Thermodynamic study of hydrolysis and esterification reactions with immobilized lipases

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ABSTRACT

The present paper investigate about the physical significance of thermodynamic constants such as change in Gibbs free energy (ΔG), enthalpy (ΔH), entropy (ΔS) for a simultaneous hydrolysis and esterification reactions. The hydrolysis of fish oils with immobilized Candida antarctica lipase-B (CAL-B) and esterification of tuna free fatty acids with immobilized lipases from Pseudomonas cepacia (PCL) and Thermomyces lanuginosus (TLL) have been studied. The catalytic efficiency of lipases has also been compared using turn-over number (k_{cat}) and thermal deactivation constant (K_d).The negative magnitude of ΔG<0, ΔH<0 and ΔS<0 have been obtained; correspond to the feasibility of these reactions with thermal stability of lipases at reaction conditions. A higher of 381.02 sec^{-1} turn-over number with immobilized CAL-B and PCL have been obtained, indicating a good shell life of lipase system whereas a lower value of thermal deactivation (K_d) corresponds to good activity of lipases over multiple uses.

Keywords: Enthalpy; entropy; gibbs free energy; thermal deactivation constant; turn-over number

1. INTRODUCTION

Diverse classes of lipases (EC 3.1.1.3) are available in present era, which not only differs in their activity but also show significantly unique mode of action as they are originated from different sources. They can be synthesized and extracted from various sources such as plant (Huang, 1884), animal (Carriere*et al*., 2005) and microorganisms (Jaeger *et al*., 1994).The type of reactions, catalyzed by them can be broadly classified as hydrolysis and esterification, depending upon the nature of substrate available. A hydrolysis reaction is one in which ester bonds break down in the presence of water to form alcohol and fatty acids whereas reverse happens in esterification, where ester bonds form with alcohol. It has been seen that same set of lipases can play important role in both hydrolysis and esterification reactions but with different rate and

mechanism (Sharma *et al*., 2001 & Sivozhelezov*et al*., 2009). Lipases do not change the position of equilibrium in a reaction but they change the amount of energy required to achieve the same position of equilibrium in both forward and reverse reactions. The magnitude of energy required can be explained in terms of various thermodynamic parameters such as Gibbs free energy (G), enthalpy (H) and entropy (S) for both reactants and products involved in a reaction. In total, the difference of energy states for converting reactant into product is defined in form of change of Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (ΔS)(Bailey&Ollis, 2010 and Fersht, 1999). For a lipase catalyzed reaction, the interaction of a protein molecule in lipase with reactant or product can be due to the formation of hydrogen, van der Walls, covalent, hydrophobic and electrostatic bonds. The type of interaction exists between two molecules which can be examined using ΔG, ΔH and ΔS(Li*et al*., 2011 & Iftikhar*et al*., 2008).The present study aims to understand the feasibility of a lipase catalyzed reaction in terms of their thermodynamic behavior to compare amount of energy released or required. Similarly, the catalytic activity of lipases is also evaluated in terms of two mathematical constants, known as turn-over number (k_{cat}) and thermal deactivation constant (K_d).

2. MATERIALS AND METHODS

2.1 Substrate and lipases

Three types of fish oils such as Tuna (refined and deodorized grade; Lot: 0038601), Salmon (blend; Lot: 029/11) and Herring (veterinary grade; Lot: 802-6980), were purchased from Jedwards International, Inc., Quincy MA 02169, USA in a packing of 3 liters each. For the hydrolysis of fish oil triglycerides, lipase-B from *Candida Antarctica* (CAL-B) immobilized on immobead-150 (0.15-0.3mm; <10% loss on drying), recombinant from yeast (activity >2000U/g) were used. For the selective esterification studies, *Pseudomonas cepacia* lipase (PCL) and *Thermomyces lanuginosus* lipase (TLL) immobilized on immobead-150 were used. The commercial properties of all the lipases are given in Table 1 as provided by Sigma-Aldrich, Saskatoon, Canada.

TABLE 1: Commercial properties of immobilized lipases

Immobilized lipases abbreviations (EC3.1.1.3)	Form activity	Microbial origin	Lot number and Manufacturer
Lipase B *Candida antarctica* (CAL-B) immobilized on immobead-150	White granular &>2000 U/g[a]	Recombinant from yeast	52583 and Sigma
Lipase, Pseudomonas (or Burkholderia) *cepacia* (PCL) immobilized on immobead-150	White powder with lumps and ≥900 U/g[a]	Gram negative bacteria	54327 and Sigma
Lipase, *Thermomyces lanuginosus*(TLL) immobilized on immobead-150	White powder with lumps and ≥3000U/g[a]	Fungus	76546 and Sigma

[a]1 U corresponds to the amount of enzyme which liberates 1 µmol butyric acid per minute at pH 7.5 and 35-40 °C (tributyrin, Fluka No. 91010, as substrate).

