Synthesis of Medically Important Ethyl Cinnamate Ester by Porcine Pancreatic Lipase Immobilized on Poly(AAc-*co*-HPMA-*cl*-EGDMA) Hydrogel

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Received 17 November 2009; accepted 4 August 2010
DOI 10.1002/app.33146
Published online 29 March 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Ethyl cinnamate was synthesized by the reaction of ethyl alcohol with cinnamic acid using hydrogel immobilized porcein pancreatic lipase (PPL). The PPL was immobilized on to a formalin (2%, v/v) activated poly(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel. The hydrogel-bound PPL was used to perform esterification of ethanol with cinnamic acid in equimolar ratio (100 mM each) in dimethyl sulfoxide. Various kinetic parameters were optimized to produce ethyl cinnamate using immobilized lipase. The maximum conversion (55 m*M*) was achieved at temperature 65°C in 27 h under continuous shaking. The hydrogel-bound PPL retained more than 50% of its original activity up to second cycle of repetitive esterification under optimized conditions.
 J Appl Polym Sci 121: 2674–2679, 2011

Key words: porcine pancreatic lipase; immobilization; poly(AAc-*co*-HPMA-*cl*-EGDMA); hydrogel; ethyl cinnamate; DMSO

INTRODUCTION

Compounds of cinnamic acid are widely present in plants, including edible vegetable staple oil of cinnamon. Their biological properties, particularly the antioxidant activity, are well known and depend on the structural characteristics of these compounds.[1] Because of their relative polar properties, important efforts have been made to increase their hydrophobicity, and therefore, to produce amphiphilic molecules of industrial value.[2] Esters of hydro-*p*-coumaric,[3] hydroferulic[4] and hydrocaffeic[5] acids, as well as alkyl coumarates[6] and ferulates,[7] have been widely reported as antioxidants in food, cosmetic, and pharmaceutical formulations. Interestingly, hydrocinnamic esters have been used in the synthesis of HIV-1 protease inhibitors or as precursors for the synthesis of 1,3,4,9-tetrahydropyrano[3,4-b]indole-1-acetic acid, which is used as an analgesic, an inflammation inhibitor, and an antipyretic compound.[8]

Biocatalytic methods are often preferred over chemical methods because their mild reaction conditions avoid unwanted side reactions and decrease the possibility of producing pollutants. As an interesting alternative, the lipase-catalyzed esterification of phenolic acids (including various hydroxycinnamic derivatives) with aliphatic alcohols in anhydrous conditions has been reported.[9] Ethyl cinnamate can be produced by esterification of cinnamic acid and ethanol catalyzed by a lipase (Fig. 1). Immobilized lipases generally offer economic incentives of enhanced thermal and chemical stability, ease of handling, recovery, and reuse relative to nonimmobilized forms. The use of tailor-made synthetic hydrogels as support for enzyme immobilization has attracted attention of scientist worldwide.[10–13] In this study, we have synthesized ethyl cinnamate using hydrogel-immobilized porcein pancreatic lipase (PPL) in DMSO.

MATERIALS AND METHODS

Chemicals

Porcine pancreatic lipase (SRL Mumbai, India), ethyl cinnamate (Hi Media, Mumbai, India), cinnamic acid, acrylic acid (AAc), cinnammic acid (sd Fine Chemicals, Mumbai, India), DMSO, ethyl alcohol, hydroxyl propyl methacrylic acid (HPMA), ethylene glycol dimethacrylate (EGDMA; Merck, Schuchardt, Germany), and ammonium per sulfate (Sarabhai Chemicals, Vadodra, India) were of analytic grade and were used as received. Porcine pancreatic lipase was immobilized on poly(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel treated with formalin (2%). Its binding efficiency was 95.58% and lipase activity was 0.7 U/mg.

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Contract grant sponsors: University Grants Commission, Department of Biotechnology, Government of India.

