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Bioreactor for the Continuous Purification of Simvastatin by Lovastatin Esterase

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ABSTRACT

This work investigates an efficient method for purification of simvastatin ammonium salt from the residual amounts of lovastatin ammonium salt. Lovastatin esterase produced by the fungus *Clonostachys compactiuscula* was chosen as a biocatalyst for selective hydrolysis of lovastatin ammonium salt. Based on specific activities, determined by a spectrophotometric assay, two enzyme preparations were chosen as suitable candidates for this transformation: lovastatin esterase immobilized on Sepharose 4 B or immobilized on diethylaminoethylcellulose. Immobilized enzyme was packed into the inner space of the steel column and examined as a bioreactor in a continuous flow process. Both reactors indicated excellent selectivity (> 100) and stability.

1. Introduction

Chemical manufacturing is performed using either batch systems[1] or continuous-flow systems [2–4]. Flow systems have several advantages over conventional batch systems, such as facile automation, reproducibility, safety, and process reliability [5,6]. Although this innovative and promising alternative is receiving more and more attention, the substantial part of pharmaceutical manufacturing still relies on batch systems because the synthesis of complex molecules such as drugs is difficult to achieve with continuous-flow systems [7,8]. To address this challenge, many immobilization approaches have been explored, including the attachment of the organic, organometallic and biocatalysts to solid supports [9]. Recent technological improvements have made it possible to synthesize relatively complex molecules using packed-bed rectors [10,11]. In this mode the reagent solution is flown through a reactor that is filled with the catalyst immobilized on a solid support, usually silica or a polymeric matrix. Particularly, the incorporation of immobilized lipase [12–15] or ω-transaminase [16,17] into a flow system has broadened the general applicability of flow chemical processes.

Immobilized enzymes, in contrast to native enzymes, have many advantages, such as higher resistance to environmental changes, recycling of used biocatalyst and easy separation of enzymes from the products. These enzymes significantly reduce the cost of whole operation. Enzymes may be immobilized on carriers by physical methods, where a weak interaction between support and enzyme exists, or by chemical methods, wherein a covalent bond is formed [18,19].

In this study, we describe the application of hydrolytic enzyme, covalently bound to a solid support, packed in a continuous flow reactor for purification of simvastatin ammonium salt from residual lovastatin ammonium salt. Simvastatin, known for its selective hydroxymethyl glutaryl-coenzyme A (HMG-CoA) reductase inhibition activity [20], is a semi-synthetic lovastatin derivative, possessing a 2,2-dimethylbutyryloxy side chain at the 8′ position. It is mainly obtained from lovastatin by direct C-methylation of the (*S*)-2-methylbutyryloxy side chain[21–23] or *via* a multistep synthesis that includes protection and deprotection steps[24]. Unfortunately, simvastatin contains residual amounts of lovastatin, that prohibits its use as a drug. Due to the structural similarity, they are difficult to separate. Only selective hydrolysis of lovastatin ammonium salt (**A**) followed by crystallization enables ready separation from simvastatin ammonium salt (**B**) (Fig. 1) [25].

Lovastatin esterase produced by the fungus *Clonostachys compactiuscula* was reported as a suitable biocatalyst for selective cleaving of an ester bond of the 2-methylbutyryloxy side-chain and could be applied for purification (Fig. 1) [26]. Moreover, monacolin J ammonium salt (**C**) generated during hydrolysis, also inhibits HMG-CoA reductase activity[27] and may be used as a cholesterol-lowering agent, as well as a valuable intermediate in the synthesis of other semi-synthetic statins [28]. For these reasons, to date hundreds of microbial strains were screened toward lovastatin esterase activity [25]. Unfortunately, lovastatin esterase is unstable and requires a modification method which would increase its stability and activity. Versatile and broadly applicable immobilization methods are the current methods of first choice for enhancing enzymes stability [29]. Moreover, a rapid, reproducible and sensitive method for detection and quantification of

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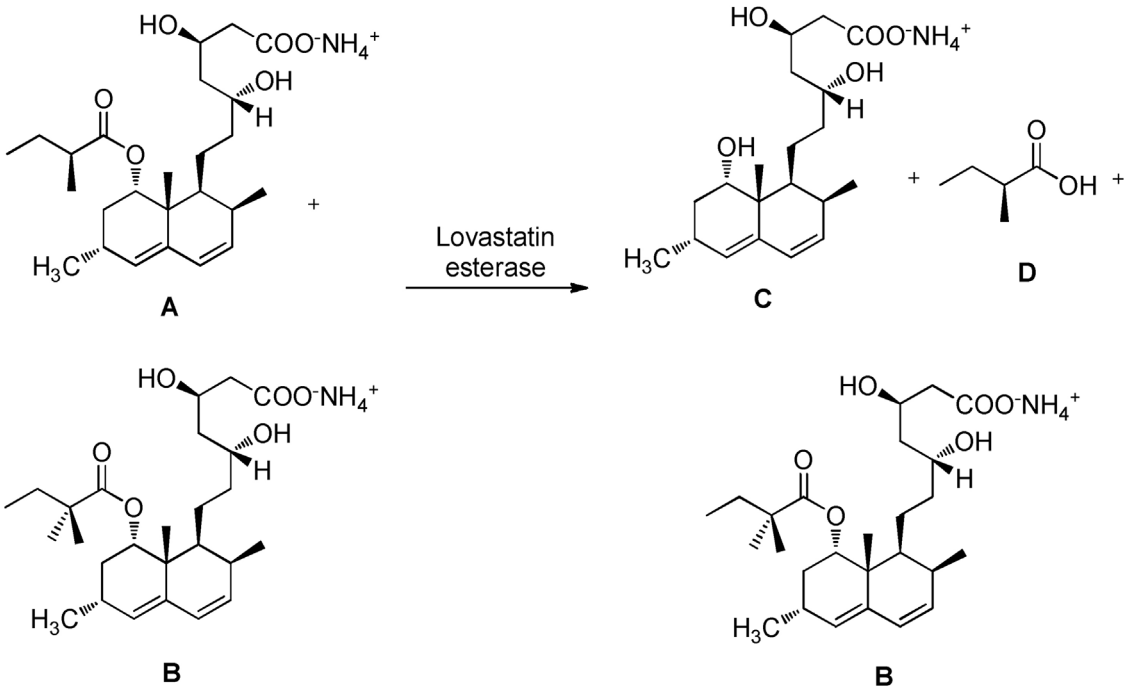

