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RESEARCH ARTICLE

Extraction and biodegradation of ginkgolic acids from *Ginkgo biloba* sarcotestae

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Abstract Ginkgolic acids are unwanted constituents in standard *Ginkgo biloba* leaves extracts. Thus, for the quality control of ginkgo extracts, it is important to establish an effective degradation method, with high catalytic efficiency and safety, to remove ginkgolic acids. Laccases are oxidases with potential for application in elimination of hazardous phenolic compounds. In this study, single-factor and orthogonal experiments were used to optimize extraction of ginkgolic acid from *G. biloba* sarcotestae. The results showed that ethanol was the best solvent, with the highest extraction rate for ginkgolic acid at 85% ethanol. On this basis, we measured ethanol volume fraction, extraction time, temperature and solid-liquid ratio using an orthogonal experiment. By using absorbance of 310 nm as standard, the optimal extraction conditions were 85% ethanol with, solid-liquid ratio of 1:14 at 40°C for 12 h. These conditions gave a ginkgolic acid yield of 73.1 $mg \cdot g^{-1}$. Subsequently, recombinant laccase was used to degrade the ginkgolic acid in several laccase/mediator systems, of which LacC was the best. At 50°C, pH 4.5, enzyme concentration of 0.01 $U \cdot mL^{-1}$, 0.5 $mmol \cdot L^{-1}$ mediator ABTS and reaction time of 3 h, the degradation rate of ginkgolic acid reached 100%. These results lay the foundation for research on and application of biological enzymes for detoxification of *G. biloba* extracts.

Keywords biodegradation, extraction, ginkgolic acid, laccase, orthogonal method

1 Introduction

Ginkgo biloba L., ginkgo, originally known as gongsun tree in China, is a relict plant that coexisted with dinosaurs and is thus recognized as a living fossil. Ginkgo has been used as an edible, medicinal and ornamental plant[1–5]. The main active compounds in ginkgo are ginkgolide and ginkgo flavonoids, as well as ginkgolic acid, which is considered a mutagen and carcinogen, causing skin, mucous membrane and other allergic inflammatory responses[6,7]. Therefore, one quality standard limits the concentration of ginkgolic acid in *G. biloba* extracts to 5 $\mu g \cdot g^{-1}$[8]. Ginkgolic acid is abundant in *G. biloba* sarcotestae, leaves and fruits, with the highest levels reported from 5% to 13%[9]. The majority of *G. biloba* sarcotestae are discarded, thereby polluting the environment and resulting in resource wastage. Ginkgolic acid can be extracted from ginkgo sarcotestae and used as a biological pesticide. Selective degradation and removal of ginkgolic acid would improve the safety of *G. biloba* extracts and ginkgo food. Common ginkgolic acid removal methods include fermentation, traditional Chinese medicine compatibility attenuation, high temperature pyrolysis and supercritical CO_2 detoxification[10–12]. However, these methods not only reduce the yield of other active substances, they also increase production costs. As an alternative, laccase is an enzyme that can efficiently degrade phenolic compounds. It has the potential advantages of high specificity, high catalytic efficiency, mild reaction conditions and low pollution, and has been extensively used in organic pollutant biodegradation, biological bleaching, industrial wastewater purification and soil remediation[13–15].

In the present study, we investigated the effects of organic solvents, temperature, time and solid-liquid ratios on extraction of ginkgolic acid from *G. biloba* sarcotestae using single-factor and orthogonal experiments, and determined the optimal extraction conditions. Moreover, we studied the effects of a series of recombinant laccase isoenzymes on ginkgolic acid degradation, thereby laying the foundation for research on and application of biological enzymes for ginkgo extract detoxification.

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2 Materials and methods

2.1 Strains and medium

A series of laccase isozymes (GS115-LacA, GS115-LacB, GS115-LacC, GS115-Lcc1 and GS115-Lcc2 from *Trametes versicolor*) was prepared and preserved by the Microbial Technology Research Laboratory of Nanjing Forestry University[16–18]. YPD and BMGY media were prepared as described in *Pichia* expression manual (Invitrogen, San Diego, CA, USA).