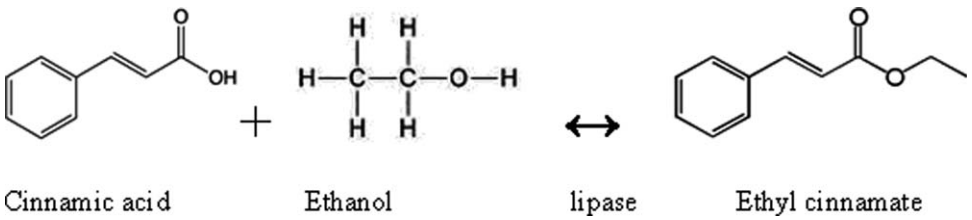

Figure 1 Synthesis of ethyl cinnamate using lipase.

Matrix

The polymeric matrix was prepared by taking AAc and HPMA in the ratio of 5 : 4 (w/w) and 5% (w/w) of a crosslinker (EGDMA).[14] The reaction mixture was heated in a water bath at 50°C for 30 min with ammonium per sulfate as the initiator. The dried network (xerogel) was synthesized as a single piece. The sol fraction if any, trapped in the body of the network was separated by a polarity gradient method by the treatment of network with water, methanol, and acetone separately in a Soxhlet apparatus from a solvent of higher polarity to one of lower polarity. Finally, the polymer was dried in an air oven for 24 h to obtain a constant weight.

Swelling capacity

Swelling capacity of dry matrix (xerogel) was determined by incubating the preweighed (3.15 g) dry matrix in distilled water for 15 h at 8°C. The swollen matrix (hydrogel) was momentarily sedimented by centrifugation (10,000 g for 1 min). The aqueous phase was decanted completely. The hydrogel was reweighed (5.6 g). The swelling capacity was found to be 0.78 times.

Immobilization of PPL on hydrogel

The xerogel was grinded in a pastel and mortar to obtain a fine powder. The powdered polymeric hydrogel was activated by exposure to formalin (2%, v/v). The hydrogel (2 g) was pretreated with Tris-HCl (0.05*M*, pH 8.5) to get rid of the activating agent formalin. This hydrogel was suspended in a solution containing PPL, and the suspension was incubated for 1 h at 37°C under shaking. The unbound protein was assayed for protein and lipase activity by standard methods. The unbound protein was subtracted from the total amount of protein used for immobilization, and the binding efficiency of the hydrogel matrix was calculated. The binding efficiency of the matrix poly(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel for PPL was found to be 97% with specific activity 0.7 U/mg. The hydrogel-bound PPL was extensively washed with Tris-HCl (0.05*M*, pH 8.5). The immobilized matrix was kept suspended in Tris-HCl (0.05*M*, pH 8.5) at 4°C till further use.

Assay of lipase activity

The lipase was assayed by a colorimetric method[15] using *p*-nitrophenyl palmitate (*p*-NPP). The reaction mixture contained 80 μL of *p*NPP stock solution (20 mM *p*-NPP prepared in 2-propanol), 10 mg of hydrogel-bound PPL, and Tris buffer (0.05*M*, pH 8.5) to make final volume to 3 mL. The reaction mixture was incubated at 45°C for 10 min in a water bath. The reaction was stopped by keeping the reaction mixture in −20°C for 7 min. The A_{410} of *p*-nitrophenol released was measured spectrophotometrically. The unknown concentration of *p*-nitrophenol released was determined from a reference curve of *p*-nitrophenol (2–50 μg/mL final concentrations in 0.05*M* Tris buffer, pH 8.5). Each of the assays was performed in triplicate, unless otherwise stated, and mean values were recorded. One unit (U) of lipase activity was defined as micromole(s) of *p*-nitrophenol released per minute by hydrolysis of *p*NPP by 1 mL soluble enzyme or 1 g hydrogel bound enzyme (weight of matrix included) at 45°C under assay conditions. Specific activity was expressed as μmol (s) of the *p*-nitrophenol released per min by 1 mg of protein.

Ester analysis using gas liquid chromatography

A reference curve was plotted between molar concentration of ethyl cinnamate (20–100 mM) and the area under the peak (retention time 1.25 min). A sample size of 2 μL was used for gas liquid chromatography (GLC) analysis. The GLC (Michro-9100, Netel Chromatographs, Thanne, India) was programmed for oven temperature 250°C, injector 260°C, and FID temperature 270°C. The detection of ethyl cinnamate was performed on 10% SE chromo WHP column (2 m × 1.8 in.) using N_2 as a carrier gas at a flow rate 30 mL/min.

