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Effect of temperature on Candida antartica lipase B activity in the kinetic resolution of acebutolol

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Abstract. Thermodynamic studies of free *Candida antartica* lipase B in kinetic resolution of acebutolol have been carried out to characterize the temperature effects towards enzyme stability and activity. A decreased in reaction rate was observed in temperature above 40°C. Thermodynamic studies on lipase deactivation exhibited a first-order kinetic pattern. The activation and deactivation energies were 39.63 kJ/mol and 54.90 kJ/mol, respectively. The enthalpy and entropy of the lipase deactivation were found to be 52.12 kJ/mol and -0.18 kJ/mol, respectively.

1. Introduction

Generally, chiral drugs including beta blockers are available as racemic mixture consists of (S) and (R)-enantiomers. However, the beta blockage properties of most beta blocker drugs reside on the (S)-enantiomer. The (R)-enantiomer is not active and sometimes responsible for the undesired effect. Unfortunately, until recently, most of the beta blocker drugs are still market in racemic form. Among these is acebutolol [N-{3-acetyl-4-[(2-hydroxy-3-isopropylamino)propoxy] phenyl}butanamide, which is effectively used in treating hypertension and cardiac arrhythmias [1,2].

A large number of studies have been done with various strategies and routes to produce pure enantiomer of beta blocker such as asymmetric synthesis, biotransformation and chromatography methods as well as kinetic resolution. Most of the enantiomeric resolution of beta blockers performed by direct and indirect method of high performance liquid chromatography HPLC [3-7]. Although these techniques are capable of producing high enantiomeric excess, they are costly, time consuming and required more rigid condition to be met.

Asymmetric synthesis of acebutolol have been reported earlier. Wang *et al.,* synthesized acebutolol from 4-aminophenol via Fries rearrangement, acetylation of the amino and hydroxyl group, hydrolysis and butyrylation. 51.5% of overall yield was achieved [8]. Acebutolol pure enantiomers have also been synthesized through hydrolytic kinetic resolution (HKR), condensation and followed by reaction with propyl amine, obtained overall yield and optical purity of 47.3 and >98%, respectively [9]. However, both techniques involved various intermediate product couple with multi step and lengthy reaction.

A kinetic resolution (KR) is one of the promising method in preparation of pure enantiomer of chiral drugs including beta blockers. This method offers advantages of less energy consuming and lower production cost [10]. In kinetic resolution, the two enantiomers of a racemate are transformed

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into products at different rates [11]. Generally, there are two methods employed in enzymatic kinetic resolution of racemic drugs, which are esterification and hydrolysis. As for beta blockers, enantioselective acylation or transesterification is the common method of enzymatic kinetic resolution that has been applied in the production of its pure enantiomers.

Various types of lipase and solvent was studied for the resolution of propranolol by Chiou *et al.*, [12]. Another example of enzymatic acylation of beta blocker was conducted by Quan *et al.,* which demonstrated a control of the enzymatic acylation position at the hydroxyl or amino group of propranolol [13]. Recently, preparation of pure enantiomer of propranolol [14] and atenolol [15] have been carried out. In both studies, the effects of reaction parameters on the reaction rate and enantiopreference of immobilized *Candida antartica* lipase B (CALB), as well as the effects of different immobilization protocol has been investigated. However, up to now, there is no published data on the thermodynamic study of beta blockers enzymatic kinetic resolution using CALB. Therefore, in this paper, an attempt is made to investigate the thermodynamic properties of acebutolol kinetic resolution catalyzed by CALB. Temperature effect on enzymatic reaction can be determined by understanding the thermodynamic properties such as entropy, free energy change and enthalpy change.

2 .Material and Methods

2.1 Material
The lipase from *Candida antarctica* fraction B (Novozym CALB L) was obtained from Novo Nordisk (Bagsvaerd, Denmark). All other lipases was supplied by Sigma-Aldrich (Michigan). Racemic acebutolol was purchased from Suzhou Yuanfang Chemical Co., Ltd (Shanghai, China). All other chemicals used in this work were of analytical grade and were supplied by Fisher Scientific (Nepean, Ont., Canada) and used without further purification.

2.2 Kinetic resolution of racemic acebutolol
The kinetic resolution of racemic acebutolol through enantioselective transesterification was conducted in a 250 ml Erlenmeyer flask. Racemic acebutolol as substrate was added to 20 ml of selected solvent followed by acyl agent and then by enzyme. The mixture was continuously shaken at 200 rpm and 37 °C in an incubator shaker. Periodically, 1 ml aliquot was withdrawn from the reaction medium for HPLC analysis.