2.2 Reagents and equipment

Two oven-dried (60°C) *G. biloba* sarcotestae collected from Pizhou, Jiangsu Province and ginkgolic acid standards were used in this study. In the standard purchased from China Pharmaceutical and Biological Products Institute, the main components of total ginkgolic acid A were ginkgolic acid C15:0 and C17:1, the main components of total ginkgolic acid B were ginkgolic acid C13: 0, C15:1, and C17:2, the ratio of ginkgolic acid A and B, and 1:2, with the concentration of standard compounds of ginkgo phenolic acids was 100 mg·L^{-1}. Methanol, ethanol, glacial acetic acid, n-hexane, cyclohexane and petroleum ether were of the analytical grade, and the water used was degassed and redistilled. ABTS [2,2'-Azide-bis (3-ethylbenzothiazole-6-sulfonic acid)] was purchased from Sigma (St. Louis, MO, USA), and HOBT (1-hydroxybenzotriazole), VA (violet acid), guaiacol and vanillin were pure and locally-produced. Citric acid and trisodium citrate were locally-produced, analytical grade.

Equipment used included a U-1800 spectrophotometer (Hitachi, Tokyo, Japan), plant shredder FZ102 type (Tianjin Tai Site Instrument Co., Ltd., Tianjin, China), digital constant temperature water bath HH-4 (Guohua Electric Co., Ltd., Changzhou, China), IKA RV 05 basic rotary evaporator (IKA-Werke GmbH & Co. KG, Staufen, Germany), electric thermostatic blast oven DHG-9203A (Shanghai Jinghong Experimental Equipment Co., Ltd., Shanghai, China) and HPLC Agilent 1260 (Agilent, Santa Clara, CA, USA).

2.3 Experimental methods

2.3.1 Extraction of ginkgolic acids

G. biloba sarcotestae were cleaned and de-cored, oven-dried at < 60°C and crushed with a pulverizer. *G. biloba* sarcotesta powder was accurately weighed to 10 g and extracted with an organic solvent at specific solid-liquid ratios and times, and filtered. The filtrate was evaporated using a rotary evaporator to remove the organic solvent, water was added to a final volume of 100 mL, and then extracted with 100 mL of n-hexane. The water layer was discarded, the n-hexane layer was evaporated to dryness using a rotary evaporator, methanol was added to dissolve the powder to a final ginkgo phenolic extract volume of 3 mL.

$$\text{Yield of ginkgol phenolic acid (mg·g}^{-1}) = (C \times V)/M \times 100\%$$

where C is the content of the total ginkgo phenolic acids calculated by the standard curve, V is the volume of methanol, M is the quality of the powder of *G. biloba* sarcotesta.

The standard curve was $y = 4713.0x + 0.0475$, $R^2 = 1.000$, where, y is peak area of the total ginkgo phenolic acids, x is content of the total ginkgo phenolic acids.

2.3.2 Chromatographic conditions

The content of ginkgolic acid was determined by HPLC-UV (Agilent 1260). The chromatographic conditions were Eclipse XDB-C18 column (4.6 nm × 250 nm, 5 μm), mobile phase of methanol and 3% glacial acetic acid at 92:8, column temperature 40°C, detection wavelength of 310 nm, flow rate of 1.5 mL·min^{-1}, injection volume of 20 μL and run time of 10 min.

2.3.3 Effects of different factors on the extraction of ginkgolic acids

Using the extraction method described above, we studied the effects of different solvents, solvent concentrations, extraction times, temperatures and solid-liquid ratios on the extraction of ginkgolic acid from *G. biloba* sarcotestae in single-factor experiments. Each experiment was repeated three times. We analyzed the resulting crude extract by HPLC, investigated the effects of various factors on the extraction of ginkgolic acid, and selected three optimal levels of each factor for orthogonal optimization experiments.

To determine the optimal conditions for the extraction of ginkgolic acid from *G. biloba* sarcotestae in the orthogonal experiment, we selected four factors: ethanol volume fraction (80%, 85% and 90%), extraction time (10, 12 and 14 h), temperature (30, 40 and 50°C), solid-liquid ratio (1:10, 1:12 and 1:14) for combination testing based on single-factor experiments, and used an L9 (3^4) orthogonal table.

2.3.4 Determination of enzyme activity of recombinant laccase

A 3-mL reaction system contained 1 mmol·L^{-1} substrate ABTS and 100 mmol·L^{-1} citrate-trisodium citrate buffers (pH 3). We immediately added the appropriate amount of enzyme solution after incubation at 50°C for 5 min, measured the absorbance at 1, 2 and 3 min at 420 nm, and

defined the amount of enzyme required to oxidize 1 μmol of ABTS per minute as one enzyme unit (U)[19].