Determination of amount of ethyl cinnamate

After the completion of esterification reaction at 55°C at specified time intervals (0–33 h, 120 rpm), the reaction mixture was withdrawn (2 μL) and subjected to analysis of ethyl cinnamate by GLC (Fig. 2).

Effect of bound lipase concentration

The effect of bound lipase concentration on ester formation was evaluated by using increasing amount of

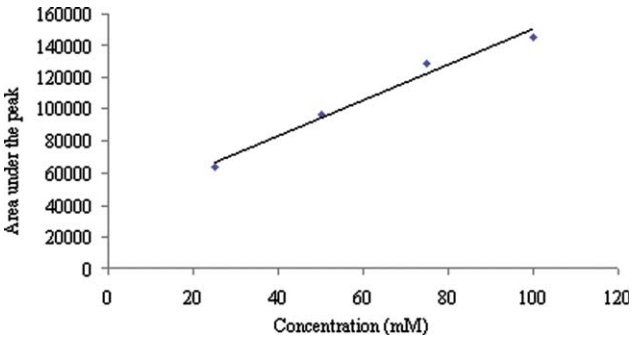

Figure 2 Standard profile of ethyl cinnamate in DMSO. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

bound lipase (5, 10, 15, 20, 25, and 30 mg) in the reaction mixture comprising 100 m*M*: 100 m*M* of cinnamic acid and alcohol, respectively, at 55°C, under shaking for 24 h.

Effect of relative proportion of reactant on ethyl cinnamate synthesis

The immobilized matrix was washed twice in 1 mL of DMSO solvent at room temperature. Thereafter, the matrix was recovered by decantation of DMSO and used to catalyze the esterification of ethyl cinnamate. The effect of concentration and relative molar ratio of ethyl alcohol and cinnamic acid on the synthesis of ethyl cinnamate was determined by keeping the concentration of one of the reactants (ethyl alcohol or cinnamic acid) at 100 m*M* and varying the concentration of second reactant (25–100 m*M*) in a reaction volume of 1 mL in DMSO. The esterification was carried out using matrix bound lipase (20 mg) at 55°C in a 5-mL teflon-coated screw-capped vial for 24 h under continuous shaking (120 rpm). The ethyl cinnamate formed in each of the combinations of the reactants was determined by GLC analysis.

Effect of reaction time for synthesis of ethyl cinnamate

The reaction mixture (2 mL) contained 20 mg of hydrogel-bound lipase and 100 m*M* each of ethyl alcohol and cinnamic acid in DMSO. The reaction mixture was incubated at 55°C in an incubator under shaking (120 rpm) up to 33 h. The reaction mixture was sampled (2 μL) in duplicate at an interval of 3 h and subjected to analysis by GLC for the formation of ethyl cinnamate.

Effect of reaction temperature

Temperature for the esterification reaction was studied at 25, 35, 45, 55, 65, 75, and 85°C and for 27 h in DMSO using 20 mg bound lipase under shaking (120 rpm). The ethyl cinnamate formed in each case was determined by GLC.

Effect of addition of molecular sieves on synthesis of ethyl cinnamate

Molecular sieves were used to study the effect of water quenching on the synthesis of ethyl cinnamate by hydrogel-bound lipase. To the above reaction mixture prepared in DMSO, varying amounts (25–500 mg) of molecular sieves were added. The esterification was carried out in duplicate by adding 20 mg of bound lipase at 65°C with continuous shaking for 27 h. Ethyl cinnamate synthesized in each case was determined by GLC.

Reusability of bound lipase on synthesis of ethyl cinnamate

The formation of ethyl cinnamate from cinnamic acid and ethyl alcohol (100 m*M* : 100 m*M*) with bound lipase was assayed for four cycles of 27 h each. After each cycle of esterification, the bound lipase was washed twice for 5 min each in 1 mL DMSO at room temperature. Thereafter, DMSO was decanted and matrix was reused for fresh cycle of ester synthesis under similar conditions.

