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Highly efficient enzymatic acetylation of flavonoids: Development of solvent-free process and kinetic evaluation

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ABSTRACT

Solubility and stability of flavonoid glycosides, valuable natural constituents of cosmetics and pharmaceuticals, could be improved by lipase-catalyzed acylation. Focus of this study was on development of eco-friendly process for the production of flavonoid acetates. By using phloridzin as model compound and triacetin as acetyl donor and solvent, 100% conversion and high productivity (23.32 g l^{-1} day^{-1}) were accomplished. Complete conversions of two other glycosylated flavonoids, naringin and esculin, in solvent-free system were achieved, as well. Comprehensive kinetic mechanism based on two consecutive mono-substrate reactions was established where first one represents formation of flavonoid monoacetate and within second reaction diacetate is being produced from monoacetate. Both steps were regarded as reversible Michaelis-Menten reactions without inhibition. Apparent kinetic parameters for two consecutive reactions (V_m constants for substrates and products and K_m constants for forward and reverse reactions) were estimated for three examined acetyl acceptors and excellent fitting of experimental data ($R^2 > 0.97$) was achieved. Obtained results showed that derived kinetic model could be applicable for solvent-free esterifications of different flavonoid glycosides. It was valid for entire transesterification course (72 h of reaction) which, combined with complete conversions and green character of synthesis, represents firm basis for further process development.

1. Introduction

Flavonoids, secondary plant metabolites, are well known for their biological, pharmaceutical and medicinal properties. It has been established that beneficial properties of flavonoids are mostly attributed to their antioxidant activity which is related to their ability to scavenge free radicals and reduce their formation. Furthermore, they can possess anti-inflammatory [1], anti-carcinogenic [2], antibacterial [3], antiviral [4], anti-allergic [5], antithrombotic, vasodilatory [6] and estrogenic activity, as well as inhibitory effect on certain enzyme systems, such as phospholipase A2, cyclooxygenase, lipoxygenase, glutathione reductase, and xanthine oxidase [7]. Considering all these properties, flavonoids are desirable constituents of food, cosmetic and pharmaceutical preparations. The majority of these compounds are present in nature in glycosylated form, so, in general, they are hydrophilic bioactive molecules.

However, wider application of these compounds in lipophilic preparations is limited due to their hydrophilic nature and low solubility and stability in hydrophobic environments. These properties can be improved by effective modification strategy involving introduction of hydrophobic groups onto the flavonoid molecule through esterification/transesterification reactions. Besides increasing solubility and stability of these molecules in lipophilic systems, the selective acylation of these polyhydroxylated compounds may also introduce beneficial properties to the molecule, such as penetration through the cell membrane [8] and improved antioxidant [9], antimicrobial [10], anti-inflammatory [11], antiproliferative [12], anticancer [9,13] and enzyme inhibition activities [14,15]. Two methods are well established for acylation of flavonoids: chemically or enzymatically catalyzed esterification. Enzymatic acylation is preferred, since it is regioselective, can be conducted under milder reaction conditions than chemi-

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cal synthesis and do not require protection/deprotection steps of numerous reactive hydroxyl groups. Various hydrolytic enzymes, such as proteases, esterases and lipases have been tested as biocatalysts for esterification of flavonoids. Lipases are most commonly used catalysts for esterification of various acyl acceptors which demonstrated their high efficiency in microaqueous environments [16,17]. Furthermore, in most studies dealing with acylation of flavonoids, lipase B from *Candida antarctica* (CAL B) is the most widely employed biocatalyst, mainly due to its selectivity and high acylation rates [8,18,19].

So far, different acyl donors (aromatic or aliphatic acids) have been tested for acylation of flavonoids, where considerable attention is attributed to fatty acids since the highest conversion degrees and reaction rates were achieved in reactions where these molecules were used as acyl donor [8,14,20,21]. Medium or long chain fatty acids are the most frequently used substrates for acylation of flavonoids [18,20,22,23], while esterification with short chain acyl groups (up to 4 carbon atoms) was rarely investigated. The importance of short chain acyl donors increases with emerging prospect of application of flavonoid esters as active compounds in cosmetic formulations [24–26]. Since it has been established that active substances with molecular weight less than 500 Da and log P between 1 and 3.5 are adequate for efficient transdermal diffusion [27], the fact that vast majority of flavonoid acylations with medium- or long chain acyl moieties results with active compounds with molecular weight and log P out of proposed ranges, brings focus on short chain acyl donors.

