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Development of an Improved Immobilized CAL-B for the Enzymatic Resolution of a Key Intermediate to Odanacatib

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ABSTRACT: An immobilized form of *Candida antarctica* lipase B (CAL-B) has been developed with enhanced stability and activity compared to commercially available preparations. The immobilized CAL-B is more active, and 15 times more stable than the previously used preparation. This permits a continuous dynamic kinetic resolution process that is significantly less expensive than the original batch process, and with a 3-fold reduction in the process *E*-factor.

INTRODUCTION

Osteoporosis affects an estimated 200 million people worldwide and is responsible for a majority of the >800,000 hip fractures that occur in the United States and Europe each year.[1−3] Cathepsin K inhibitors are an important class of antiresorptive agents that help prevent bone loss, while allowing the bone formation process to continue. Odanacatib is an orally bioavailable, potent, and selective cathepsin K inhibitor currently being evaluated in clinical trials.[4,5]

The total synthesis of odanacatib relies on an enzyme-mediated dynamic kinetic resolution[6−9] for the production of a key chiral fluoroleucine intermediate. The asymmetric synthesis of fluorinated amino acids is a valuable technology for the production of biologically active intermediates that exhibit a wide array of physiological functions, including enzyme inhibitors, receptor antagonists, and lipophilicity-enhancing agents.[10−12] Early deliveries used Novozyme435 in a batch process for the enzyme-catalyzed ring-opening ethanolysis of azlactone **1** to the desired ester (*S*)-γ-fluoroleucine ethyl ester **2**.[13] Significant yield and selectivity gains were achieved by minimizing side reactions and eliminating attrition of the solid enzyme support by switching to a continuous plug flow column reactor setup.[14,15] However, deactivation of the immobilized enzyme under the optimized reaction conditions remained a significant concern, and cost drivers led us to investigate the development of a more active and stable form of immobilized *Candida antarctica* lipase B (CAL-B). Herein we report on the development of a new immobilized form of CAL-B with greatly enhanced stability and activity compared to those of commercially available preparations.

Figure 1 shows the desired transformation along with the optimized reaction conditions. The original batch process provided 79% yield with 78% enantiomeric excess (ee) of the desired product with an enzyme (Novozyme435) to substrate ratio of 1:1 by weight.[13] This process was cost prohibitive, and development of a continuous column reactor process improved the yield and ee to 90% and 86%, respectively, with an enzyme to substrate ratio of 1:20.[14,15]

This represented a significant process improvement with a corresponding decrease in process cost, and enabled the delivery of material for clinical trials.[16] However, further cost reduction was necessary to arrive at an acceptable manufacturing route.

RESULTS AND DISCUSSION

Various immobilized CAL-B preparations from multiple vendors were evaluated in batch mode to determine initial activity under the optimized process conditions. 100 g/L azlactone substrate was added to MTBE (methyl *tert*-butyl ether) containing 60 mL/L EtOH, 2.5 mL/L TEA (triethylamine), and 50 g/L immobilized CAL-B. The reactions were agitated at 60 °C and samples were taken to determine the initial activity of each immobilized enzyme preparation. Stability was also determined by incubating the immobilized enzymes under reaction conditions with no substrate azlactone and then charging the substrate after predetermined time points to determine residual activity. Novozyme435 was found to have the highest specific activity, but stability needed to be improved to arrive at an economically viable process. None of the other commercially available immobilized CAL-B preparations that were tested provided better stability under our process conditions.

Failing to identify a superior commercially available immobilized CAL-B, our efforts turned to creating and evaluating a more active and stable enzyme preparation in-house. Five SEPABEAD resins from Mitsubishi were tested for the immobilization of CAL-B. The resins chosen represented various compositions and functional groups, and included both covalent and hydrophobic immobilization binding methodologies (Table 1).

Liquid CAL-B from Novozyme was incubated with each resin for 24 h at room temperature. The resins were then filtered off, washed with buffer and dried under vacuum with a nitrogen sweep. Optimization of enzyme loading on the resins was investigated. Pretreatment of the resins by washing with potassium phosphate buffer, incubation times longer than 24 h, and dilution of the liquid CAL-B with potassium phosphate buffer before incubation with each resin all resulted in lower enzyme loading. The optimal conditions established for immobilization were an incubation of 3 mL of liquid CAL-B for every 1 g of resin with stirring for 24 h at room temperature.

After immobilization, each resin was tested for activity and 48 h stability under the optimized reaction conditions (Figure 2). Novozyme435 is included in Figure 2 as a reference point for

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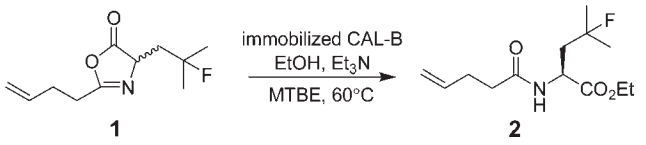


Figure 1. Enzyme-catalyzed dynamic kinetic ring-opening ethanolysis of azlactone.