RESULTS

Effect of biocatalyst load

Porcine pancreatic lipase was immobilized on poly (AAc-*co*-HPMA-*cl*-EGDMA) hydrogel treated with formalin (2%). Its binding efficiency was 97% and lipase activity was 0.7 U. The effect of varying concentration of bound lipase on ester formation was evaluated by increasing the concentration of the hydrogel-bound biocatalyst (i.e., 5, 10, 15, 20, 25, and 30 mg) in the reaction system. The esterification reaction with ethyl alcohol : cinnammic acid (100 m*M* : 100 m*M*) in DMSO was performed (Fig. 3). The formation of ester remained decreased with an increase in concentration of matrix bound lipase

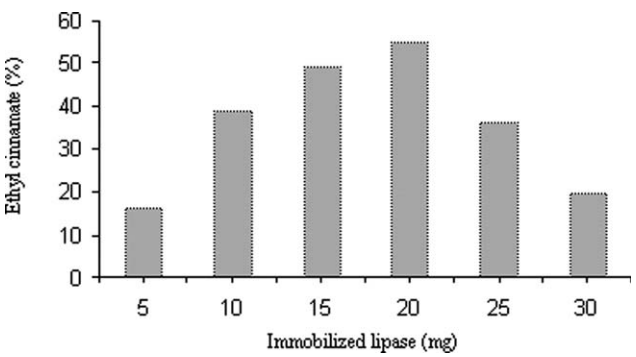

Figure 3 Effect of immobilized lipase concentration.

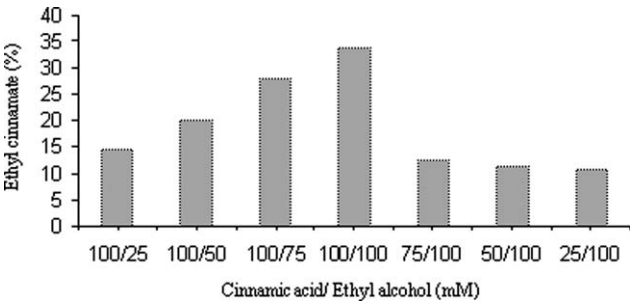

Figure 4 Effect of relative proportion of reactants.

under continuous shaking condition after 27 h at 55°C. In the subsequent esterification reactions, 20 mg of hydrogel bound lipase was used for biocatalysis in organic media.

Effect of relative proportions of reactant on synthesis of ethyl cinnamate

The effect of relative molar concentration of each of the reactants on the synthesis of ethyl cinnamate was studied. The formation of ester was higher when cinnamic acid and alcohol were used as 100 m*M* : 100 m*M* in DMSO under continuous shaking conditions after 27 h at 55°C (Fig. 4). In the subsequent reactions, same concentration of reactant was used. Amount of ethyl cinnamate was estimated from a standard profile of pure ethyl cinnamate prepared in DMSO.

Effect of reaction time on synthesis of ethyl cinnamate

The effect of reaction time on synthesis of ethyl cinnamate using immobilized lipase was studied at a temperature of 55°C in DMSO under shaking condition up to 33 h. The synthesis of the ester was time-dependent and a maximum amount of ethyl cinnamate (39.7 m*M*) was produced after 27 h of reaction when ethyl alcohol and cinnammic acid were used at 100 m*M* each in DMSO (Fig. 5). Thus, in subsequent reaction, a reaction time of 27 h was considered optimum to perform synthesis of ethyl cinnamate using bound lipase under shaking.

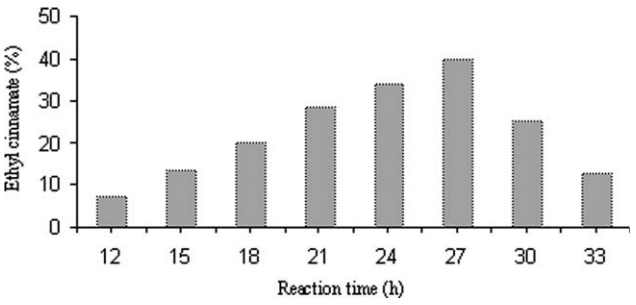

Figure 5 Effect of reaction time on synthesis of ethyl cinnamate.

