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Short communication

Efficient regioselective acylation of andrographolide catalyzed by immobilized *Burkholderia cepacia* lipase

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ABSTRACT

For the first time, PSL-C, an immobilized lipase from *Burkholderia cepacia*, was successfully applied to the regioselective acylation of andrographolide by vinyl acetate in acetone. FT-IR spectra demonstrated the occurrence of acylation reaction. The ^{13}C NMR, ESI-MS and elemental analysis confirmed that the 14-acetylandrographolide was formed exclusively. Water activity and reaction temperature had a significant effect on the initial rate and the substrate conversion, but little effect on the regioselectivity of the reaction. The optimal water activity and reaction temperature were 0.11 and 50 °C, respectively. Under these conditions, the initial rate and substrate conversion were 50.2 mM h^{-1} and 99.0%, respectively, after a reaction time of around 4 h. Besides, immobilized lipase also displayed higher operational stability and 83.5% of its original activity was maintained after being reused for eight batches.

1. Introduction

Andrographolide ($C_{20}H_{30}O_5$, Scheme 1), a diterpene lactone [1], is the main and most medicinally active component isolated from *Andrographis paniculata* (Burm.f.) Nees [2], an important herbal medicine traditionally used to treat different range of diseases in China, India and some Southeast Asian countries [3]. Andrographolide is found in the whole plant but is most concentrated in the leaves. Andrographolide has been reported to have anticancer [4,5], anti-inflammatory [6], antiviral [7], anti-allergic [8], anti-diabetic [9] and immunostimulatory activities [10]. Because of antitumor effects, andrographolide was utilized as a starting material for the synthesis of derivatives. Some acylated andrographolide products demonstrated good antitumor activity against various cancer cell lines [5,11]. However, the selective monoacylation of andrographolide is difficult to achieve by means of conventional reactions due to the similar reactivity of the hydroxyl groups. For this reason, multi-step synthesis based on protection/deprotection reactions is required [5]. The discoveries that enzymes are able to work in non-aqueous media, functioning in the reverse direction that in nature, offer hydrolytic enzymes the opportunity to catalyze efficiently a wide variety of synthetic reactions [12,13].

Up to now, no study on enzymatic modification of andrographolide has been reported. We herein describe the first effort to carry out regioselective acylation of andrographolide with vinyl acetate catalyzed by an immobilized *Burkholderia cepacia* lipase in acetone (Scheme 1) in order to develop a novel and efficient route for the preparation of a monoester of andrographolide. Effects of some major influential factors, such as water activity, reaction temperature and reaction time, on the reaction were investigated.

2. Materials and methods

2.1. Materials

Andrographolide (purity >99.0%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Lipase PSL-C "Amano" (1900 U g^{-1}, from *B. cepacia*, immobilized on ceramic) was a gift from Amano Enzyme China Ltd. Vinyl acetate and acetone were purchased from Sigma–Aldrich (USA). All other reagents were obtained from commercial sources and were of analytical grade.

2.2. General procedure for enzymatic acylation of andrographolide

In a typical experiment, 5 ml of acetone with a specified water activity (a_w) containing 0.1 mmol andrographolide, 1.0 mmol vinyl acetate and a certain amount of enzyme were incubated in an air-bath shaker under predetermined temperature reaction. Aliquots were withdrawn at specified time intervals from the reaction mixture, and then diluted five times with a methanol prior to HPLC analysis. In order to structurally characterize the product, the PSL-C-catalyzed reaction was scaled up [reaction conditions:1.0 mmol (350.0 mg) andrographolide;10 mmol vinyl acetate; 50 ml acetone; 500 U PSL-C; a_w = 0.11; 50 °C]. Upon completion of the reaction (checked by HPLC), the reaction mixture was filtered to remove the immobilized enzyme and evaporated under vacuum. The product was dissolved in methanol and then purified by silica gel chromatography with the mixture of methanol and dichloromethane (30/70, v/v) as an eluant. After crystallization from methanol, the product was obtained (yield 372.8 mg, 95.1%). A control reaction, which was performed by following the above procedure except that no enzyme was added, demonstrated that no chemical acylation reaction was detectable.

