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Kinetic and Thermodynamic Studies of Free and Alginate/ Agar-Agar Immobilized α-Amylase Catalyzed Reaction

SOUMEN PRAMANIK, KHOMENDRA KUMAR SARWA, ASHOKE KUMAR DOLUI and ATUL KUMAR*

Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh-786 004, India

*Corresponding author: E-mail: atul2008@gmail.com

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The present study deals with the immobilization of α-amylase into alginate and agar-agar medium and determination of kinetics as well as thermodynamic parameters of both free and immobilized enzyme catalyzed reaction to predict the extent of reaction and the position of equilibrium. At optimized condition, the K_m value derived from Lineweaver Burk plot for free enzyme (0.40 % w/v) was lower than the immobilized enzyme (0.52 % w/v for alginate and 0.76 % w/v for agar-agar immobilized). The free enzyme had an E_a value of 1609 cal/mol compared to those of immobilized enzyme (6495 cal/mol for alginate and 3542 cal/mol for agar-agar immobilized). Computed ΔS value for free enzyme is more negative than the immobilized enzyme. The increasing value ΔG° in immobilized enzyme system indicates that the enzyme-substrate reaction is slower during immobilization. However immobilized enzyme could be reused even after 12 days of storage.

Key Words: α-Amylase, Kinetics, Enzymes, Immobilization, Alginate, Agar-agar.

INTRODUCTION

The uses of immobilized enzymes are increasing in biotechnology, biomedicine, food technology and analytical chemistry due to their various advantages over free enzymes including easy separation of the reactants, products and reaction media, easy recovery of the enzyme and reusability.

One of the largest selling industrial enzymes is α-amylase that has several industrial applications[1-3]. Immobilization of such enzyme increases its economic value for industrial processors by substituting costly free enzyme with immobilized one[4].

Several methods of enzyme immobilization have been reported so far, such as-adsorption[5], adsorption and cross-linking[6], cross-linking[7], ion-exchange resins[8], entrapment[9], microencapsulation[10], copolymerization[11] and covalent attachment[12]. In the present study, alginate and agar-agar were chosen as carrier materials due to their cost effectiveness and good immobilization efficiency. In addition, alginate, a naturally occurring polymer consisting of mannuronic acid and gluronic acid, formes inert aqueous environment inside its matrix and high gel porosity that allows high diffusion rates of macromolecules[13]. On the other hand, agar-agar is a natural polysaccharide with a strong gelling ability and good acid stability characteristics, showing no protein reactivity.

To predict the extent of reaction and to know the position of equilibrium for any enzymatic reaction, enzyme kinetic information is necessary and that prediction has important applications in biochemistry, industrial enzymology and in metabolic control analysis also. Thermodynamics and activation parameters also provide a detailed mechanism for many chemical and biological reactions[14]. Hence in the present work kinetics as well as thermodynamic aspects for both free and immobilized amylase-catalyzed reactions has been studied.

EXPERIMENTAL

α-Amylase and soluble starch were purchased from Sigma Chemical Co., USA and Central Drug House Pvt. Ltd., Mumbai, India, respectively. Sodium alginate and agar-agar powder were from Loba Chemie Pvt. Ltd., Mumbai, India. All other chemicals used were of analytical grade.

Determination of optimum pH and temperature: To determine the optimum pH for maximum enzyme activity, the reaction was carried out at different pH using 0.2 M acetate buffer of pH 3.6, 4.0, 4.4, 4.8, 5.0, 5.2 and 5.6. Duplicate sets of seven tests tubes were arranged and 0.5 mL enzyme solution was added to each test tube containing 1.75 mL of starch, 2.25 mL of buffer (pH range 3.6, 4.0, 4.4, 4.8, 5.0, 5.2 and 5.6) and 1.0 mL of distilled water. All the test tubes were incubated at 40 ºC in a thermostatic water bath. After 20 min, 0.5 mL of 1 M HCl was added to each test tube to stop the reaction. 0.2 mL of the reaction mixture from each test tube was diluted with 14.5 mL with distilled water. Then 0.2 mL of

iodine reagent and 0.1 mL of 1 M HCl were added to each of them. The absorbance of reaction mixture was measured at 610 nm in a UV-VIS spectrophotometer (Shimadzu UV-1800) against a blank[14-17].