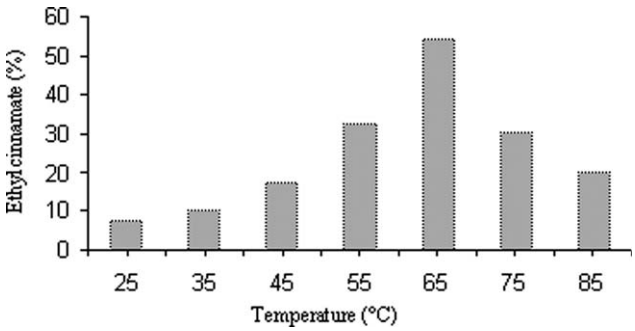

Figure 6 Effect of reaction temperature.

Effect of reaction temperature for ester synthesis

The effect of change in the reaction temperature on the synthesis of ethyl cinnamate by immobilized lipase was studied up to 85°C. Maximum synthesis (54.1 m*M*) of ethyl cinnamate was obtained at 65°C after 27 h (Fig. 6). At 75 (30.3 m*M*) and 85°C (20.4 m*M*) marked decrease in the synthesis of ethyl cinnamate was noticed that might be on account of degradation of the lipase.

Effect of addition of molecular sieves on esterification

The esterification reaction resulted in formation of water as a byproduct of the reaction, and its removal using molecular sieves might enhance the synthesis of ester by pushing the reaction equilibrium in the forward direction. However, when the effect of molecular sieves were studied by adding molecular sieves (25–300 mg per reaction volume), a gradual decline (53.7–16.1 m*M* ethyl cinnamate) in the amount of ester formed was noticed (Fig. 7). Thus, the addition of molecular sieves had a deleterious effect on the esterification reaction in this study.

Reusability of immobilized enzyme for ester synthesis

The bound lipase when repetitively used to perform esterification at 65°C under optimized conditions a steep decline in ethyl cinnamate synthesis was

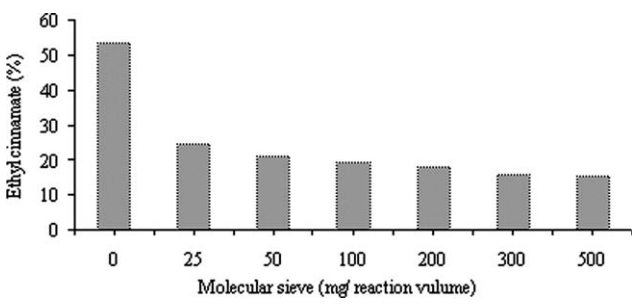

Figure 7 Effect of molecular sieves on the synthesis of ethyl cinnamate.

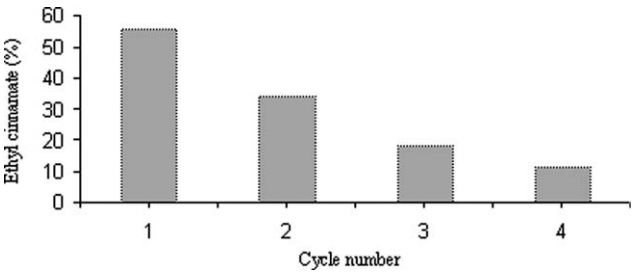

Figure 8 Reusability of bound lipase.

noticed and only 11.6 m*M* could be produced in 27 h under shaking (Fig. 8).

DISCUSSION

Lipase is a ubiquitous activity found to be possessed by most of the organisms. Lipases are very widely used enzymes in organic synthesis[16] as well as in clinical analysis and industry.[17] They can hydrolyze fats and oils or can also function in esterification or transesterification synthetic reactions.[18,19] In aqueous emulsions, they catalyze the stereospecific hydrolysis of esters, including *N*-protected amino acid methyl esters. The performance of the porcine pancreatic (commercial) lipase for hydrolysis of *p*-NPP in aqueous phase was studied by systematically evaluating the effect of various physical and chemical parameters.