2.3.5 Optimization of degradation conditions of ginkgolic acids by recombinant laccase

The total volume of degradation reaction system was 5 mL, which consisted of recombinant laccase, citric acid-trisodium citrate buffer and ginkgolic acid (final concentration 200 mg·L^{-1}). We studied the effects of different laccases (GS115-LacA, GS115-LacB, GS115-LacC, GS115-Lcc1 and GS115-Lcc2), small-molecule mediators (ABTS, HOBT, Guaiacol, VA), enzyme concentrations (0.004, 0.008, 0.01, 0.05, 0.1, 0.2, 0.3 and 0.5 U·mL^{-1}), pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0), temperatures (30, 35, 40, 45, 50, 55 and 60°C) and reaction times (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 h) on ginkgolic acid degradation by laccase.

3 Results and analysis

3.1 Effects of different factors on the extraction of ginkgolic acids

We used five kinds of organic solvents, namely, ethanol, methanol, n-hexane, cyclohexane, and petroleum ether as extraction solvents. For the extraction for 12 h at 40°C with a 1:10 solid-liquid ratio (m/v), extract yield reached 66.0 mg·g^{-1}. Figure 1a shows that the extract yield using ethanol was significantly higher than with the other four extraction solvents. Ginkgolic acid yield after extraction using n-hexane, cyclohexane or petroleum ether were not significantly different. Therefore, ethanol was selected as the extraction solvent for ginkgolic acid from *G. biloba* sarcotestae. Moreover, ethanol concentration had a significant effect on the extraction of ginkgolic acid from *G. biloba* sarcotestae. With increasing ethanol concentration, ginkgolic acid extract yield initially increased and then decreased. Figure 1b shows that when the volume fraction of ethanol was 90%, the average extract yield was 68.1 mg·g^{-1}. When the ethanol volume fraction was 85%, extract yield was not significantly different from 90% ethanol, and the extract yields of the two were higher than for the four other ethanol volume fractions. Based on these findings, 85% ethanol was selected as optimal solvent concentration for extracting ginkgolic acid from *G. biloba* sarcotestae.

Temperature also affected the extraction of ginkgolic acid. Four different temperature settings (30, 40, 50 and 60°C) were used in extraction (Fig. 2a), and the highest ginkgolic acid yield was observed at 40°C, reaching 66.3 mg·g^{-1}. Ginkgolic acid yield decreased when the extraction temperature was > 40°C. At certain temperatures, higher extraction rate was achieved because high temperature increased molecular motion and accelerated the dissolution of ginkgolic acid, but when the temperature was over 40°C, the yield declined because of the decomposition of ginkgolic acid. Therefore, 40°C was selected as the optimal extraction temperature.

Ginkgolic acid yield remained the same with the prolongation of extraction time. Different extraction times (4, 6, 8, 10, 12 and 14 h) were evaluated in this study. When the extraction time was short, no significant differences in extract yield were observed; however, the yield after a 12-h extraction time was 65.6 mg·g^{-1}, and did not significantly increase with 14 h of extraction (Fig. 2b). Considering economic factors, 12 h was selected as the optimal extraction time for ginkgolic acid from *G. biloba* sarcotestae.

A range of solid-liquid ratios (1:4, 1:6, 1:8, 1:10, 1:12, and 1:14) for the extraction of ginkgolic acid were