Optimum temperature of the enzyme was determined by above procedure using buffer of optimum pH (5.0) and incubating at different temperatures *viz*., 30, 35, 40, 45 and 50 ºC using a thermostatic water bath.

Immobilization of α-amylase with alginate: For immobilization 0.5 mL of α-amylase solution (1 % w/v) was added to 1.5 mL of 2 % sodium alginate solution and the mixture was extruded drop wise into a gently stirred 2 % $CaCl_2$ solution by maintaining a height of 2 cm. All the beads were cured for 20 min and separated by filtration. They were washed twice with distilled water[15].

Immobilization of α-amylase with agar-agar: Mixture of 0.5 mL α-amylase (1 % w/v) and 1.5 mL 3.0 % molten agar-agar at about 45 ºC was placed in the well of a microtiter plate and beads were formed when the mixture was cooled down.

Kinetics study: Duplicate sets of six test tubes were taken, each containing 2.25 mL of acetate buffer (pH 5.0), 1 mL of distilled water and 1.75 mL of different concentration *viz*., 2.4, 2.0, 1.6, 1.2, 0.8 and 0.0 % (blank) of soluble starch and maintained at 35 ºC in a thermostatic water bath. To each test tube 0.5 mL amylase solution (1 %) was added. After 10 min, reaction in each test tube was stopped by addition of 0.5 mL of 1 M HCl. 0.2 mL of the reaction mixture was diluted by addition of 14.5 mL distilled water followed by addition of 0.2 mL of 2 % iodine reagent and 0.1 mL of 1 M HCl. The absorbance was measured at 610 nm in a UV-VIS spectrophotometer. The above procedure was repeated at 40 and 45 ºC.

Above procedure was repeated for the kinetic study of immobilized enzyme but instead of using 0.5 mL free enzyme, alginate or agar immobilized enzyme beads prepared from 0.5 mL 1 % enzyme were used.

Reusability: Starch degradation properties of the prepared immobilized enzyme beads were determined using 1.2 % w/v starch as substrate at optimum pH (5.0) and temperature (40 ºC). Then the beads were filtrated through filter paper, washed thrice with distilled water and stored in a refrigerator (4 ºC). On 6th day and 12th day again the starch degradation activities of the beads were determined. The beads were also visually inspected for any sign of physical deformity.

RESULTS AND DISCUSSION

Enzyme activities at different pH and temperature are presented in Figs. 1 and 2, respectively and from the figure optimum pH and temperature of α-amylase are found to be 5.0 and 40 ºC, respectively.

Starch degradation activities of the free enzyme (α-amylase), alginate immobilized and agar-agar immobilized enzyme at different substrate concentration and different temperature are presented in Tables 1-3, respectively. For enzyme kinetic study, Michaelis-Menten equation *i.e*., $V = V_m \frac{S}{K_m + S}$ is hyperbolic, Lineweaver Burk transformed the hyperbolic

