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Enzyme-assisted extraction of flavonoids from *Ginkgo biloba* leaves: Improvement effect of flavonol transglycosylation catalyzed by *Penicillium decumbens* cellulase

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ABSTRACT

We report a novel enzyme-involved approach to improve the extraction of flavonoids from *Ginkgo biloba*, in which the enzyme is employed not only for cell wall degradation, but also for increasing the solubility of target compounds in the ethanol–water extractant. *Penicillium decumbens* cellulase, a commercial cell wall-degrading enzyme with high transglycosylation activity, was found to offer far better performance in the extraction than *Trichoderma reesei* cellulase and *Aspergillus niger* pectinase under the presence of maltose as the glycosyl donor. TLC, HPLC and MS analysis indicated that *P. decumbens* cellulase could transglycosylate flavonol aglycones into more polar glucosides, the higher solubility of which led to improved extraction. The influence of glycosyl donor, pH, solvent and temperature on the enzymatic transglycosylation was investigated. For three predominant flavonoids in *G. biloba*, the transglycosylation showed similar optimal conditions, which were therefore used for the enzyme-assisted extraction. The extraction yield turned to be 28.3 mg/g of dw, 31% higher than that under the pre-optimized conditions, and 102% higher than that under the conditions without enzymes. The utilization of enzymatic bifunctionality described here, naming enzymatic modification of target compounds and facilitation of cell wall degradation, provides a novel approach for the extraction of natural compounds from plants.
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1. Introduction

Enzyme-assisted extraction of natural functional compounds from plants is widely investigated in recent years for its advantages in easy operation, high efficiency, and environment friendship [1]. Most of the works in this field utilize cellulase, pectinase and β-glucosidase to hydrolyze and degrade plant cell wall constituents to improve the release of intracellular contents [2–4]. However, another important factor in the extraction – the intrinsic property especially the solubility of the target compound – has seldom been concerned according to our knowledge. Low solubility of target compounds in the extractant leads to low extraction yield and require large amount of solvents, which largely impedes the economic efficiency in industry. Therefore, here we propose a novel enzyme-assisted extraction approach, in which the extraction is improved not only from enhanced cell wall degradation, but also from the increased solubility of target compounds in the extractant. The extraction of plant flavonoids, which generally have poor solubility in mild solvents such as ethanol–water solution, is selected as an example to validate our proposal.

Flavonoids are natural compounds showing high physiological activities in therapies for inflammations, heart diseases and cancers [5,6]. There are three predominant flavonol aglycones, quercetin, kaempferol, and isorhamnetin (Fig. 1), in *Ginkgo biloba*, a Chinese medicinal plant well known for its high content of flavonoids [7]. Extract from *G. biloba* is among the most popular phytomedicines and herbal dietary supplements [8], whose primary active components are flavonoids (24%) and small amount of terpene lactones (6%) [9].

Various methods have been used to assist the extraction of flavonoids from plants, such as ultrasonication [10], supercritical fluids [11], microwave [12], membrane adsorption [13] and molecular imprinting [14]. There are also several reports on the enzyme-assisted extraction of plant favonoids. Pectinase and protease were employed for the extraction of anthocyanins from black currant juice press residues [15]. Luteolin and apigenin were enzymatically extracted from pigeonpea leaves with pectinase, cellulase and β-glucosidase [16]. In another study, extraction of antimicrobial and antioxidant phenolics, mainly anthocyanins, from berries was studied, and the enzyme was found to effectively hydrolyze

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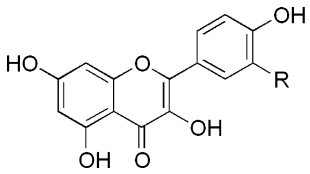

R	Compound
OH	Quercetin
H	Kaempferol
OCH_3	Isorhamnetin

Fig. 1. Chemical structures of three predominant flavonol aglycones in *G. biloba*.

glycosides to their corresponding aglycones [17]. Although the enzyme-assisted approach has largely improved the extraction rate, due to the intrinsic low polarity of flavonol aglycones, the extraction after enzymatic treatment still has to be performed with solvents like hexane, acetone and butanone in order to achieve high productivity. Nevertheless, regarding toxicological limitations, there is a clear trend in the industry to substitute these organic solvents for alternative nontoxic solvents, particularly in products for human consumption, with the first option being water or alcohols [18]. Therefore, large amount of high concentration ethanol–water solution is often used. However, this unfavorably leads to a high production cost, making it desirable to increase the solubility of flavonoids without affecting their physiological activities.