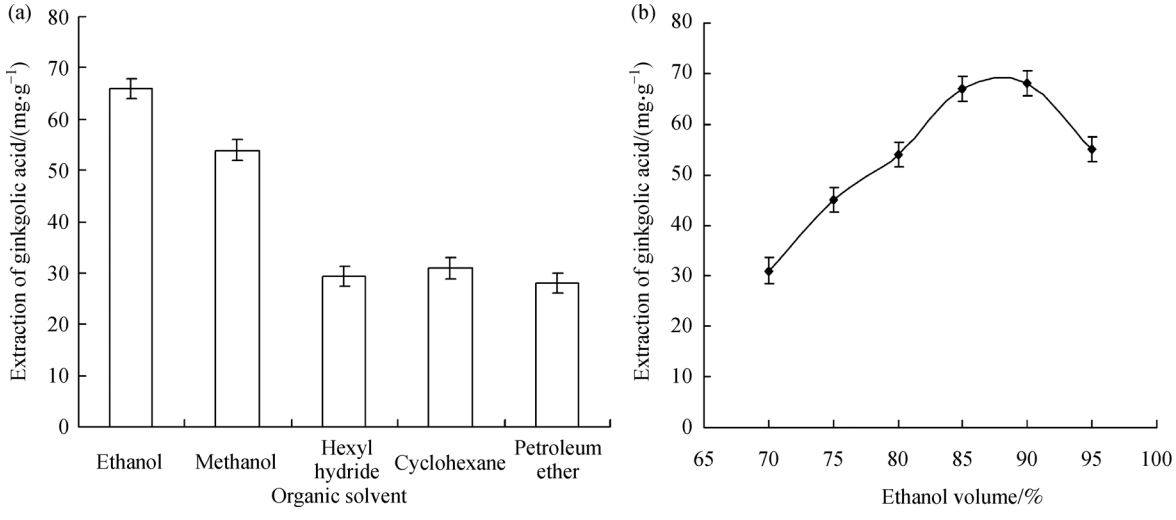

Fig. 1 Effect of different organic solvents (a) and ethanol volume (b) fractions on extraction of ginkgolic acid from *Ginkgo biloba* sarcotestae

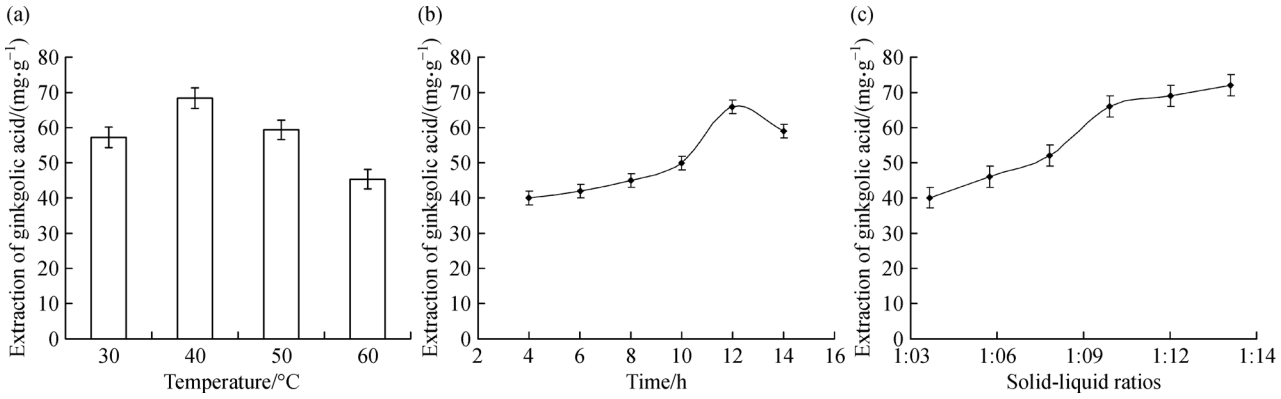

Fig. 2 Effect of different extraction temperatures (a), time (b), and solid-liquid ratios (c) on extraction of ginkgolic acid from *Ginkgo biloba* sarcotestae

examined in this study (Fig. 2c). Extraction using a lower amount of solvent generated a low yield. When the solid-liquid ratio was relatively low, the organic solvents volume is not good for dispersion of solid powder in the solution, inhibiting the contact between the ginkgolic acid molecules and the extracting solution. Ginkgolic acid yield significantly increased with higher volumes of the solvent. When the solid-liquid ratio reached 1:10, ginkgolic acid yield reached 67.3 mg·g^{-1}, and yield did not increase at higher ratios. When the solid-liquid ratio was 1:14, extract yield was the highest (72.5 mg·g^{-1}). Also considering on extraction efficiency and cost, a solid-liquid ratio of 1:14 was determined to be optimal for extracting ginkgolic acid from *G. biloba* sarcotestae.

3.2 Orthogonal experiment

An orthogonal experiment with four factors at three levels of ethanol volume fraction, extraction time, extraction temperature, and solid-liquid ratio, and the horizontal range was conducted based on results of the single-factor experiments (Table 1). Comparison of the extreme value (R) indicated the order, A > D > B > C, where the greater the extreme value, the greater the impact of the factor on ginkgo acid yield. The higher the K value, the more appropriate the factor is. Comparison of K values indicated that the combination A2B2C3D2 was optimal for extraction, which consisted of the following conditions: ethanol volume fraction, 85%; extraction time, 12 h; solid-liquid ratio, 1:14; and extraction temperature, 40°C. Using these settings, the extract yield for ginkgolic acid from *G. biloba* sarcotestae was 73.1 mg·g^{-1}.

