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Porcine pancreas lipase catalyzed synthesis of lauryl laurate in organic solvent media: A kinetic study

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The esterification of lauric acid with lauryl alcohol was studied using lipase from *Porcine pancreas,* with particular emphasis on the effect of the pertinent variables and kinetic aspects of the reaction. The reaction was studied in eight different solvents having hydrophobicity (the logarithm of octanol-water partition coefficient, log *P*) values ranging from 0.6 to 3.5 with constant water content in the reaction mixture and *n*-hexane was the most suitable solvent. The initial rates of the reaction were attempted to correlate with solvent properties and a significant good correlation was obtained with solvent hydrophobicity (log *P*) and water solubility (log *Sw*). The kinetics of the esterification reaction conformed to the so-called Ping-Pong Bi-Bi mechanism with alcohol inhibition.

Keywords: *Porcine pancreas* lipase, Lauryl laurate, Kinetics, Mechanism, Ping-Pong Bi-Bi

Enzymatic synthesis offers various advantages over chemical synthesis such as lower energy requirement, enhanced selectivity, substrate specificity and recoverability of the enzymes and quality of products[1,2]. In recent years, non-aqueous enzymatic catalysis has grown considerably for its potential applications in flavor, perfumery, fine chemical, agrochemical, pharmaceutical and drug industries. In non-aqueous enzymatic catalysis, solvent selection is an important factor for the successful application of enzymes. The use of organic solvents in such reactions increases the solubility of organic substrates, which are insoluble or poorly soluble in water[3], as well as facilitates the enzyme and product recovery. However, a large-scale use requires that the solvent must be able to dissolve high substrate concentrations to obtain high productivity[4]. Enzymes used predominantly in organic media require some water to achieve good catalytic activity[5]. In some esterification reactions, the initial activity of the enzyme exhibits an optimum value at certain water content in the reaction medium[6]. Lipases (EC 3.1.1.3) are the most widely used enzymes for synthesis of esters through some esterification and transesterification reactions due to their greater stability at high temperatures and over a wide range of *p*H and easy handling.

Lauryl laurate, a cosmetic ester imparts unique conditioning, emolliency, moisturization, emulsification, thickening, slip and superfatting in skin and hair care applications. Lipase-catalyzed synthesis of esters is gaining importance in view of the environmentally benign process conditions involved, mild temperature and pressure as well as possibility of integration of reaction-separation system for process intensification. *Porcine pancreas* lipase is one of the cheap and commercially available non-microbial enzymes, which has high thermostability and activity in anhydrous reaction media.

Although lipases are known to be potential and environmentally benign catalyst for producing a number of commercially important esters[7-9], detail kinetic studies, which can provide a better insight of the mechanism of catalyzed reactions and appropriate kinetic models have not been reported for several reactions. For the design of suitable reactors, kinetic information on the rate of product formation and the effect of change in system conditions is needed.

In the present work, kinetics and mechanism of esterification of lauric acid with lauryl alcohol catalyzed by *P. pancreas* lipase has been investigated. In order to optimize the esterification process, the effects of various parameters such as solvent properties, reaction temperature, concentration of

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lipase and substrate on the initial rate have been studied. The reactions that have been studied are shown in Fig. 1.

Materials and Methods

Enzyme and chemicals

Porcine pancreas lipase (specific activity 41 U/mg protein) was procured from Sigma Chemicals, USA. Lauric acid, lauryl alcohol and solvents of analytical grade were procured from CDH Pvt Ltd., Mumbai, India. The solvents were distilled, prior to use and their water content was determined by Karl-Fischer Titrator (Spectralab MA 101-B, Alpha Instruments, New Delhi).

Reaction procedure

Standard lauryl laurate was prepared by the procedure reported in our previous communication[10]. The solvent effect experiments were carried out under optimized reaction conditions with 200 m*M* lauric acid and 200 m*M* lauryl alcohol dissolved in 10 mL anhydrous solvent in presence of 10 mg/mL lipase. The reactions were carried out in a 50 mL round bottom flask by mixing the reaction substrates at a speed of 200 rpm with a magnetic stirrer for about 3 h. The reaction temperature was maintained at 27°C. Aliquots of samples were withdrawn periodically at 30 min interval, diluted with the solvent to a known level, the enzyme was filtered out and the samples were analyzed by gas chromatography (GC) for determination of the product concentration. All experiments were conducted in duplicate and the reproducibility was found to be ± 5%.