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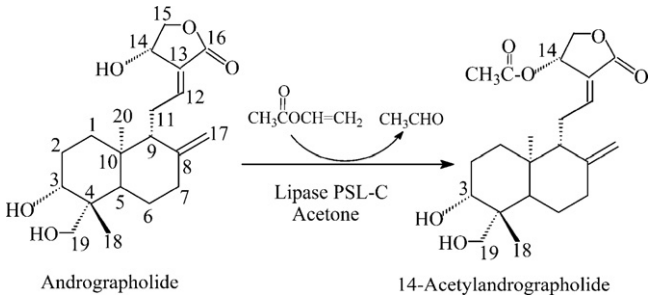

Scheme 1. PSL-C-catalyzed regioselective acylation of andrographolide with vinyl acetate in acetone.

2.3. Control of the initial water activity (a_w)

The a_w of the reaction media, the substrates and the enzyme were controlled by gaseous equilibrium with different saturated salt solutions in separate closed containers at 25 °C [14,15].

2.4. Thermal stability of enzyme

For the thermal stability of enzyme, 100 mg aliquots of PSL-C were added into 5 ml acetone with fixed a_w (0.11) and incubated for 12 h at variable temperatures from 30 to 70 °C. Then the immobilized enzyme was recovered by filtering off from the reaction medium, washed three times with warm (50 °C) acetone, and added into the above-mentioned fresh reaction medium containing 0.1 mmol andrographolide and 1.0 mmol vinyl acetate. The mixture was incubated at 150 rpm and 50 °C, followed by assay of enzyme's activity for catalyzing the reaction. The initial rate of acylation was used to evaluate the enzymatic acylation activity. The relative activity was expressed as the ratio of retained activity after incubation to the original activity of the enzyme in the same reaction system.

2.5. Operational stability of enzyme

The stability of PSL-C during batch acylation reactions was investigated. After each batch reaction (reaction conditions: 0.1 mmol andrographolide; 1 mmol vinyl acetate; 5.0 ml acetone; 100 U PSL-C; a_w = 0.11; 50 °C), immobilized lipase was recovered by filtration, washed three times with acetone and used in the next batch reaction composed of new substrates. The residual activity determined after 4 h was expressed as relative conversion. The conversion achieved in the first batch was set to 100.

2.6. HPLC analysis

The reaction mixture was analyzed by RP-HPLC on a 4.6 mm × 150 mm (5 μm) Zorbax Eclipse XDB-C18 column (Agilent Technologies Co., Ltd, USA) using an Agilent G1311A pump and a UV detector at 225 nm. The mobile phase was a mixture of water and methanol (30/70, v/v) at a flow rate of 1.0 ml min^{-1}. The retention times for andrographolide and 14-acetylandrographolide were 1.93 and 2.62 min, respectively. Regioselectivity was defined as the ratio of the HPLC peak area corresponding to the indicated product to that of all the products formed [16]. The initial rate (V_0) and the substrate conversion (C) were calculated from the HPLC data. The average error for this assay is less than 0.5%. All reported data are averages of experiments performed at least in triplicate.

2.7. Structure determination

The position of acylation in enzymatically prepared ester was determined by NMR. ^{13}C NMR spectra were recorded on a Bruker AVANCE AV-500 instrument (Bruker Co., Switzerland) at 500 MHz. CD_3OD was used as a solvent and TMS was used as an internal reference. Chemical shifts (δ) were expressed in ppm shift. Mass spectra were recorded on Agilent 1100 LC-MSD-Trap-SL mass spectrometer (Agilent Technologies Co., Ltd, USA). FT-IR spectra were recorded in a transmittance mode from 4000 to 400 cm^{-1} using a Vector 33 spectrometer (Brucker Company, Germany). Elemental analysis was measured using CHN–O-rapid elemental analysis apparatus (Heraeus, Germany). Accurate is within 0.3%.

Andrographolide: ^{13}C NMR, δ (ppm), 38.2 (C1), 29.0 (C2), 80.9 (C3), 43.7 (C4), 56.4 (C5), 25.2 (C6), 38.9 (C7), 148.8 (C8), 57.4 (C9), 39.9 (C10), 25.7 (C11), 149.4 (C12), **129.8** (C13), **66.7** (C14), **76.1** (C15), 172.6 (C16), 109.2 (C17), 23.4 (C18), 65.0 (C19), 15.5 (C20). FT-IR (cm^{-1}), 3407 (ν_{OH}), 2868–2925 (ν_{CH}), 1727 (γ-lactone, $\nu_{C=O}$), 1674 ($\nu_{C=C}$), 1220/1032 (ν_{C-O-C}). 14-Acetylandrographolide: ^{13}C NMR, δ (ppm), 38.1 (C1), 29.0 (C2), 80.9 (C3), 43.6 (C4), 56.3 (C5), 25.1 (C6), 38.9 (C7), 149.1 (C8), 57.2 (C9), 39.9 (C10), 26.3 (C11), 151.5 (C12), **125.7** (C13), **69.3** (C14), **73.1** (C15), 172.0 (C16), 108.9 (C17), 23.4 (C18), 64.9 (C19), 15.5 (C20); acetyl: 171.5 (C=O), 20.6 ($-CH_3$). FT-IR (cm^{-1}), 3409 (ν_{OH}), 2865–2932 (ν_{CH}), 1770 (acetyl, $\nu_{C=O}$), 1751 (γ-lactone, $\nu_{C=O}$), 1679 ($\nu_{C=C}$), 1218/1209/1021 (ν_{C-O-C}). ESI-MS, (m/z: 392, M^+, 100%).