The PPL was efficiently immobilized on poly(AAc-*co*-HPMA-*cl*-EGDMA). The bound lipase was subsequently used to synthesize ethyl cinnamate esters under optimized conditions in a water-free organic solvent system. Ester synthesis in the water-free media/organic solvents could be achieved very easily.[20,21,22] Thermostable lipases can overcome the problem of degradation of lipase at high temperature. In this study, a poly(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel was used for immobilization of thermotolerant commercial porcine pancreatic lipase. When the effect of amount of concentration of biocatalyst was studied; it was observed that the ester synthesis was maximum at 20 mg concentration of biocatalyst. Further increase in the concentration of biocatalyst prompted a decline in the yield of ester. Such inhibitory effect of increasing biocatalyst concentration might be on account of rapid accumulation of product (ester) in the vicinity of the biocatalyst thereby causing a product-mediated feedback inhibition mechanism.

In the esterification reaction, the hydrogel bound lipase optimally produced ethyl cinnamate at 65°C in 27 h. At the extended reaction, it was observed that ester synthesis declined, which might be because of degradation of its accumulated ethyl cinnamate ester into the reaction mixture. However, further increase in the reaction temperature to 75 or 85°C drastically decreased the esterification rate. The alteration in temperature of reaction mixture might interfere with the porosity, hydrophobic character, and diffusion of the reactants and/or products at the catalytic site of enzyme or hydrogel. It has been previously reported that cinnamic acid is unstable at high temperature.[23] At temperature more than 65°C, there was no further increase in the amount of ester synthesized, which might be on account of denaturation of the lipase as well as alteration in the native structure of lipase. The synthesis of ethyl cinnamate in the presence of bound PPL appeared to be optimum (54.1 m*M*) when ethyl alcohol and cinnamic acid were used at 100 m*M* each in DMSO at 65°C in 27 h. A relative excess molar concentration of either acid or alcohol would denature or precipitate the protein, and such effect would inactivate the biocatalyst and thus would decrease the ester synthesis. Also, it was likely that excess of acid might have partially inactivated the bound lipase because of charge alteration/charge rearrangement at the catalytic site of the hydrogel-bound biocatalyst. In past, we have reported that optimal synthesis of ethyl laurate and ethyl propionate at an equimolar proportion of reactants (100 m*M* each) in *n*-nonane.[24] In another study, effect of acetic acid concentration on esterification reaction using lipase SP435 was studied.[25]

Since reactants/products are not soluble in *n*-alkanes, and were only soluble in DMSO, we have chosen the DMSO as a model solvent in the present study. Thus, it appeared that choice of an appropriate solvent system is critical for the synthesis of ester using immobilized PPL. Water and alcohol that are produced as byproduct(s) of the esterification or *trans*-esterification reaction respectively performed by biocatalysts in organic media have several adverse effects on the reaction and enzyme activity/performance. Esterification is generally a water-limited reaction because the equilibrium catalyzed by hydrolytic enzymes is in favor of hydrolysis.[26] The addition of molecular sieves inhibited the forward reaction. Molecular sieves are an important class of synthetic adsorbents, which possess high porosity with pores of uniform size and essentially of molecular dimensions.[27] When molecular sieves were added in the reaction mixture, and the effect of its concentration in the reaction system on rate of esterification by hydrogel-bound lipase was studied, less than 50% yield was noticed, and with the increasing concentration of molecular sieves the esterification decreased. Thus, the presence of molecular sieves in the reaction mixture was redundant to improve the esterification between ethyl alcohol and cinnamic acid.

This study thus showed that poly(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel network-immobilized-lipase (porcine pancreatic lipase) efficiently performed esterification of ethyl alcohol and cinnamic acid into

ethyl cinnamate at a temperature of 65°C, addition of molecular sieves in conjunction with a hydrophobic matrix caused a decline in the rate of esterification; and an equimolar ratio of ethyl alcohol as well as cinnamic acid was necessary for achieving an optimal rate of esterification in a reasonable period of 27 h under shaking.

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