Therefore, the aim of this study was to develop efficient green enzymatic process for acetylation of flavonoids, using phloridzin as model flavonoid, as well as to examine kinetics of reaction and determine key kinetic parameters. Phloridzin (phloretin-2′-*O*-glucoside), the member of the class of dihydrochalcones, is flavonoid which is naturally produced in some plants, primarily in apples, *Malus* species [28]. It has a role as regulator of glucose transport, so it can be applied in treatment of type 2 diabetes. Furthermore, it possess good antioxidant activity, as well as protective role against UV radiation, hence it is a constituent of various commercial cosmetic preparations [29]. In initial experiments, reactions were performed with different acyl donors in acetonitrile which was previously proven as suitable reaction medium [23]. However, application of organic solvents, particularly if product is intended for human use, should be avoided, hence synthesis of phloridzin derivatives was investigated in solvent-free system. This strategy represents alternative, eco-friendly approach for lipase-catalyzed acylation of flavonoids whose implementation can avoid finding even traces of organic solvents in final product and provide significantly higher productivities due to high initial substrates concentrations [30,31]. Additional increase of ester concentration in solvent-free synthesis was accomplished by further optimization of key experimental factors (phloridzin and enzyme concentration). Wider applicability of triacetin-based solvent-free enzymatic synthesis was tested on members of other classes of flavonoids (Scheme 1), naringin and esculin, also being glycosylated molecules with physiological activity. Finally, based on reversible Michaelis–Menten kinetics, a two-step, eight-parameter kinetic model of reactions was developed and validated with the model-predicted results by performing time course simulation.

2. Material and methods

2.1. Materials

Lipase B from *Candida antarctica* (CAL B) immobilized on acrylic resin, Novozym® 435, was purchased from Novozymes (Bagsvaerd, Denmark). Phloridzin (>97% pure) and naringin (>90%) were purchased from TCI Europe N.V., Zwijndrecht, Belgium and esculin (>97%) was purshed from Acros Organics, New Jersey, USA. As acyl donors acetic acid (99.8%, Beta Hem, Belgrade, Serbia), acetic anhydride (97%, Merck-Alkaloid, Skoplje, Macedonia), and triacetin (99%, Sigma-Aldrich, St. Luis, USA) were used. Acetonitrile (p.a., Kemika, Zagreb, Croatia) was used as a reaction medium.

2.2. Enzymatic esterification of flavonoids

Acylation reactions were performed in sealed orbitally shaken flasks in a thermostated shaker (IKA KS 4000i Control, Staufen, Germany) at 70 °C and 150 rpm. Reaction mixtures in organic solvent were composed of phloridzin (10 mM) and a certain amount of different acyl donors (acetic acid, acetic anhydride and triacetin). Concentrations of used acyl donors, triacetin, acetic anhydride and acetic acid were 30, 45 and 90 mM, respectively, which corresponds to 1:9 molar ratio of acyl acceptor to acyl group in reaction mixture. Acetonitrile was added to reaction mixtures to reach 5 ml and reactions were initiated by adding 1% (w/v) of enzyme. The measurement of initial water contents in reaction mixtures were performed on Karl-Fisher apparatus [32] (Mettler Toledo, USA) and determined values ranged from 0.02–0.03%. For solvent-free systems reaction mixtures were prepared with appropriate amount of flavonoid dissolved in 5 ml of acyl donor. Reaction was started by adding different amount of enzyme. Average water contents in this set of experiments were 0.14%, 0.04% and 0.06% for acetic acid, acetic anhydride and triacetin, respectively. Samples for HPLC analyses (50 μl) were taken at predefined times during reaction period. All experiments were performed in duplicate. The mean values are presented in the following figures and for all of them standard deviations were less than 5%. Control samples (without enzyme) were prepared in a same way, subjected to the same temperature treatment and product was not detected in them.

2.3. HPLC analyses

Quantitative analysis of samples were done by Dionex Ultimate 3000 Thermo Scientific (Waltham, USA) HPLC system and a reverse phase column (Hypersil GOLD C18, 150 mm × 4.6 mm, 5 μm). Elution system was: A – H_2O:HCOOH = 100:0.1%(v/v) and B – MeOH:HCOOH = 100:0.1%(v/v). It was conducted as follows – 0–15 min gradient step from 30% to 100% B, than 15–20 min 100% B, 20–20.1 min gradient from 100% to 30% B, and 20.1–25 min isocratic step of 30% B. Elution flow rate was 1.0 ml min^{-1} and column was thermostated at 30 °C. Depending on sample, reaction mixtures were ten or twenty times diluted and injection volumes were in range from 5 to 15 μl. Detection was achieved by UV detector at 280 nm (phloridzin and naringin) and 346 nm (esculin).

Concentrations of products were calculated using following equation:

$$C_{product}\,(mM) = \frac{A_{product}}{A_{flavonoid} + A_{product1} + A_{product2}} * C^{0}_{flavonoid} \qquad (1)$$

Where $C^{0}_{flavonoid}$ is initial concentration of flavonoid used, A (mAU min) is area of products and flavonoid in HPLC chromatograms.

2.4. Mass spectrometry

UPLC–MS analysis was performed on Waters ACQUITY UPLC H Class system (BEH C18 1.7 μm column) coupled with Waters QqQ mass analyzer. All analyses were performed using ESI ion source in positive ion mode.