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Highly efficient and regioselective synthesis of dihydromyricetin esters by immobilized lipase

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ABSTRACT

Dihydromyricetin is the principle component of the Chinese herbal tea Teng-cha and a promising ingredient for functional food and nutraceuticals, but its low solubility limits its application potentials. This study explored enzymatic acylation of dihydromyricetin to improve its solubility in lipid systems. Acylation was achieved with several lipases with the synthesis of a major (>86%) product and a minor product. Isolation and purification of the products by preparative HPLC followed by LC–MS, ^{13}C NMR, ^{1}H NMR and 2 D-HSQC NMR analyses showed that the major product was a dihydromyricetin monoester with the acylation site at the 3-OH group of C ring. Quantum chemical calculations revealed that the 3-OH had the lowest antioxidant activity, and therefore acylation at this site was expected to have minimum impact on the antioxidant activity. Several factors, including solvent, acyl donor, enzyme origin, molar ratio of substrates and reaction temperature and time, exhibited significant effects on the initial rate, conversion yield and regioselectivity of the reaction. Acylation occurred only with vinyl acetate as the acyl donor, and highest conversion yields were achieved with immobilized *Penicillium expansum* lipase and Novozyme 435 with DMSO and acetonitrile being the best solvents. In general, the acylation results were found to be superior to previous reports on acylation of aglycone flavonoids with respects to conversion yield and regioselectivity.

1. Introduction

Dihydromyricetin (DMY) is a natural aglycone flavonoid (Fig. 1) and one of the principal active components in the traditional Chinese medicinal plant, Teng-cha (*Ampelopsis grossedentata*) leaves (Du et al., 2002; Gao et al., 2009). Like many flavonoids, DMY has been found to possess numerous bioactivities with potential beneficial effects to the human body. Reported health-promoting functions of DMY include antioxidant, anti-inflammatory, analgesic, antitussive, expectorant, antibacterial, anti-thrombotic and anti-tumor activities and the ability to protect liver and alleviate hangover (Xia et al., 2014; Liu et al., 2009; Zeng et al., 2014). Furthermore, DMY exists abundantly in *A. grossedentata*, with young leaves containing up to 20% (dry weight) of this compound (Li et al., 2011). These properties make DMY a promising and easily accessible bioactive ingredient that can potentially be applied to many nutraceutical and pharmaceutical products. However, like many flavonoids, DMY is poorly soluble in both aqueous and non-aqueous systems, which limits its processability and application potential.

A number of studies have attempted to improve the solubility of flavonoids in organic solvents and lipid systems by acylation (Plaza et al., 2014). Although acylation of flavonoids can be accomplished by both chemical and enzymatic methods, the former is thought to be impractical as flavonoids typically contain numerous reactive hydroxyl groups that would require elaborate protective steps to achieve target acylation. Enzymatic acylation, on the other hand, has been shown to be more regioselective and capable of achieving desired acylation outcomes (Chebil et al., 2006). Most studies on enzymatic acylation of flavonoids are conducted with glycosylated forms with acylation occurring on the hydroxyl groups of the sugar moieties (as reviewed by Plaza et al., 2014; Chebil et al., 2006). Only a few studies on acylation of aglycone flavonoids have

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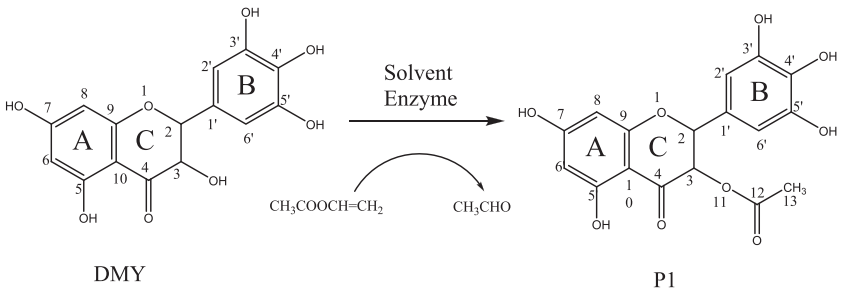

Fig. 1. Regioselective route to the synthesis of dihydromyricetin esters by enzymatic acylation using vinyl acetate as acyl donor.

been reported and they are conducted with only a small number of flavonoids (Lambusta et al., 1993, 2003; Chebil et al., 2007). To date, there is no report on enzymatic acylation of the aglycone flavonol, DMY.

The antioxidant and associated biological activities of flavonoids is determined by their chemical structure, with the number and arrangement of the hydroxyl groups on the aromatic backbone being the crucial factor (Plaza et al., 2014). In the case of DMY, the pyrogallol group (the three adjacent hydroxyl groups at 3′-, 4′- and 5′-positions) is especially important for its antioxidant activity, and therefore needs to be protected during the acylation process. For aglycone flavonoids such as DMY, because acylation occurs on the aromatic backbone (A, B and C rings), in contrast to glycosylated flavonoids where acylation occurs on the sugar moieties, it is desirable for the substitution to occur at a minimal number of hydroxyl groups and at the positions with least impact on the antioxidant activity (Chebil et al., 2006; Lambusta et al., 1993). Previous research shows that conditions of enzymatic acylation, such as the source of lipase, type of solvent and acyl donor, ratio of acyl donor to flavonoid and reaction temperature and time, can strongly influence outcomes of the process, including regioselectivity, acylation rate and conversion yield (Chebil et al., 2006, 2007; Lambusta et al., 1993, 2003).