2.2 Chemicals and solvents

Analytical grade chemicals such as methanol (CH_3OH), potassium hydroxide (KOH), phenolphthalein indicator, anhydrous sodium sulfate, sodium chloride, BF_3/methanol (~1.3 M) 10% solution, lauryl alcohol were purchased from Sigma-Aldrich, Canada and used in the experimental work. All the chemicals used

were of AR grade. Industrial grade N_2 gas cylinder was supplied by Pyrex, Saskatoon, SK (Canada) for maintaining inert atmosphere during the reaction.

2.3 Experimental details

For studying the hydrolysis reaction, fish oils (1.5 g) were dissolved in one g solvent. 0.13 g of immobilized CAL-B along with the distilled water (3.0 g) and 1 g of pH 7.0 phosphate buffer was added to prepare the reaction mixture in a 125 ml Erlenmeyer flask. Standard AOCS test methods were used for estimating the acid value (AOCS, 1997 & ASTM, 2004) of product samples at different reaction times to determine µmoles of free fatty acids (FFAs) formed per ml of reaction mixture. The tuna free fatty acids (TFFAs) have been extracted and purified from tuna fish oil by the enzymatic hydrolysis with immobilized Candida *antarctica* lipase-B (CAL-B). Therefore, the extracted tuna free fatty acids have been further utilized as substrate for the selective esterification reaction. Whereas for esterification reaction, TFFAS (1.0 g) were dissolved in one g of iso-octane, 0.53 g of lipase along with the lauryl alcohol (3.0 g), 0.5 g of buffer (pH 8.0) at 50 ºC and 800 rpm speed up to duration of 56 h. The reaction was conducted continuously in a small glass reactor, placed in an oil bath on hot plate which was equipped with electrically driven high rpm motor, thermometer for monitoring stable temperature of reaction mixture. The activity of lipase for esterification has been expressed as µmoles of fatty acid esters formed per g of immobilized lipase per min. The experiments were performed in repeated sets and a variation in TFFAs ester formation <±5 %was observed.

2.4 Standards and Analysis

An Agilent make Gas Chromatography system (model 7890A)has been used, equipped with flame ionization detector (FID, 260°C) and capillary column DB-23 (dimensions: 60 m length, 0.25 mm ID, 0.25 µm film) for analysis of free fatty acids in methyl ester form (FAMEs). The initial composition of TFFAs was analyzed by converting the free fatty acidsintomethyl ester form with BF_3/methanol solution under inert N_2 purging atmosphere. Throughout the experimentation, the GC was operated at constant conditions (Carrier: hydrogen gas with flow rate 20 cm/min & 23.148 psi pressure; Oven: 140 to 240 ºC at 4°C/min. and Injection: 1µl sample, 260 ºC & split: 20:1). Supelco 37 component FAME mixture (Catalog No. 47885 Supelco and Lot No: LB-85810; 10mg/ml in methylene chloride) was purchased from Sigma-Aldrich, Canada for GC calibration and analysis.

3. RESULTS AND DISCUSSION

The thermodynamics is concerned with the change in energy as the chemical or biochemical process takes place. Thermodynamic study for simultaneous hydrolysis and esterification reactions (Scheme 1) were carried out at optimized reaction conditions. In connection with this, initially dependency of rate constant on temperature was studied at 25, 30 & 35 ºC for hydrolysis and 30, 40 & 50 ºC for esterification, respectively. Then, equilibrium rate constant have been determined using rate constants of forward and reverse reactions.

$$Tri\ esters\ of\ fish\ oil\ +\ water \xrightarrow{hydrolysis} Free\ fatty\ acids\ +\ alcohol$$

$$Free\ fatty\ acids\ +\ alcohol \xrightarrow{esterification} Esters\ of\ fatty\ acids\ +\ water$$

Scheme 1: reaction scheme for hydrolysis and esterification of tri esters of fish oil

To understand the physical significance of these reactions, different thermodynamic constants were determined such as change in Gibbs free energy (ΔG), enthalpy (ΔH), entropy (ΔS). Additionally, kinetic constants such as turn-over number (k_{cat}) and thermal deactivation constant (K_d) for different set of lipases

have also been evaluated. For hydrolysis and esterification reactions, the rate dependency on temperature was determined using Arrhenius law as given below in Eq. (1).