Table 1. Immobilization resins and their composition and method of enzyme binding

resin name	resin composition	resin functional group/binding method
EC-EP	polymethacrylate	epoxide/covalent
EC-HFA	polymethacrylate	amino epoxide/covalent
EXA252	styrene/DVB	porous structure/hydrophobic
EXE119	polymethacrylate	epoxide/covalent
EXE120	polymethacrylate	octadecyl/hydrophobic

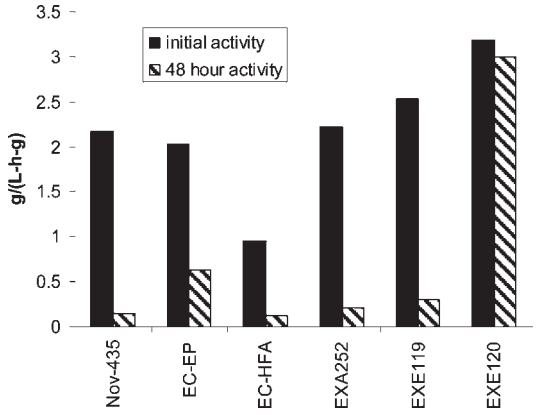


Figure 2. Initial activity and 48 h stability of CAL-B immobilized on SEPABEAD resins compared to Novozyme435.

comparison to the standard process. Novozyme435 exhibits an activity of 2.2 g/L·h·g_{enzyme}. Higher specific activity by 50% was observed with the CAL-B immobilized on EXE120. Additionally, Novozyme435 retained only 6% of its initial activity after 48 h under process conditions compared to 94% activity retention for the EXE120 immobilized CAL-B (MRK-CALB-EXE120).

Novozyme435 and MRK-CALB-EXE120 were then compared head to head in continuous packed bed plug flow reactor (PFR) mode.[14] As expected from the batch reaction studies, MRK-CALB-EXE120 exhibited much greater (15×) stability compared to Novozyme435 (Figure 3). Additionally, higher product yield and ee is obtained using the higher specific activity MRK-CALB-EXE120 compared to Novozyme435 (95% yield vs 90% and 88% ee vs 86%). The greater stability of the new immobilized CAL-B prep also provides for a significant reduction in enzyme to substrate loading, from 1:20 for Novozyme435 to <1:100 for MRK-CALB-EXE120.

Next, the ability to recharge MRK-CALB-EXE120 that had lost its activity over time under process conditions was investigated. The immobilized enzyme was allowed to deactivate under process conditions for 250 h until 70% residual activity was observed. The resin was then rinsed with 50 mM potassium phosphate buffer and resuspended in liquid CAL-B following the same immobilization protocol outlined earlier. After workup, the

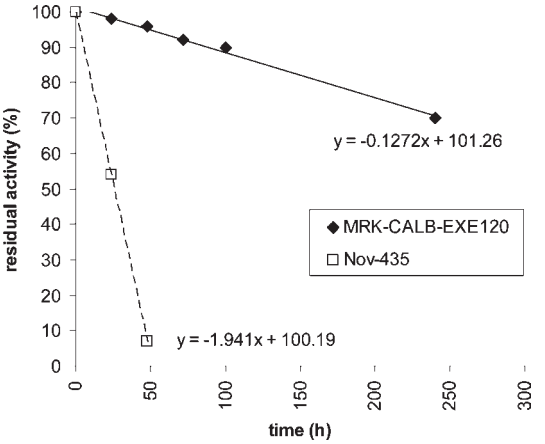
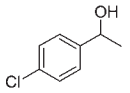
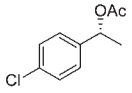
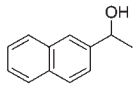
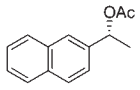
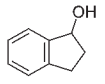
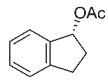
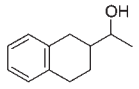
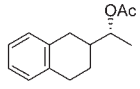
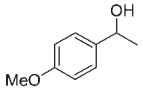
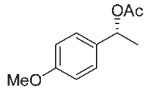
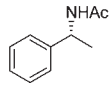


Figure 3. Stability of MRK-CALB-EXE120 and Novozyme435 in plug flow reactor (PFR).