Fig. 1. Generation of monacolin J ammonium salt (**C**) and 2-methylbutanoic acid (**D**) during selective hydrolysis of lovastatin ammonium salt (**A**) in the presence of simvastatin ammonium salt (**B**).

the lovastatin esterase activity is highly valuable during identification, purification, or immobilization.

Thus, based on our previous studies [30,31], we designed chromogenic probes for determination of lovastatin esterase activity. Synthesized substrates showed high specific reactivity with lovastatin esterase and satisfactory stability in the presence of another hydrolytic enzyme. Next, lovastatin esterase immobilized on different solid supports was assayed, packed into steel column and evaluated as a bed in a continuous flow bioreactor for purification of simvastatin ammonium salt from residual lovastatin ammonium salt.

2. Experimental

2.1. Materials and Methods

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Gemini 400 MHz Spectrometer. Chemical shifts are expressed in parts per million using TMS as an internal standard. UV–vis spectra and spectrophotometric time profiles were recorded at 37 °C on a U-1900 spectrophotometer (Hitachi) in 1 cm plastic cuvettes. Commercial enzymes were purchased from Sigma–Aldrich. All chemicals were commercial products of analytical grade. For determination of lovastatin and simvastatin conversions, the efflux from bioreactor was analyzed by HPLC, using a ProStar HPLC instrument (Varian/Agilent Technologies) coupled to a UV–vis detector. An RP LiChro Cart 55-4 column (55 mm × 4 mm × 3 µm) was used with the following conditions: eluent, acetonitrile:water:phosphotic acid (936:400:1, v/v/v); room temperature; flow 1.5 mL min^{-1}; λ = 238 nm. Retention times for monacolin J ammonium salt 0.28 min; lovastatin ammonium salt 1.35 min; simvastatin ammonium salt 2.0 min.

2.2. Enzyme Assay

Substrates **1-7** were prepared as stock solutions in acetone and tested with native lovastatin esterase and lovastatin esterase immobilized on various supports. Assays were initiated by an addition of solution of chromogenic substrate (0.5 mL in acetone) to a thermostated Tris-HCl buffer (10 mL, 20 mM, pH 7.8, 37 °C) then enzyme solution was added (0.5 mL). Spectrophotometric time profiles were recorded in plastic cuvettes in the U-1900 spectrophotometer (Hitachi). The spectrophotometric data for all experiments were acquired over 5 min. Spectrophotometric data were converted to a chromophore concentration by using a calibration curve. The linear portion of each curve was used to generate the reaction rates.

2.3. General Procedure for Lovastatin Esterase Purification

Lovastatin esterase is produced by the fungus *Clonoslachys compacliuscula* (ATCC 38009). The fungus was harvested according to the literature protocol (U.S. Pat. No. 5,223,415) and 236 g of the mycelium was triturated with glass beads (1.2 g) in liquid nitrogen for 8 h, then it was extracted with the phosphate buffer (240 mL, pH 6.5), and the solid was centrifuged off (12,000 rpm; 10 min; 40 °C). The supernatant was separated and the extraction procedure with buffer (160 mL) was repeated. Supernatants were combined and filtered *via* a qualitative filter to yield 440 mL ($c_{protein}$ = 4,180 µg mL^{-1}). Next, ammonium sulfate was added to achieve the concentration of 40% (w/v, 101.6 g, 1 h, 0 °C), left for 1 h (0 °C) and centrifuged (12,000 rpm; 20 min; 40 °C). The supernatant was separated and ammonium sulfate was added to achieve the concentration of 85% (w/v, 132 g; 1 h; 0 °C), and centrifuged (12,000 rpm; 20 min; 4 °C). The resulting precipitate was collected and dissolved in a phosphate buffer (pH 7.8; 20 mM; 0.5 mol NaCl) to provide 15.84 mL of the lovastatin esterase solution ($C_{protein}$ = 980 µg mL^{-1}) having the specific activity of 9.8 nmol mg^{-1} 1 min^{-1}. A hydrophobic interaction column packed with Phenyl Sepharose 6-fast flow (26 mL) was subjected to equilibration with a phosphate buffer (pH 6.5; 1 mL min^{-1}; 5 h). Next, the lovastatin esterase solution (15 mL) was fed to the column, and eluted, respectively, with a phosphate buffer (pH 6.5; 1.4 mL min^{-1}) and redistilled water (1.4 mL min^{-1}). Fractions containing the lovastatin esterase were pooled and the carbonate buffer (1 mL, pH 9.4; 50 mM) was added to yield 12.5 mL of the solution ($c_{protein}$ = 21 µg mL^{-1}), having a specific activity of 313 nmol mg^{-1}min^{-1}.