All the kinetic experiments were carried out in a 50 mL round bottom flask using *n*-hexane as the solvent. The temperature, substrate and lipase concentrations were varied systematically from 20-60°C, 250-450 m*M* and 5-30 mg/mL, respectively. The reaction mixture was agitated vigorously with a magnetic stirrer. In all experiments, the water concentration was maintained constant and determined with a Karl Fischer Titrator.

Analytical methods

Lauric acid and lauryl laurate concentrations were determined by GC analysis, performed on a Chemito Model 8510 FID GC equipped with GC data processor. A fused silica wide bore GC analytical column NUKOL (15 m × 0. 53 mm i.d. × 1.0 μm film thickness) with hydrogen flow rate of 7 mL min^{-1} was used for separation of components of reaction mixture. The column oven temperature was programmed from 120-210°C at the rate of 5°C min^{-1}. The injector and detector ports temperatures were maintained at 230°C each.

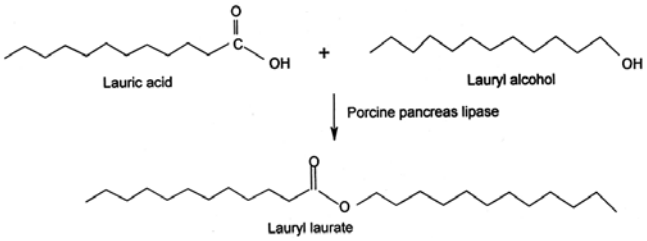

Fig. 1—Synthesis of lauryl laurate by lipase in organic media

The initial reaction rates were determined from the percentage conversion of the product *versus* reaction time profiles corresponding to the first 10% conversion and expressed as the amount of substrate converted per unit time and weight of the lipase (mmol/min g). The enzyme activity was determined at 27°C according to previously described method[10]. One unit of lipase activity was defined as the amount of enzyme that produced 1 μmol of lauryl laurate per min under the assay conditions.

Results and Discussion

Effect of solvent on initial rate

Solvent effect, in general, of lipase-catalyzed reactions was interpreted from the structural and physico-chemical properties of the solvent. The most important solvent properties were hydrophobicity (log *P*), water solubility (log S_w), electron-pair acceptance and donation index (E_T^N + DN^N), polarizability (ρ) and dielectric constant (n_d). The solvents were selected on the basis of their log *P* values (Table 1), the range of which lies between 0.6-3.5, as the activity and stability of the enzymes were reported to be optimal in this range[7]. The log *P* value was proposed as a quantitative measure of solvent polarity[11] and the enzyme activity for lipase catalyzed reactions, in general, increases with increasing log *P* value of the solvent. It was apparent that the initial rate (r) increased almost linearly with an increase of solvent log *P* value (Fig. 2a) and the lipase could catalyze the esterification in a wide variety of solvents.

The increased lipase activity with log *P* of the solvent in the present study was similar to that reported for enantioselective esterification of racemic glycidol with butyric acid by *P. pancreas* lipase[12] and

Table 1—Properties of solvents used in the study[a]

Solvent	log *P*	log *Sw*	E_T^N	DN^N	Dielectric constant	Polarizability (in units of 10^2)
Dichloromethane	0.60	-0.84	0.321	0.03	8.90	6.48
Ethyl acetate	0.70	0.21	-	-	6.00	9.70
Diethyl ether	0.85	-1.19	0.124	0.49	4.34	10.20
Chloroform	2.00	-1.12	0.260	0.10	4.81	9.50
Toluene	2.50	-1.80	0.096	0	2.38	11.80
Carbon tetrachloride	3.00	-1.930	0.09	0	2.24	11.20
Cyclohexane	3.20	-2.25	-	-	2.00	11.00
n-Hexane	3.50	-2.39	0.074	0	1.88	11.90

[a]log *P* is the logarithm of partition coefficient in octanol-water system[5]. log *S*w is the logarithm of saturated solubility of water in solvent on molar basis, E_T^N is the normalized electron pair acceptance index and DN^N is the Gutmann's donor number.