3.3 Analysis of ginkgo acid components

HPLC analysis of the crude extract (Fig. 3a) of ginkgolic acid generated using optimal extraction conditions was performed and compared to the total ginkgolic acid standard (Fig. 3b). The five peaks in the crude extract reached the baseline separation; peak shape was suitable,

Table 1 Orthogonal experiment on extraction of ginkgolic acids

No.	Ethanol/%	Time/h	Solid-liquid ratio /(g·mL^{-1})	Temperature/°C	Ginkgolic acid/(mg·g^{-1})
1	1 (80)	1 (10)	1 (1:10)	1 (30)	41.325
2	1 (80)	2 (12)	2 (1:12)	2 (40)	48.357
3	1 (80)	3 (14)	3 (1:14)	3 (50)	40.635
4	2 (85)	1 (10)	2 (1:12)	3 (50)	56.265
5	2 (85)	2 (12)	3 (1:14)	1 (30)	68.649
6	2 (85)	3 (14)	1 (1:10)	2 (40)	71.112
7	3 (90)	1 (10)	3 (1:14)	2 (40)	55.728
8	3 (90)	2 (12)	1 (1:10)	3 (50)	48.909
9	3 (90)	3 (14)	2 (1:12)	1 (30)	48.192
K_1	144.970	170.353	179.273	175.740	
K_2	217.807	184.350	169.793	194.663	
K_3	169.810	177.710	183.347	162.010	
R	73.010	13.997	13.554	32.650	

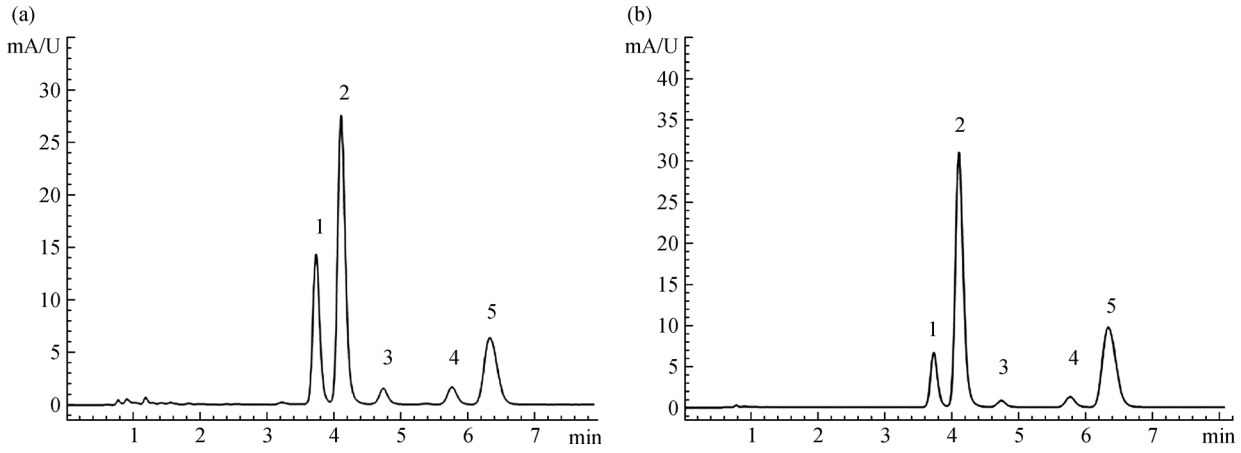

Fig. 3 HPLC analysis of ginkgolic acid extracts; crude extract from *Ginkgo biloba* sarcotestae (a) and a total ginkgolic acid standard (b). The hydrocarbon side chains 1 to 5 are C13:0, C15:1, C17:2, C15:0 and C17:1, respectively.

and analysis time adequate, thereby meeting the requirements for HPLC analysis. The peaks also matched the five target peaks of the standard.