We come up with an idea to utilize the transglycosylation activity of glycosidases to introduce hydrophilic groups, e.g. glycosides, into flavonoids to improve their polarity in the extraction process, while utilizing the enzyme's activity in cell wall hydrolysis at the same time. Although commercial cellulases are often selected and modified to minimize their transglycosylation activity for more complete degradation of cellulose chains in biomass transformation [19], many cellulase systems in microorganisms show both hydrolysis and the reverse transglycosylation activities [20]. Enzymatic transglycosylation by various glycosidases, including glucosidases [20–22], galactosidases [20], xylosidases [23], rhamnosidases [24], etc., has already gained importance to be an efficient and low cost way to synthesize oligosaccharides, alkyl glucosidases, and glycoconjugates. In a study by Gao et al. [25], catechin, a flavonoid, was successfully glycosylated into its glycoside form by several glycosidases. In our case, the transglycosylated product of ginkgo flavonoids, naming the flavonol glycosides, present physiological activities on almost the same level with flavonol aglycones, and can be much more readily absorbed by the human body [6] directly in the small intestine or after hydrolysis by bacterial enzymes in the intestine [5]. A commercial cell wall-degrading enzyme, cellulase from *Penicillium decumbens*, was used in our research for flavonol glycosylation. It is composed of endoglucanase, cellobiohydrolase and β-glucosidase [26,27]. Besides high activity for cellulose degradation, *P. decumbens* cellulase shows high transglycosylation activity probably originating from its β-glucosidase activity as indicated in our previous research [28].

In this work, we aim to make it clear whether the enzymatic transglycosylation of flavonoids could improve their extraction from ginkgo leaves. For this purpose, the effect of *P. decumbens* cellulase on the extraction yield was compared with several other cell wall degrading enzymes with little transglycosylation activities under the addition of glycosyl donors. After validated for its improving effect on the extraction, flavonol transglycosylation by *P. decumbens* cellulase was confirmed by analyzing the catalytic products of the three predominant flavonoids in *G. biloba*. Then we optimized the enzymatic conditions, studied the transglycosylation kinetics, and examined whether the extraction could be improved under the optimal transglycosylation conditions. To the best of our knowledge, this is the first report to utilize enzymatic bifunctionality – for cell degradation and target product transformation – in the extraction of natural compounds from plants. This is also the first report on enzyme-assisted extraction of flavonoids from ginkgo leaves.

2. Materials and methods

2.1. Plant material

Dried leaves of *G. biloba* were bought from Henan Medical Limited (Zhengzhou, China) and ground into powder. The particle size was controlled within 280 and 600 μm using sifters.

2.2. Chemicals and enzymes

Quercetin, kaempferol and isorhamnetin were purchased from Sigma–Aldrich (Steinheim, Germany). Cellulase from *Trichoderma reesei* ATCC 26921 (Celluclast 1.5 L, ≥700 U/g) was bought from Novozymes A/S (Bagsvaerd, Denmark). Pectinase from *Aspergillus niger* (>1 U/mg) was bought from Sigma–Aldrich (Steinheim, Germany). *P. decumbens* cellulase was provided by Ningxia Cellulase Preparation Plant, China. All other reagents were of analytical grade and commercial available.

2.3. Enzyme-assisted extraction of total flavonoids

Distilled water was added to enzymes to obtain stock solutions at the concentration of 2 mg/mL. For each batch of enzyme-assisted extraction, 50 mL enzyme stock solution and certain amount of glycosyl donor were diluted with ethanol–water and adjusted with acetate buffer to obtain 500 mL extractant with the desired pH, ethanol–water ratio and glycosyl donor concentration. 30 g ginkgo leaf powder was added to the extractant in an Erlenmeyer flask covered with aluminum foil. The mixture was incubated under 200 rpm stirring for 30 h on a multichannel magnetic stirrer with temperature controller (Guohua Electronics Co., Changzhou, China). After incubation, the mixture was filtered and the filtrate was forwarded to analysis. For the comparison of extractions assisted by different enzymes, incubation was done at 40 °C in the extractant with an ethanol–water ratio of 3:7 (v/v) and at pH 6.0.

2.4. Determination of total flavonoids

The aluminumchloride colorimetric method described by Chang et al. [29] was used to determine the total content of flavonoids. 0.5 mL extract or standard solution was mixed with 1.5 mL methanol, 0.1 mL of 10% aluminum chloride (substituted with distilled water in blank probe), 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After 30 min incubation, absorbance at 415 nm was determined against a distilled water blank on a UV-1206 spectrophotometer (Shimadzu, Kyoto, Japan). All samples were made in triplicate, and mean values of total flavonoid content are expressed as milligrams of quercetin equivalents per gram of dry weight (dw) calculated according to the standard calibration curve.

2.5. TLC analysis

Extract from ginkgo leaves or enzymatic reaction mixture was loaded to a silica gel plate (10 cm × 20 cm, GF254, Qingdao Haiyang Chemical Co. Ltd., China) and developed with the mobile phase of benzene–ethyl acetate–acetone–acetic acid (10:8:2:2, v/v/v/v).

2.6. Enzymatic transglycosylation reaction

The reaction system for the enzymatic transglycosylation consisted of 2 mL stock solution of *P. decumbens* cellulase, 25 mg flavonoid substrate and 25 mg glycosyl donor in 48 mL ethanol–water

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