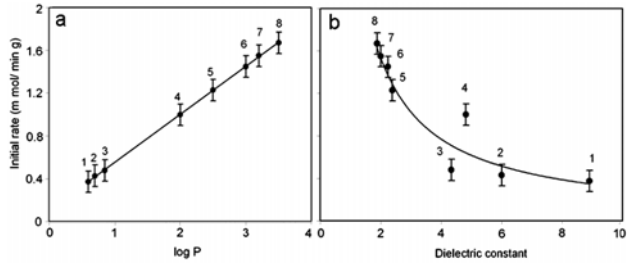

Fig. 2—**(a):** Initial rate as a function of solvent hydrophobicity; and **(b):** Initial rate as a function of dielectric constant [L. acid] = 200 m*M*; [L. alc] = 200 m*M*; lipase = 10 mg/ml; Temp. = 27°C

2-chloropropionic acid with *n*-butanol by *Candida cylindracea* lipase[11]. For esterification of butyric acid with propanol in immobilized *Pseudomonas cepacia* lipase at constant water activity, the relation of initial rate and log *P* was found to be sigmoidal in nature[13]. A good correlation of log *P* and mole fraction of ester at equilibrium was also observed for *Chromobacterium viscosum* lipase-catalyzed esterification of decanoic acid with glycerol[14].

A good correlation was developed between biocatalytic activity and solvent log *P* value[15] and the Hildebrand solubility parameter[16,17]. The correlation between polarity and activity paralleled the ability of organic solvents to distort the essential water layer that stabilized the biocatalyst. Biocatalysis in organic solvents was low in more hydrophilic solvents such as dioxane, tetrahydrofuran and chloroform; having a log *P*< 2 may strips-off the essential hydration layer on the enzyme surface and thus caused the enzyme inactivation. On other hand, in hydrophobic solvents such as cyclohexane, *n*-hexane and *n*-pentane, having a log *P* values between 2 and 4, biocatalytic activity was moderate, as they preserved the microaqueous layer around the enzyme, thereby retaining the activity and was high in apolar solvents having a log *P* > 4.

The water solubility of the solvent (log S_w) was recognized as the most useful parameter of the solvent polarity for correlating the rates of esterification reactions[18]. A good relationship between initial reaction rate with water solubility (log *Sw*) for this esterification reaction was observed. However, initial reaction rate decreased with log *Sw* of the solvent, which was in agreement to esterification of oleic acid with ethanol by *P. pancreas* lipase[7], suggesting that solvents with low water solubility value favoured esterification reaction.

Non-polar solvents were also chosen from the well accepted rules for the effects on biocatalytic activity[3]. For predicting performance of reaction media using polarity as the criteria, there is also other fundamental basis, which seems to rely on the donor-acceptor interactions of the solvent including hydrogen bonding capability. Solvation of water requires both donation and acceptance of hydrogen bonds (or electron pair) or other dipole-dipole interactions. Accordingly, an attempt was made to correlate the initial rate with the sum of normalized electron pair acceptance index (E_T^N) and Gutmann's donor number (DN^N).

The correlation for the present system seemed to be rather weak in comparison to that reported in early study[18], wherein a good correlation was established between log *Sw* and (E_T^N + DN^N) of several organic solvents. The weak correlation might perhaps be attributable to the lack of data points for two solvents, whose (E_T^N + DN^N) values were not known. However, the observed trend on decrease of initial rate with (E_T^N + DN^N) might be considered reasonable.

The polarizability (ρ) is another property that represents the ability of a solvent to stabilize the charge of a dipole in solution. In order to understand the effect of important solvent properties on reaction

rates, we attempted to correlate the reaction rate with solvent ρ, but no good correlation was obtained. When solvent ρ combined with log *P* and expressed in terms of log *P* divided by ρ, a representative effect of this parameter on initial rate could be deduced. This relationship again indicated the important role of solvent hydrophobicity on initial rate.

Since n_d is a function of ρ, we attempted to correlate initial rate with n_d. It was apparent that initial rate decreased exponentially with increasing n_d of solvents (Fig. 2b). Hence, n_d did not represent a statistically sound correlation, but the observed trend of decreasing rate appeared to be reasonable.

Effect of lipase concentration on initial rate

In enzymatic reactions, one of the main factors is the cost of enzyme. Thus, it is important to achieve high esterification at low enzyme concentration. Therefore, the effect of lipase concentration on initial rate for the esterification reaction was studied using equimolar concentrations of lauric acid and lauryl alcohol, varying the lipase concentrations. It was observed that with the increase of lipase concentration increased the initial rate almost linearly (Fig. 3) suggesting that the reaction might be kinetically controlled. Earlier, similar observations were reported for esterification reactions catalyzed by lipase from *Rhizomucor miehei*[19] and *Penicillium simplicissimum*[20], respectively.