Table 2. Resolution of alcohol and amine substrates using MRK-CALB-EXE120

Entry	Substrate	Product	conversion	%ee
1			47%	>99
2			48%	>99
3			50%	>99
4			49%	>99
5			48%	>99
6			45%	>99
7			48%	>99

MRK-CALB-EXE120 returned to full activity. This cycle was repeated for a total of three resin recharges. The ability to recharge the resin with active enzyme reduces both process cost and total waste.

Finally, we evaluated the generality of this new immobilized CAL-B preparation *via* the resolution of a variety of alcohol and amine substrates. Twenty grams per liter of each substrate was dissolved in MTBE with 5 equiv of vinyl acetate and 50 g/L MRK-CALB-EXE120. The reactions were run overnight for 18 h. Excellent enantioselectivity (>99% ee) was obtained for each of the substrates tested (Table 2).

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CONCLUSION

In summary, the development of a new immobilized CAL-B (MRK-CALB-EXE120) exhibiting significantly greater activity and stability under our process conditions compared to the original enzyme preparation (Novozyme435) has led to a more efficient and less expensive manufacturing process for a key fluoroleucine intermediate in the synthesis of odanacatib. Factoring in the reusability of the new immobilized CAL-B preparation along with cost to manufacture the immobilized enzyme,, the MRK-CALB-EXE120 catalyzed process is 99.9% less expensive than the process utilizing Novozyme435, with a 3-fold lower *E*-factor.[17] This process has been demonstrated several times at 100 kg scale (>90% yield and 88% ee).

EXPERIMENTAL SECTION

Conversion Analysis. Analysis of the extent of conversion was carried out by isocratic reverse phase Agilent (Palo Alto, CA) HPLC using a Zorbax SB-C18 (75 mm × 4.6 mm) column and a 70% acetonitrile/30% water (containing 0.5% H_3PO_4) mobile phase at 1 mL/min and 25 °C. UV absorbance was monitored at 210 nm. The ester and azlactone were quantified using their characteristic retention times of 1.0 and 1.3 min, respectively, during elution.

Enantiomeric Excess Analysis. Enantiomeric excess of the fluoroleucine ester product was determined by isocratic normal phase high performance liquid chromatography (HPLC) using a Chiralcel OD-H (250 mm ×4.6 mm) column and a 98% hexanes/2% isopropanol mobile phase at 1.75 mL/min and 25 °C. UV absorbance was monitored at 210 nm. The undesired (*R*)-ester and desired (*S*)-ester were quantified using their characteristic retention times of 10.3 and 21 min, respectively, during elution.

Reagents and Enzymes. Commercial grade reagents and solvents were purchased from Sigma-Aldrich and used without further purification. Liquid CAL-B and Novozyme435 were purchased from Novozymes. SEPABEADS resins were purchased from Mitsubishi.

Batch Reactions for Immobilized Enzyme Activity Determination. Immobilized CAL-B (50 g) was rinsed with 500 mL of MTBE for 10 min. This process removes unwanted water that can lead to background hydrolysis. The enzyme was then added to 500 mL of MTBE in a reaction vessel. Next, the reaction was heated to 60 °C, and then 50 g of substrate azlactone was added followed by 1.25 mL of triethylamine and 30 mL of EtOH.

CAL-B Immobilization Procedure. No pretreatment of the immobilization resin was necessary. Immobilization resin (50 g, Mitsubishi SEPABEADS) was added to 150 mL of Novozymes liquid CAL-B. The mixture was stirred with overhead stirring for 24 h at room temperature. The liquid was then drained from the resin, and the resin was then rinsed with 3× volumes of 50 mM pH 7.5 potassium phosphate buffer. Next, the resin was dried over a filter with a nitrogen sweep for 5 h. The immobilized enzyme can then be stored at 4 °C.

Continuous Plug Flow Reactor (PFR). One g of immobilized CAL-B was slurry packed in a column with MTBE under gravity. Two feed solutions were then made. The first solution contained 200 g/L azlactone substrate in MTBE (500 mL total volume). The second solution contained 120 μL/mL EtOH and 5 μL/mL triethylamine in MTBE (500 mL total volume). Both solutions were pumped at equal flow rates of 2.5 mL/h each to a T-junction just before entering the top of the column, which was preheated to 60 °C. It is important to keep the streams separate until just before they enter the column to minimize background (non-catalyzed and nonselective) reaction with ethanol. The column effluent passed through a back pressure regulator set at 50 psi and then to a quench tank containing 1 M H_2SO_4. The column was run until the feed solutions were depleted. Ten milliliters of MTBE was then passed through the column to flush out the remaining product. The organic phase was then taken from the quench tank and washed with 500 mL of saturated sodium bicarbonate. It was then dried to an oil by vacuum distillation (95% yield, 88% ee). Further experimental detail on the workup and analysis is found in reference13.

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(17) *E*-factor = total waste (kg)/product (kg).