2.3 HPLC analysis
The concentration of the optically pure product from the reaction is determined by HPLC. The HPLC analysis is based on the method explained by Ekelund *et al.,* [16]. Chiralcel OD (25 cm x 4.6 mm I.D, 10 um) type of column from Daicel Chemical Industries is used with a UV detector with a wavelength of 320 nm. The mobile phase used is hexane-ethanol-diethylamine with the ratio of 90:10:0.1. The injection volume and mobile phase flowrate is 1 uL and 1.0 ml/min, respectively.

2.4 Thermodynamic studies on CALB
The thermodynamic studies for kinetic resolution of acebutolol were divided into two parts, mainly on the effect of temperatures on lipase activity and stability. The first part consists of studies on the effect of temperatures on the production rate implemented at various reaction temperatures of 30, 35, 40, 45, 50, 55 and 60 °C. In the second part, the irreversible denaturation rate for CALB under different thermal treatment was studied to determine half-life time as well as the thermal resistance strength. The kinetics of irreversible denaturation was studied by incubating CALB in the substrate without vinyl acetate at temperatures of 45, 50, 55 and 60 °C for variable times of 1, 2, 3 and 4 days. The residual activity of the lipase was then examined by implementing typical kinetic resolution as described in section 2.2.

The thermodynamic properties such as $\Delta G,$ free activation enthalpy (kJ/mol) is given by equation (1) and equation (2).

$$\Delta G = -RT \ln K \qquad\qquad (1)$$

$$\Delta G = \Delta H - T.\Delta S \qquad\qquad (2)$$

R represents Universal Gas Constant with the value of 8.314 J/mol K. Whereas, ΔH and ΔS represent activation enthalpy (kJ/mol) and activation entropy (J/mol.K), respectively.

Combining equation (1) and equation (2) and solving for ln k gives;

$$\ln K = \frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad\qquad (3)$$

The thermal deactivation constant (k_d) used in the estimation of kinetic and thermodynamic parameters is a function of temperature as given by Eyring equation,

$$k_d = T^{\frac{k_B}{h}} e^{\Delta S/R} e^{-\Delta H/R} \qquad\qquad (4)$$

The linearized form of equation (4) gives

$$\ln\left(\frac{k_d}{T}\right) = \ln\left(\frac{k_B}{h}\right) + \left(\frac{\Delta S}{R}\right) - \left(\frac{\Delta H}{R}\right)\left(\frac{1}{T}\right) \qquad\qquad (5)$$

Where k_d, T, k_b, h and R represent deactivation constant, absolute temperature, Boltzmann constant, Planck's constant, and gas constant, respectively. From this equation, it can be observed that, by plotting log of k_d as a function of the inverse of absolute temperature, the energy of deactivation E_d can be obtained as the product of angular coefficient of the adjusted straight-line multiplied by the value of universal gas constant R. This approach has been successfully applied by several authors in order to study the thermodynamic properties of various lipases [17-19].

3. Result and discussion

3.1 Effect of temperature on kinetic resolution of racemic acebutolol
The effect of temperature on the kinetic resolution of acebutolol was studied. Figure 1 shows that the conversion increased gradually with the increase of temperature. At low temperature, CALB unable to exhibit its highest activity. The maximum conversion of 42% was obtained when the reaction was carried out at 40°C. Further increase in the temperature brings a significant decrease in the conversion. At high temperature, the enzyme was partially deactivated. Similar behaviours have been reported previously for CALB applied in esterification of lactic acid [20], 1-phenylethanol [21] and ketoprofen [22].
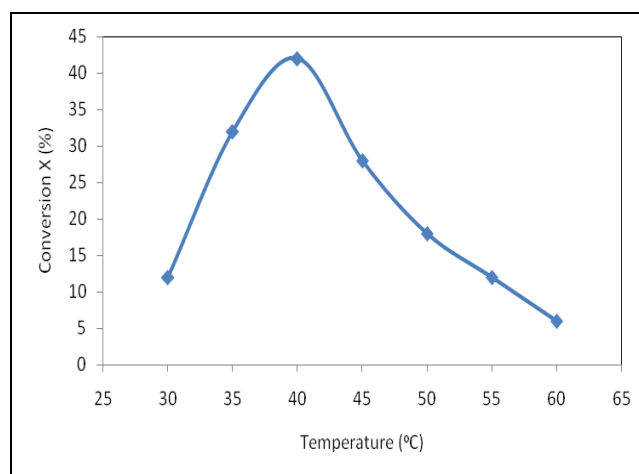
Figure 1. Effect of reaction temperature on the conversion.