Effect of temperature on initial rate

The initial rate of esterification increased exponentially with the increase of reaction temperature from 25° to 50°C, without catalyst inactivation. Similar observation was reported for the synthesis of oleic acid esters catalyzed by *Rhizomucor miehei* lipase immobilized on a macroporous anion-exchange resin[21]. The reaction rate started decreasing at temperature higher than 50°C, as thermal deactivation of the enzyme occurred which was in agreement with those reported in earlier studies[22-24].

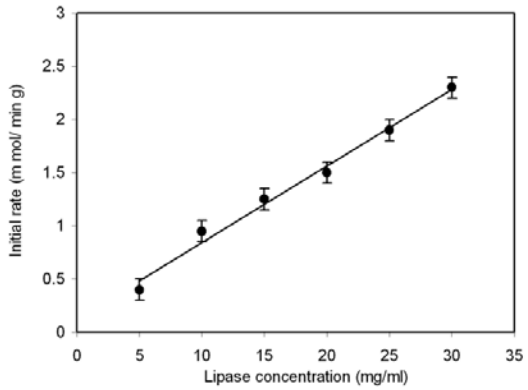

Fig. 3—Initial rate as a function of lipase concentration: [L. acid] = 200 m*M*; [L. alc] = 200 m*M*; Temp. = 27°C

The effect of temperature on the esterification reaction rate constant (*k*) for lauryl laurate synthesis was studied under same reaction conditions. The reaction rate constant (*k*) was obtained from the Arrhenius rate equation:

$$k = A \exp(-E_a/RT) \quad \ldots (1)$$

where A is the pre-exponential factor; E_a, is the activation energy and T, is the temperature.

The E_a value for esterification of lauric acid with lauryl alcohol was estimated to be 12.8 Kcal/mol. In immobilized system, the E_a value was an apparent value and due to diffusional limitations in immobilized lipase, the apparent value might be expected to be higher than the true value[25]. The reported values of E_a for the synthesis of ethyloleate[7] and oleyl cinnamate[26] were reported to be 24.0 and 43.6 Kcal/mol, respectively. The E_a value for the esterification of oleic acid was higher than lauric acid, which might be attributed to higher carbon number of oleic acid. The highest E_a value for cinnamic acid was perhaps attributable to its aromatic ring, which usually has lower reactivity.

Effect of substrate concentration on initial rate

To perform a subsequent kinetic analysis, two series of experiments were carried out, one at different constant lauryl alcohol concentrations to study the change in reaction rate with lauric acid concentration and second at different constant lauric acid concentrations to study the dependence of the reaction rate on lauryl alcohol concentration at different acid and alcohol molar concentrations and at 50°C temperature. Fig. 4 shows the variation of initial rate of esterification reaction as a function of lauric acid concentration for various concentrations of lauryl alcohol. The figure shows no inhibition by lauric acid at all lauryl alcohol concentrations tested and the initial rate increased with increase of lauric acid concentration at a constant lauryl alcohol concentration. However, for alcohol concentrations higher than 350 m*M*, the reaction rate decreased, suggesting the alcohol inhibition effect.

Fig. 5 shows dependence of initial rate of esterification on lauryl alcohol concentration for various lauric acid concentrations tested. The initial rate increased proportionally up to an alcohol

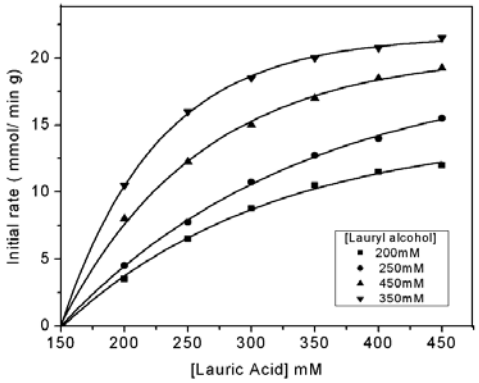

Fig. 4—Initial rate as a function of lauric acid concentration at various concentrations of lauryl alcohol:lipase = 30 mg/ml; temp. = 50°C