$$k = A_0 . e^{-E_a/RT} \quad (1)$$

$$\ln k = lnA_0 + \left(\frac{-E_a}{RT}\right) \quad (2)$$

Where, k = rate constant; A_o = pre-exponential factor; E_a = activation energy (J/mol); R = gas constant and T = temperature (ºKelvin).The linear form of Arrhenius equation is given in Eq.(2).The percentage conversion of triglycerides into free fatty acids and free fatty acids into esters have been plotted with time (up to 2 h) to determine the value of rate constant (k) at varying temperatures for hydrolysis of fish oils and esterification of TFFAs assuming a pseudo first order reversible reaction model. To determine activation energy, ln k was plotted with respect to the reciprocal of temperature with data in Table 2. The study of activation energy for both hydrolysis and esterification has been conducted separately. The activation energy for hydrolysis of tuna, salmon and herring fish oils with immobilized CAL-B were found as 26.1, 16.6 and 32.1 (kJ/mol), respectively. Whereas, for esterification of tuna free fatty acids activation energy have been obtained with immobilized PCL and TLL are 22.8 and 24.9 (kJ/mol), respectively.

Table2: Effects of different temperature on rate constant (k) for hydrolysis of fish oils with immobilized CAL-B and esterification of TFFAs

Temperature T (ºK)	**Rate constant k (µmoles of FFAs per ml)**			**Temperature T (ºK)**	**Rate constant k (µmoles of FFAs per ml)**	
	Tuna oil	Salmon oil	Herring oil		Immobilized PCL	Immobilized TLL
298	0.11	0.064	0.031	303	0.38	0.29
303	0.12	0.072	0.063	313	0.41	0.275
308	0.19	0.086	0.089	323	0.63	0.38

Thermodynamic functions such as Gibbs free energy, enthalpy and entropy, are functions of state. This means that they depend only on the state of the system being considered and not on how that system came into being. Changes in the functions of state between two states depend only on the initial and final states and not on the route between them (Fersht, 1999). For a chemical reaction, the change in the Gibbs free energy function (ΔG) is the energy which is available to do work as the reaction proceeds from the given concentrations of reactant and products to chemical equilibrium. The enthalpy change (ΔH) is defined as the quantity of heat adsorbed by the system under the given conditions. The increase in the entropy of surroundings is represented by (-ΔH/T) and the increase in the entropy of system is (ΔH/T). For any spontaneous process at constant temperature and pressure, the increase in the free energy of the system (ΔG) is defined as shown in Eq. (3).According to Eq. (4), ln K is equilibrium constant and depends upon the ratio of the rate constants for forward (hydrolysis) and reverse (esterification) reactions. Hence, a plot of ln K against 1/T, will give a straight line whose slope will be equal to ΔH/R and intercept will be (-ΔS/R)(Pogaku*et al*., 2012).The thermodynamic catalytic constant of an enzyme which is a measure of its catalytic efficiency can be defined as given below in Eq. (5).This quantity is also known as the turn-over number (k_{cat}) of an enzyme because it is the number of reactions catalysed by enzymes per active site in unit time (Liese*et al*., 2006). When using expensive catalyst, the turn-over number should be as high as possible so as to reduce the cost of the product (Price and Stevens, 1999)**.** Immobilized enzymes lose their catalytic activity upon reuse over time. The constant of deactivation (K_d) was calculated with Eq. (6) where T_{max} is

the maximum reaction temperature (in ºKelvin).The dissociation constant value shows the deactivation nature of enzyme for a set of reaction system. The magnitude of K_d can also be determined according to a thermodynamic study conducted by Gitinet al. in 2006 for the thermal deactivation of Novozym 435 assuming enzyme may be reversible, irreversible or a combination of the two. In this study, it was concluded that the smaller values of K_d is a indicative for the more active enzyme (Gitin*et al.*, 2006 &Primožič*et al.*, 2003).

$$\Delta G = \Delta H - T.\Delta S \quad (3)$$

$$ln\ K = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \quad (4)$$

$$K = \frac{k_{forward}}{k_{reverse}} = \frac{k_{hydrolysis}}{k_{esterification}} \quad (5)$$