3. Results and discussion

3.1. PSL-C-catalyzed regioselective acylation of andrographolide

The FT-IR spectroscopy data revealed the characteristic absorptions in common with andrographolide still existed in the product spectrum, such as peaks at 3409 cm^{-1} (ν_{OH}), 2865–2932 (ν_{CH}), 1679 cm^{-1} (C=C) and 1751 ($\nu_{C=O}$ from γ-lactone). Moreover, the absorption at 1770 cm^{-1} corresponding to the carbonyl group of the acetyl moiety appeared in the product spectrum, indicating that andrographolide has been acylated.

High selectivity represents the most attractive characteristic of enzymatic reactions. The product structure of PSL-C-catalyzed acylation was further characterized by ^{13}C NMR spectroscopy and it was found that, compared to andrographolide, the spectrum of the product exhibited two additional carbon signals at δ 171.5 (C=O) and 20.6 ($-CH_3$), characteristic of an acetyl group (details in Section 2.7). Moreover, the C-14 (δ 66.7) of the andrographolide shifted downfield by 2.6 ppm and the resonance due to its neighboring carbon atom C-13 (δ 129.8) and C-15 (δ 76.1) also showed an upfield shift of 4.1 and 3.0 ppm, respectively, which suggested that the acyl group was attached to the –OH at C-14. Thus the product was identified as 14-acetyl andrographolide. Acetylation of andrographolide was further confirmed by the ESI-MS of the product (m/z: 392, M^+, 100%; calculated for $C_{22}H_{32}O_6$). Elemental analysis of the acetyl andrographolide found: C, 67.47%; H, 8.24%. $C_{22}H_{32}O_6$ requires C, 67.34%; H, 8.22%. Synthesis of 14-acetylandrographolide by chemical protection/deprotection reactions had been reported [5]. Reactions need more catalysts and reagents and yield is also low (40.8%).

To better understand PSL-C-mediated acylation reaction conducted in acetone and further optimize the reaction, the initial reaction rate, substrate conversion and the regioselectivity of the reaction were investigated in acetone as a function of the water activity, reaction temperature and reaction time.

3.2. Effect of water activity

Water is essential for enzymatic reactions in non-aqueous media as it is associated with the maintenance of enzyme's active conformation or the "loosening up" of the rigid structure of an enzyme [12,13]. However, the presence of water in reaction medium disfavors synthetic reactions while encouraging hydrolytic reactions such as hydrolysis of acylated products and acyl donors (e.g., activated esters) [14,15]. It is therefore particularly important to pay attention to water control in the case of lipase-mediated andrographolide acylation in acetone.

Table 1 depicts the clear a_w dependence of the acylation reaction performed in acetone. Both the initial rate and the substrate conversion of the acylation increased markedly with increasing a_w up to 0.11. Further increase in a_w, however, led to a lower reaction rate and a lower substrate conversion. Although hydrolysis reaction of the both the acyl donor and the product (andrographolide ester) could be inhibited when a_w was very low,

Table 1
Effect of initial water activity on the enzymatic acylation of andrographolide[a].

a_w	V_0 (mmol/L h)	C (%)
~0	12.0 ± 0.5	41.0 ± 0.9
0.07	35.0 ± 1.1	90.0 ± 1.3
0.11	45.5 ± 1.5	95.5 ± 1.0
0.33	19.4 ± 0.7	55.4 ± 1.5
0.53	5.6 ± 0.3	26.1 ± 1.2
0.75	2.5 ± 0.3	16.3 ± 0.8

[a] The reaction conditions: 0.1 mmol andrographolide; 1.0 mmol vinyl acetate; 100 U PSL-C; 5 ml acetone; 45 °C; 150 rpm; 4 h.