The objectives of this study were to explore the feasibility of enzymatic acylation of DMY and to investigate the influence of operating conditions (enzyme origin, acyl donor, solvent, substrate molar ratio, time and temperature) on the regioselectivity, reaction rate and conversion yields of the reaction.

2. Materials and methods

2.1. Chemicals and enzymes

Crude powder of PEL (Lipase from *Penicillium expansum*), RCL (lipase from *Rhizopus chinensis*; ANL (lipase from *Aspergillus niger*) and PCL (lipase from *Pseudomonas cepacia*) were from Shenzhen Leveking Bioengineering Co. Ltd., Shenzhen, Guangdong province, China. Novozym 435 (an immobilized lipase B from *Candida antarctica*), Lipozyme TLIM (an immobilized lipase from *Thermomyces lanuginosus*) and Lipozyme RMIM (an immobilized lipase from *Rhizomucor miehei*) were purchased from Novozymes Co., Ltd., Shanghai, China. LPC (enzyme powder from *Penicillium camemberti*) was from Amano Enzyme Inc., Japan. PPL (lipase from *Porcine pancreas*) and RNL (lipase from *Rhizopus niveus*) were purchased from Sigma-Aldrich. Resin D4020 was from Chemical Co. of Nankai University, Tianjin, China. DMY was from Aladdin (Shanghai, China). Vinyl esters used as acyl donors (vinyl acetate, vinyl propionate, vinyl butyrate, vinyl caprylate, vinyl benzoate, vinyl 10-undecenoate, vinyl laurate and vinyl stearate) were purchased from Sigma-Aldrich and TCI Co. Ltd. (Shanghai, China). Other chemicals were of analytical grade unless stated otherwise.

2.2. Immobilization of lipase

The lipase PEL was immobilized following the procedure of Yang et al. (2010). Crude PEL powder (10 g) was mixed with 25 ml 0.05 M glycine–NaOH buffer (pH 9.4) and the enzyme suspension was incubated with stirring (150 rpm) for 1 h at 35 °C. After that, the suspension was centrifuged at 5000 × *g* and 4 °C for 10 min and the supernatant, which contained the lipase, was collected. Resin D4020 (1 g), which had been soaked in 95% ethanol for 24 h and then washed repeatedly with deionized water, was added to the supernatant. The mixture was stirred (150 rpm) constantly for 4 h at 35 °C, filtered with sintered disk filter funnel and the beads with immobilized lipase were collected. The immobilized enzyme was lyophilized after washing three times with glycine–NaOH buffer (pH 9.4). The loading of protein on the support was 30.2 mg protein/g resin, as determined by the Bradford assay.

2.3. Determination of lipase hydrolysis activity

Lipase activity was assayed as described by Mak et al. (2009) with some modifications. Briefly, an appropriate amount of enzyme was added into a solution containing 0.6 ml of 50 mM phosphate buffer (pH 8.0) and 0.1 ml of 10 mM *p*-nitrophenyl acetate in 2-propanol. The reaction was carried out at 45 °C for 20 min with constant stirring at 200 rpm, and then stopped by adding 5.3 ml 0.1 of M Na_2CO_3. The mixture was centrifuged at 12,000 × *g* for 5 min (4 °C) and the absorbance of the supernatant was measured at 410 nm. The reaction without enzyme was used as the control. One unit of lipase activity was defined as the amount of enzyme required to catalyze the release of 1 μmol *p*-nitrophenol per min under the assay conditions. The specific activities of PEL, RNL, ANL, LPC, PPL, RCL, PCL, Novozym 435, Lipozyme RMIM, Lipozyme TLIM, and immobilized PEL were determined to be 0.30, 0.91, 0.83, 0.42, 0.66, 0.54, 0.17, 2.6, 0.55, 0.62, and 1.25 U/mg, respectively.

2.4. General procedure for enzymatic acylation of DMY

In a typical experiment, 50 U of a lipase preparation was added to 2 ml of an anhydrous organic solvent, followed by the addition of DMY and a fatty acid vinyl ester to the final concentrations of 20 mM and 500 mM, respectively. The mixture was incubated at a predetermined temperature in an orbital shaking water bath (200 rpm) for up to 48 h. Aliquots were withdrawn at specified time intervals from the reaction mixture and diluted 20-fold with the mobile phase of HPLC prior to HPLC analysis. To select the best reagents for acylation, 11 different lipase preparations, five different organic solvents and eight different fatty acid vinyl esters were tested.

To study the effect of temperature on the reaction, acylation experiments were conducted in 2 ml anhydrous acetonitrile containing 0.04 mM DMY, 0.4 mM vinyl acetate (which was found to be the only acyl donor capable of DMY acylation; details described later) and 50 U immobilized PEL while the reaction temperature