$$k_{cat} = \frac{V_{max}}{[E_t]} \quad (6)$$

$$K_d = \frac{E_a + R.T_{max}}{\Delta H - (E_a/R.T_{max})} \quad (7)$$

To determine various thermodynamic constants for both hydrolysis and esterification, equations (3) to (7) have been used simultaneously. For a set of lipase systems to carry out simultaneous hydrolysis and esterification reactions, equilibrium rate constants (K) have been determined using Eq. (5) and results are shown in Table 3. The change in enthalpy (ΔH) and entropy (ΔS) were first determined from the slope and intercept of a plot drawn between ln K with respect to the reciprocal of temperature (T) using Eq. (4) as shown in Figure 1. Further, change in Gibbs free energy (ΔG) was estimated with Eq. (3).The turn-over number (k_{cat}) was further calculated using maximum rate of reaction (V_{max}) and total enzyme concentration [E_t] (as shown in Eq. 6) which were determined separately with the kinetic study for these two reactions. The maximum rate of reaction was found to be 0.093(µmoles FFAs/sec) per unit volume of reaction mixture for hydrolysis of fish oils respectively at 7.4 x 10^{-4}(µmoles/ml) concentration of immobilized CAL-B. Similarly, 2.8 and 0.58 µmoles fatty acid esters per sec per unit volume (ml) of reaction mixture with immobilized PCL and TLL, respectively at 4.4 x10^{-3}(µmoles/ml) lipase concentration have been obtained for esterification. The deactivation constant was determined at optimized maximum reaction temperature i.e. 35 and 50 ºC for hydrolysis and esterification reactions, respectively using Eq. (7). The results obtained for thermodynamic constants are presented in Table 4 for different lipase systems.

Table 3:Effects of different enzyme system on equilibrium rate constant (K) with respect to average reaction temperature

$T_{average}$ (ºK)	Equilibrium rate constant,K		1/T (ºK^{-1})	ln K	
	Immobilized CAL-B and PCL	Immobilized CAL-B and TLL		Immobilized CAL-B and PCL	Immobilized CAL-B and TLL
303.5	0.289	0.379	0.0033	-1.24	-0.97
308	0.292	0.436	0.0032	-1.23	-0.83
315.5	0.301	0.501	0.0031	-1.2	-0.69

Table 4: Thermodynamic constants for hydrolysis and esterification reactions with different lipase system

Thermodynamic constants	Immobilized CAL-B and PCL	Immobilized CAL-B and TLL
Enthalpy, ΔH (kJ/mol)	-11.6	-1.7
Entropy, ΔS (kJ/mol.K^{-1})	-0.03	-0.005
Gibbs free energy, ΔG (kJ/mol)	-2.3	-0.16
Turnover number, k_{cat} $(sec)^{-1}$	381.02	128.7
Thermal deactivation constant, K_d(kJ/mol)	-2.98	-2.96

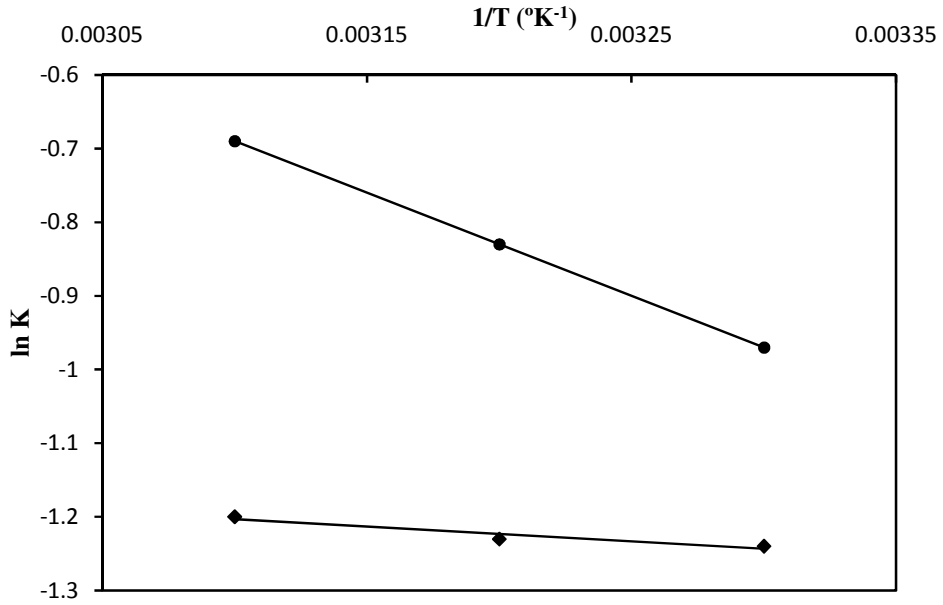

Fig. 1: Equilibrium rate constant (K) with respect to reaction temperature for hydrolysis and esterification reactions using immobilized CAL-B and PCL (♦) &immobilized CAL-B and TLL (●)