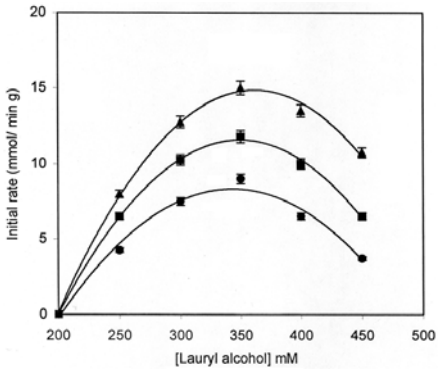

Fig. 5—Initial rate as a function of lauryl alcohol concentration at various concentrations of lauric acid: [L. acid] (-•-): 250 m*M*; (-!-): 350 m*M*; (-7-) : 450 m*M*

concentration of 350 m*M,* however, at higher alcohol concentrations, a decrease of initial rate for all lauric acid concentrations tested was observed, suggesting that lauryl alcohol was a substrate inhibitor for esterification reaction. Similar inhibition effect was also observed for *P. pancreas* lipase catalyzed synthesis of ethyl oleate and ethyl myristate using immobilized lipase in *n*-hexane[7,27-30] and in supercritical carbon dioxide[31] respectively.

Reaction mechanism and kinetics

The reaction mechanism was elucidated by Lineweaver-Burk plot (Fig. 6). A set of parallel lines was obtained, indicating the Ping-Pong Bi-Bi mechanism with alcohol inhibition, similar to that reported for synthesis of tetrahydrofurfuryl butyrate and butyl isobutyrate in *n*-heptane by immobilized *Candida antarctica* lipase[32,33].

In Ping-Pong Bi-Bi mechanism, only one substrate is bound to the enzyme at any time to form the acyl enzyme complex. As soon as one product is formed and released, subsequently, the other substrate binds to the modified enzyme (acylated enzyme) to form the second product. For this mechanism, the initial velocity equation is represented as:

$$V = \frac{V_{max}[Acid][Alcohol]}{K_{m(acid)}[Alcohol](1+[Acid]/K_i) + K_{m(alcohol)}[Alcohol][Acid]} \quad \ldots (2)$$

where [Acid] and [Alcohol] represent the initial molar concentration of lauric acid and lauryl alcohol respectively. $K_{m(acid)}$ and $K_{m(alcohol)}$ are the respective affinity constants, K_i is the inhibition constant for lauryl alcohol and V_{max} is the maximum reaction rate. The kinetic parameter values can be determined from

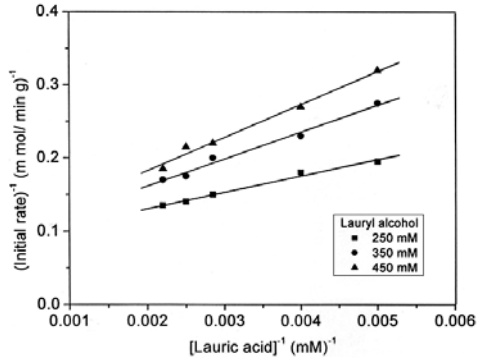

Fig. 6—Reciprocal plot of lauric acid concentrations and initial rate at various concentrations of lauryl alcohol

Lineweaver-Burk plot (Fig. 6). However, for better accuracy, these values were computed from the equation for initial rates according to Eq. 2 and the values of parameters calculated using Gauss-Newton algorithm of error minimization. The K_m value was determined to be 46.55 m*M*, whereas V_{max} value was 13.30 mmol min^{-1} g^{-1}. The results of the present study revealed a higher value of V_{max} in comparison to those obtained for other lipase-catalyzed reactions[7,27-30]. It seemed that *P. pancreas* lipase might be considered somewhat more reactive for this reaction system. However, its activity was considerably lower than that of bovine liver catalase[34].

In conclusion, a systematic solvent effect study revealed that the reaction rate of the esterification between lauric acid and lauryl alcohol catalyzed by *P. pancreas* lipase was affected by such properties as solvent hydrophobicity, water solubility, electron pair acceptance and donation index, polarizability and dielectric constant. Hydrophobic solvents were found to be more preferable than hydrophilic ones. The solvent properties also affected the thermodynamic

equilibrium position of the reaction. The important factors that affected the initial rate of reaction were the lipase and substrate concentrations and the reaction temperature. The kinetics of the reaction conformed to the Ping-Pong Bi-Bi mechanism with lauryl alcohol inhibition effect.

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