3.2 Thermodynamic properties of CALB in kinetic resolution of racemic acebutolol

In this study, the activation energy was determined to be 39.63 kJ/mol. Activation energy is associated with normal enzymatic reaction in formation of product from the substrate [23]. The enzymes with low values of activation energy is favorable for industrial application [24]. Previously, Xin *et al.,* [25] obtained the activation energy of 65.04 kJ/mol based on the Arrhenius law in enzyme mediated transesterification using CALB. By using the same type of lipase, Sun *et al*., [20] estimated the activation energy for esterification of glyceryl ferulate to be 67.4 kJ/mol.

In the present work, the energy for deactivation was found to be 54.90 kJ/mol. According to Baptista *et al.,* [26], the weakening and breakage of non-covalent bonds that maintain the protein tertiary structure in the more stable folded state may cause reversible thermal unfolding of proteins. In this work, it was found that the deactivation energy was higher as compared to the activation energy. This is similar with the results obtained in other works using different types of lipase [27-29]. This finding shows the predominant effect of deactivation process taking place. In order for the unfolding of the lipase to occur, more energy was needed to overcome the higher energy barrier [28].

It was found that the lipase has an enthalpy of 52.12 kJ/mol.K and entropy of -0.18 kJ/mol.K. Similarly, negative entropy was observed during stability studies of *T. lanuginosus* lipase in transesterification reaction [27].The unique negative entropy attributed to the compaction of enzyme molecule in the system upon denaturation can only be found in biocatalytic systems [30].
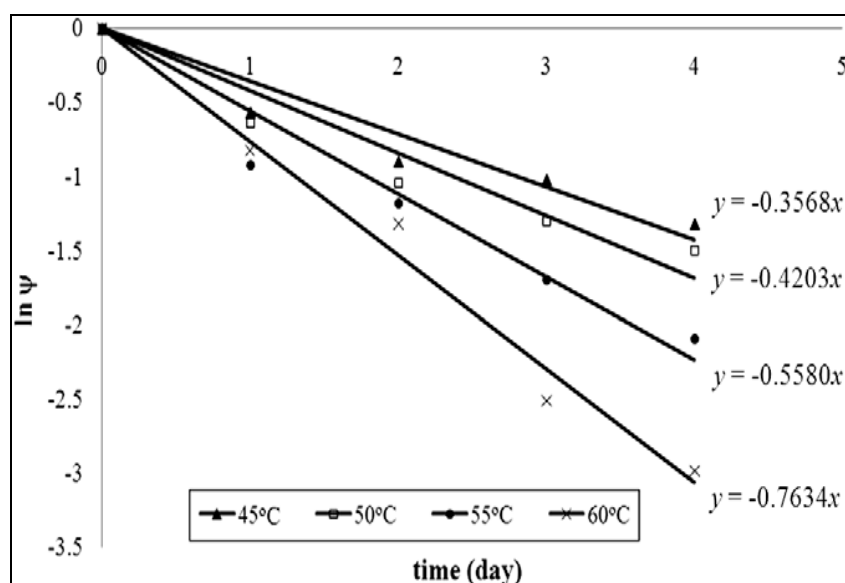

Figure 2. Semi-log plot for determination of denaturation constant

The denaturation constant, k_d at different temperatures can be estimated by plotting ln (v) against time as shown in figure 2, where v represents residual activity. It can be observed that the lipase followed the first-order denaturation pattern. The constant of CALB denaturation progressively increased from 0.012-0.031 h^{-1} with temperature. The deactivation rate was slow at low temperature but it increased rapidly with the increase in temperature. The larger the value of k_d at a particular temperature, the less stable was the enzyme. The same pattern of denaturation constant has been observed for *T.lanuginosus* lipase [27], Aspergillus *oryzae* lipase [28] and *Aspergillus niger* amyloglucosidase [29].

Another important parameter to consider in justifying enzyme stability is the enzyme half-life, $t_{1/2}$. The calculated values for half-life of the lipase were 46.73, 39.60, 29.81 and 21.79 h for 45, 50, 55 and 60 ^{o}C , respectively. From the observation, the lipase was considered to have moderate stability. At high temperatures, the irreversible denaturation effect was accelerated and more pronounced. However, the enzyme was strongly deactivated at higher temperatures.

4. Conclusion

Thermal deactivation of *Candida antartica* lipase B occurred due to enzyme distortion disturbing the active conformation of the lipase. Higher value of deactivation energy compared to activation energy implied a predominant effect of deactivation process taking place. The lipase showed good stability at lower range of temperatures but underwent a considerable deactivation at high temperatures.

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