3.4 Degradation of ginkgolic acid by recombinant laccase

Isozymes are enzymes that differ in structure and specificity for various substrates. In this investigation, we studied the specificity of five laccase isozymes cloned from *T. versicolor* on ginkgo acid (Fig. 4a). Using the conditions, enzyme concentration 0.5 $U \cdot mL^{-1}$, pH 4.5, 50°C, and reaction time of 12 h, the degradation rates of the five laccases on ginkgolic acid ranged from 4.1% to 27.0%, with LacC showing the highest degradation efficiency. However, degradation of ginkgolic acid using laccase alone was not very effective; therefore, we added small molecular mediators to participate in laccase degradation of ginkgolic acid (Fig. 4b). After introducing 0.5 $mmol \cdot L^{-1}$ of ABTS, guaiacol, HOBT and VA, the degradation rates of laccase on ginkgolic acid ranged from 57.5% to 100%, which were significantly higher than in the absence of a mediator. The degradation enhancement was in the order, ABTS > Guaiacol > VA > HOBT.

The combination of LacC-ABTS was selected for the degradation of ginkgolic acid, and the enzyme concentration, temperature, pH and degradation time were optimized (Fig. 5). The degradation rate of ginkgolic acid reached 100% after the addition of 0.01 $U \cdot mL^{-1}$ of laccase LacC. Degradation also improved within the pH range 4.0 to 6.0 and temperature range of 45 to 60°C, with the optimal pH and temperature being 4.5 and 50°C, respectively. After the addition of ABTS, the degradation time of LacC was significantly reduced from the original 12 h (27% degradation) to 3 h (100% degradation rate). The observed reduction in this time was thus associated with a significant enhancement in degradation. The common mediator, ABTS is usually used to degrade xenobiotics and can significantly enhanced degradation[14,20]. The structure-

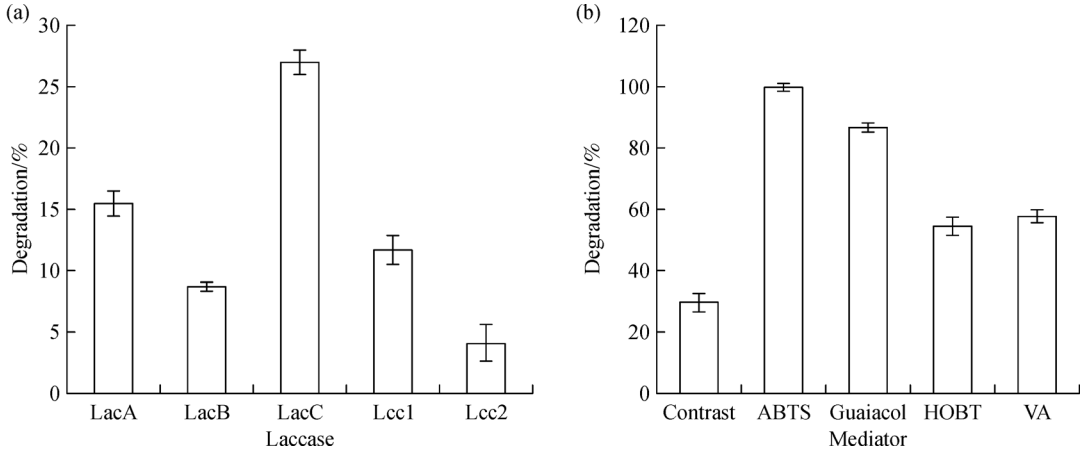

Fig. 4 Effect of different laccase isoenzymes (a) and small-molecule mediators (b) on degradation of ginkgolic acid in extracts from *Ginkgo biloba* sarcotestae

