_{105}, C_{110}, C_{115}, and C_{120}) were purchased from Larodan Fine Chemicals Co., Ltd. (Malmö, Sweden). ABTS, DPPH, pyrogallic acid, butylated hydroxytoluene (BHT), ascorbic acid (Vc), and seven tested enzymes (cellulase, pectinase, hemicellulase, β-glucosidase, amylase, glucoamylase, and neutral protease) were purchased from Aladdin Chemicals (Shanghai, China). A hydroxyl radical determination kit was purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

2.2. Selection of enzyme

Cellulase, pectinase, hemicellulase, β-glucosidase, amylase, glucoamylase, and neutral protease have different enzyme activities. Therefore, the same enzyme activity (75,000 U) was used to destroy the cell wall of GBL to identify the most effective enzyme. Pulverized GBL powder (5 g) was taken in 500-mL conical flasks, and 10 enzymes with the same enzyme activity were also added. The solid-to-liquid ratio was set at 1:20, and the conical flasks were placed in an ultrasonic cleaner for 30 min. They were then placed in a water-bathing constant-temperature vibrator for 3 h, and enzymolysis was performed at the optimum temperature and pH of each enzyme. The filtrate and residue were obtained by filtration. The filter residue was dried at 50 °C. The dried residue was then loaded into a Soxhlet extractor, with petroleum ether as the extractant, and the extraction process was carried out for 6 h. A GBL ointment was obtained by concentration. NaOH–EtOH (5%) was put into the GBL ointment, and saponification was performed at 70 °C for 3 h. The resulting product was cooled and stewed to obtain GBL lipids. Considering the yield of the GBL ointment and the GBL lipids as well as the content of polyprenols as the indices, the enzyme with the highest cell wall disruption efficiency was selected. The yield of GBL ointment and GBL lipids and the content of polyprenols were calculated with the following equations:

$$\text{The yield of GBL ointment} = \frac{\text{the quality of GBL ointment}}{\text{the quality of GBL}} \quad (1)$$

$$\text{The yield of GBL lipid} = \frac{\text{the quality of GBL lipids}}{\text{the quality of GBL}} \quad (2)$$

$$\text{Content of polyprenols (\%)} = (\%) = \frac{[(Y - B) \times \rho \times \eta \times 100]}{(A \times V \times W)} \quad (3)$$

where Y represents the total area of five main polyprenol peaks, V the sample size (μL), W the quantitative concentration of sample (mg/mL), ρ the quantitative concentration of the standard substance, η the purity of the standard substance, A the slope of standard curve regression equation, and B the intercept of standard curve regression equation.

2.3. Plackett–Burman test design

The following factors with a significant impact on the content of polyprenols were considered for investigation and screening: time and temperature of ultrasound; solid-to-liquid ratio; enzyme quantity; time, temperature, and pH of enzymolysis; and mass ratio of cellulase and pectinase.

2.4. Box–Behnken design

The BBD is a novel analytic method for the optimization of processes. It presents the relationship between factors and responses involved during the optimization of analytic systems. In this work, BBD was used to predict the levels of temperature of ultrasound (X_2), enzyme quantity (X_4), and enzymolysis pH (X_8); in addition, the low and high levels of X_2, X_4, and X_8 were set as (40 °C, 50 °C), (0.25 g, 0.75 g), and (4, 5), respectively. The experimental design consisted of a set of points lying at the midpoint of each edge and the replicated center points of the multidimensional cube. The Design-Expert (version 7.1.3) statistical software (Stat-Ease, Minneapolis, MN, USA) was used to analyze the experimental data.

2.5. Collection of different GBL polar lipid fractions

GBL (5 kg) was used in the scale-up experiment, and extraction, concentration, as well as saponification were conducted according to the optimal process conditions of the enzymolysis-based ultrasound extraction of GBL lipids. Concentration A (the purity of polyprenols was 33.52%) was obtained from this process. A certain quantity of concentration A was taken in a 500-mL conical flask, and a certain volume of acetone was also added. Then, the conical flask was placed in the refrigerator (below 0 °C) for 3 h, and this step was repeated thrice. Afterward, the supernatant was collected by filtration, and concentration B (the purity of polyprenols was 38.61%) was obtained by the concentration of the supernatant. A certain quantity of concentration B was taken for silica gel purification, and different proportions of ethyl acetate and petroleum ether were used as the eluant system; the flow rate was set at 1.0 mL/min, and 10 mL of the eluant was collected in a tube. Using thin-layer chromatography (TLC), similar polar fractions were identified and mixed together; the concentrations of ethyl acetate, petroleum ether, and absolute ethyl alcohol were in the ratio 12:87:1. At the