According to the results obtained from the theoretical calculations of thermodynamic parameters for hydrolysis and esterification reaction, it was seen that the values of change in Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) were negative in magnitude in all the cases. In the literature various explanations have been given by different researchers in this regard. According to Ferreira et al.2010 the negative magnitude for ΔG and ΔH correspondence to the spontaneous and exothermic nature of the lipase catalyzed reactions (Ferreira*et al*., 2010 &Gohri*et al*., 2011).Graber *et al*.,2002 have studied the effect of water on the alcoholysis of methyl propionate and n-propanol catalyzed by immobilized *Candida antarctica* lipase-B in a continuous solid/gas reactor and in organic liquid medium. According to them, the change in the magnitude of entropy (ΔS) for lipase catalyzed reactions may be either positive or negative. A negative value indicates the fast and convenient formation of enzyme-substrate complex when enzyme binds with substrate causing considerable loss in entropy. The consumption of reactant with the progress of reaction combined with release of translational and rotational energies are mainly responsible for the loss in entropy, which is most common for enzymatic reactions (Graber*et al*., 2002 &Lonheinne*et al*., 2000). Li et al., 2011 have also reported the negative magnitude of the three parameters (ΔG, ΔH and ΔS) for the interaction of

flavonoids with pancreatic lipase, which is mainly due to the formation of an enzyme-substrate complex. A maximum turn-over number (k_{cat})381.02 sec^{-1} was found for the hydrolysis with immobilized CAL-B and esterification with immobilized PCL system than immobilized CAL-B and TLL system, corresponding to the highest catalytic effectiveness of immobilized CAL-B for hydrolyzing fish oils and immobilized PCL for esterification reaction. In case of both reaction systems including immobilized CAL-B and PCL and immobilized CAL-B and TLL, a small and almost same value of thermal deactivation i.e. -2.98 and -2.96 (kJ/mol) were found respectively, indicating a good activity of all lipases. Turn-over number (k_{cat}) and thermal deactivation constant (K_d) for both lipase systems have also been compared graphically as shown in Figure 2. Asimmobilized enzymes lost their activity over multiple uses, Gitinet al. 2006 have shown in their studies that the lower magnitude of thermal deactivation constant is desired for achieving good activity of enzyme during reaction course.

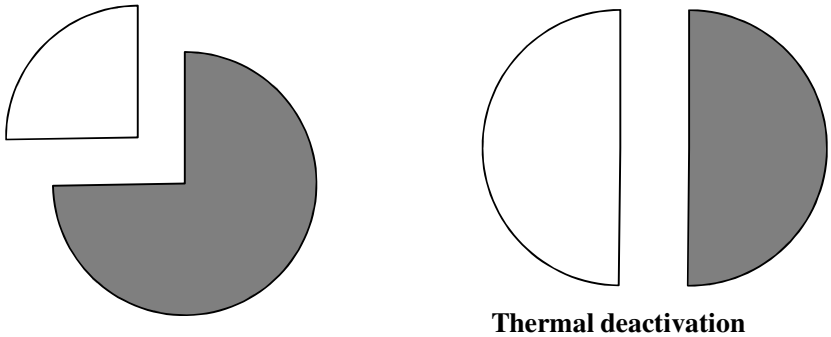

Fig. 2: Graphical comparison of Turnover number (k_{cat}) and Thermal deactivation constant (K_d) for immobilized CAL-B and PCL (grey) with immobilized CAL-Band TLL (white) lipase systems

4. CONCLUSIONS

The present study concludes that the hydrolysis and the esterification reactions catalyzed by different lipase systems are thermodynamically favorable according to the results obtained for thermodynamic constants (ΔG, ΔH and ΔS).The negative magnitude of these parameters indicates that spontaneous and exothermic nature of lipase catalyzed reactions with fast and convenient formation of enzyme-substrate complex. The higher catalytic efficiency and lower thermal deactivation of lipases have been proven, indicating thermal stability of lipases at reaction temperature by the results obtained from present study for hydrolysis and esterification reactions.

5. NOMENCLATURE

ΔG	Change in gibbs free energy	**K**	Equilibrium rate constant
ΔH	Change in enthalpy	**k**	Rate constant
ΔS	Change in entropy	**k_{cat}**	Turn-over number
CAL-B	*Candida antarctica* lipase-B	**K_d**	Thermal deactivation constant
PCL	*Pseudomonascepacia*lipase	**E_a**	Activation energy
TLL	*Thermomyces lanuginosus*lipase	**E_t**	Total enzyme concentration
TFFAs	Tuna free fatty acids	**A_0**	Pre-exponential factor
DHA	Docosahexaenoic acid	**R**	Gas constant
T	Temperature	**T_{max}**	Maximum reaction temperature
V_{max}	Maximum reaction velocity	**$T_{average}$**	Average reaction temperature

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