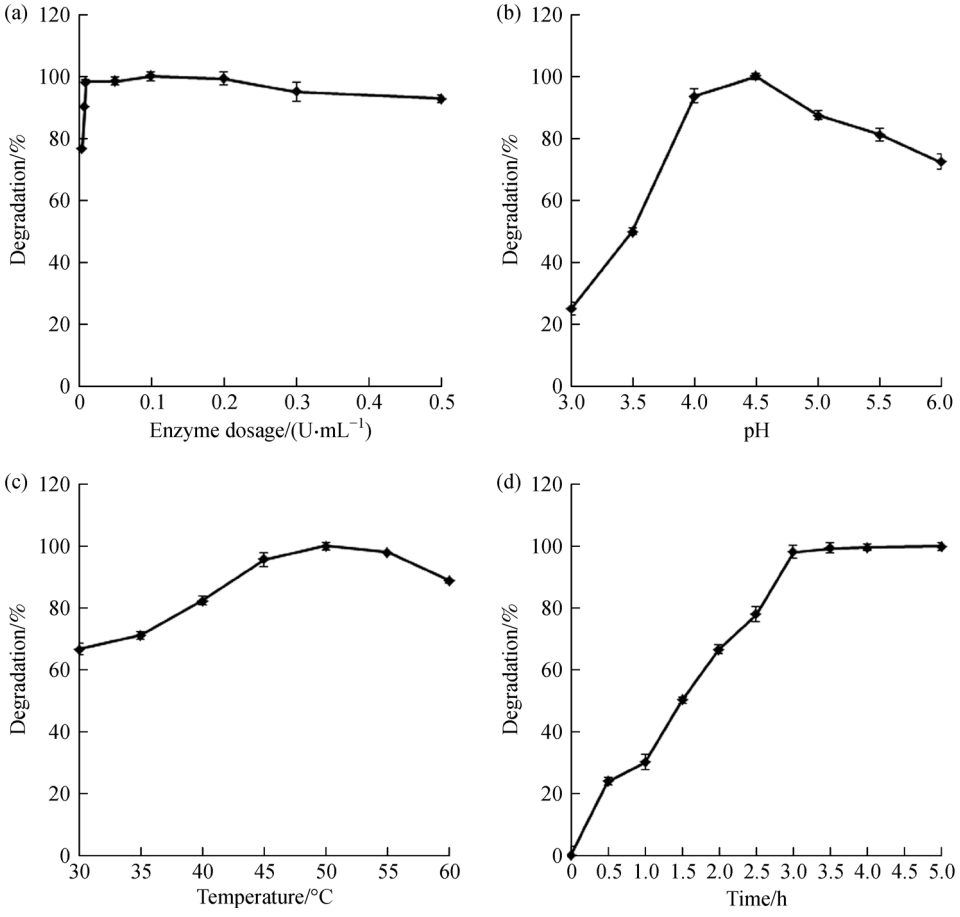

Fig. 5 Optimization of degradation conditions of ginkgolic acid in extracts from *Ginkgo biloba* sarcotestae. (a) Enzyme concentration; (b) pH; (c) temperature; (d) time.

effect relationships of mediators in the degradation of ginkgolic acid requires further study.

4 Discussion

Sarcotestae of *G. biloba* are the fleshy seed coats that are often discarded during harvest. Each year, at least 30000 to 40000 t of fresh sarcotesta resources are thrown away, which in turn becomes an environmental pollutant. Recent studies have shown that ginkgolic acid may be utilized as plant-derived pesticides, and the sarcotestae have been identified as the plant part that has the highest ginkgolic acid concentration. These discoveries were then followed by investigations on how to maximize the extraction and use of ginkgolic acid[21–23]. Established extraction methods for ginkgolic acid include boiling, chromatography, impregnation extraction, press extraction, reflux extraction and ultrasonic extraction. Of these, the impregnation extraction method gives the highest yield[24–26]. The present study examined the effects of different organic solvents, solvent concentrations, extraction times, extraction temperatures, and solid-liquid ratios using a single-factor and orthogonal experiments. Based on the findings of the present study, the optimal extraction conditions are: ethanol volume fraction of 85%, solid-liquid ratio of 1:14 and extraction temperature of 40°C. After 12 h of extraction, the final extraction yield of ginkgolic acid was 73.1 mg·g^{-1}. Our method provides a technical basis for large-scale preparation of ginkgolic acid, and raw materials for the biodegradation of ginkgolic acid. HPLC was performed to detect ginkgolic acid in the *G. biloba* sarcotesta extract, and at least five kinds of ginkgolic acids were identified, with hydrocarbon side chains of C13:0, C15:1, C17:2, C15:0 and C17:1. These findings were in agreement with the results of previous studies on ginkgolic acid in extracts from *G. biloba* sarcotestae and leaves[27].

Ginkgolic acid is a lacquer-type substance that has sensitizing and cytotoxic effects[28,29], and is thus included in ginkgo preparations at limited concentrations. The international standard for ginkgolic acid content in *G. biloba* extract is that it should not exceed 5 ppm. China provides more than 70% of the world's ginkgo resources, and the phenolic acid content *G. biloba* products largely exceed that standard, thereby potentially harming human health. Research investigations have focused on

establishing methods to effectively removing ginkgolic acid from *G. biloba* extracts. Current ginkgolic acid removal methods include fermentation, resin adsorption, traditional Chinese medicine compatibility attenuation, high temperature volatilization and supercritical CO_2 detoxification. Resin adsorption has also been extensively studied; however, this method reduces not only the content of ginkgolic acid, but also that of terpene lactones and ginkgolides[30].

Laccase is a copper-containing polyphenol oxidase that mainly catalyzes oxidation-reduction reactions. It can also catalyze the oxidation of various phenolic compounds. The addition of some small molecular mediator substances can mediate the oxidation between laccase and substrate, thereby further expanding the range of activity of laccase, including the oxidation of non-phenolic organic compounds, as well as significantly improving the degradation efficiency of phenolic compounds. Laccase/redox media have various potential applications, and thus have gained increased attention in China and internationally. Compared to chemical methods, biodegradation with laccase has the advantages of strong selectivity, high catalytic efficiency, mild reaction conditions and no pollution. It has extensive prospects for application in the food, pulp and paper, and biological detection industries. However, laccase has various isozymes, which have different specificities and catalytic efficiencies. The present study is the first to explore and compare the degradation methods of ginkgolic acid using laccase isozymes, which determined that LacC has the highest degradation efficiency, and the addition of a small-molecule mediator further improves the degradation rate, with ABTS providing the most significant enhancement. We also established a suitable process for the degradation of ginkgolic acid using laccase LacC-ABTS, pH 4.5, 50°C, 5 mmol·L^{-1} of ABTS and 0.01 U·mL^{-1} of LacC incubated for 3 h. This process gave complete degradation of ginkgolic acid at 200 mg·L^{-1}. After degradation, no peak appeared in the HPLC chromatogram, and the pathway of degradation of ginkgo phenolic acids leads possibly to complete degradation to water and carbon dioxide. Further study is required to identify the chemical structure of byproducts and prove the complete degradation of ginkgo phenolic acids using laccases. The above results show that our method can safely and efficiently use laccase in the removal of ginkgolic acid, thereby serving as a novel method for the detoxification of ginkgo products.

The present study has established a better process for the degradation of ginkgolic acid using laccases. However, further research is needed to determine (1) the mechanism of laccase-mediated ginkgolic acid degradation, (2) whether the laccase-mediator process negatively impacts flavonoids, lactones, and other active compounds in the ginkgo extracts, and (3) if the costs of using enzymes to degrade ginkgolic acid can be sufficiently reduced to make this option economically viable. The immobilization of laccase, and its directed transformation and efficient heterologous expression may be potential ways to achieve these goals.

5 Conclusions

In this study, we optimized a method for extracting ginkgolic acid, as well as established a safe and efficient degradation method for ginkgolic acid using laccase isozymes. The findings lay a technical foundation for developing novel applications for *G. biloba* and for improving the quality of *G. biloba* products. Therefore, we make the following specific conclusions:

(1) We investigated the effects of different kinds of organic solvents, solvent concentration, extraction time, temperature, and solid-liquid ratios on target extraction yield using single-factor and orthogonal experiments. Based on the findings, the optimal extraction conditions were an ethanol volume fraction of 85%, extraction time of 12 h, solid-liquid ratio of 1:14 and extraction temperature of 40°C.

(2) HPLC analysis detected ginkgolic acid in the extract of *G. biloba* sarcotestae. At least five kinds of ginkgolic acids were present in *G. biloba* sarcotestae, and the hydrocarbon side chains were C13:0, C15:1, C17:2, C15:0 and C17:1, which is in agreement with the findings of previous studies on ginkgolic acid in *G. biloba* sarcotestae and leaves.

(3) We compared the effects of five laccase isoenzymes on the degradation of ginkgolic acid. LacC showed the best degradation efficiency, and using a small-molecule mediator improved the degradation rate, with ABTS providing the most significant enhancement. We established a suitable process for the degradation of ginkgolic acid using laccase LacC-ABTS; pH 4.5, 50°C, 5 mmol·L^{-1} of ABTS and 0.01 U·mL^{-1} of LacC incubated for 3 h. This process gave complete degradation of ginkgolic acid at 200 mg·L^{-1}.

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Compliance with ethics guidelines Qi Li, Wei Sun, Yan Jiang, Fuliang Cao, Guibin Wang, and Linguo Zhao declare they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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