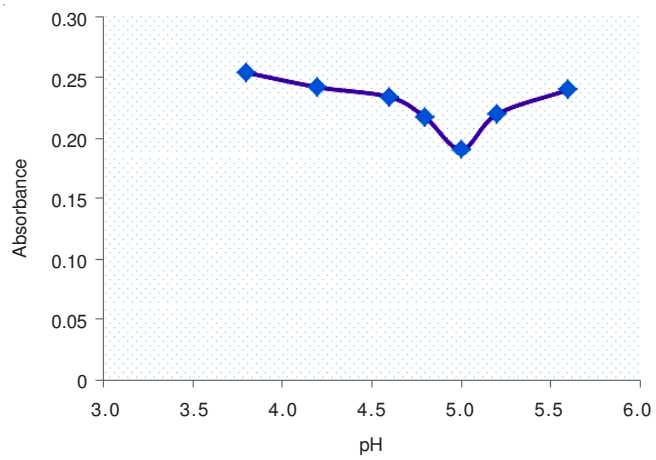

Fig. 1. Determination of optimum pH of α-amylase

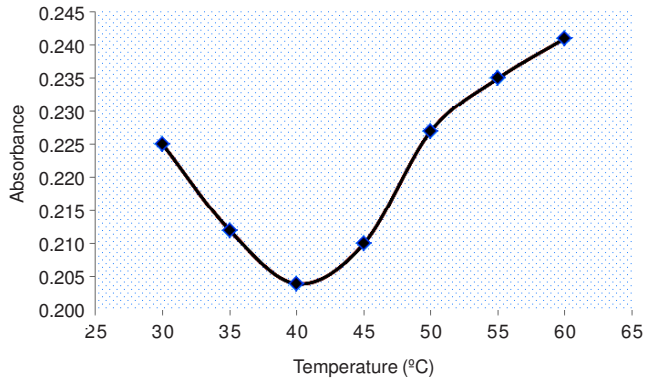

Fig. 2. Determination of optimum temperature of α-amylase

equation into a linear equation which is $\frac{1}{V} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{S}$ where, V is the velocity of reaction, V_m is maximum attainable reaction velocity, [S] is the substrate concentration and K_m is Michaelis-Menten constant. The reciprocal velocity (1/V) was plotted against the reciprocal substrate concentrations 1/[S] in Figs. 3-5 and extrapolation of the line gave the respective values of K_m which are found to be 0.40 % w/v for the free enzyme and 0.52 % w/v and 0.76 % w/v for alginate immobilized and agar-agar immobilized enzyme, respectively. The value of free amylase is lower than that of the immobilized amylase in alginate or agar-agar. Low value of K_m indicate high affinity of the enzyme for the substrate[16]. The K_m value mainly deals with the affinity of the substrate for the active site of an enzyme. This result indicates that affinity of free enzyme is more for the substrate than by the immobilized enzyme. The increase in apparent K_m value might be either due to structural changes in the enzyme induced by the applied immobilization procedure or due to the lower accessibility of the substrate to the active site of the immobilized enzyme[17].

Different thermodynamic parameters like energy of activation (E_a), Arrhenius factor (A), standard enthalpy change (ΔH), Gibbs free energy (ΔGº), standard entropy change (ΔS), probability factor (P) and equilibrium constant (K) values of enzyme catalyzed reaction for both free and immobilized enzyme were evaluated from the slope and intercept of the Arrhenius plots. The following equations were used to get the computed data.

TABLE-1
STARCH DEGRADATION ACTIVITY OF FREE α-AMYLASE

Temperature (ºC)	Concentration of soluble starch (% w/v)				
	2.4	2.0	1.6	1.2	0.8
	Absorbance				
45	1.088 ± 0.010	0.982 ± 0.005	0.811 ± 0.004	0.582 ± 0.003	0.356 ± 0.006
40	0.899 ± 0.007	0.812 ± 0.001	0.619 ± 0.014	0.437 ± 0.001	0.257 ± 0.010
35	1.009 ± 0.009	0.839 ± 0.004	0.711 ± 0.021	0.507 ± 0.004	0.322 ± 0.001

The data were represented as mean ± SD.

TABLE-2
STARCH DEGRADATION ACTIVITY OF SODIUM ALGINATE IMMOBILIZED α-AMYLASE

Temperature (ºC)	Concentration of soluble starch (% w/v)				
	2.4	2.0	1.6	1.2	0.8
	Absorbance				
45	1.179 ± 0.005	1.082 ± 0.012	0.909 ± 0.002	0.652 ± 0.007	0.402 ± 0.005
40	0.980 ± 0.001	0.896 ± 0.025	0.696 ± 0.003	0.491 ± 0.019	0.291 ± 0.004
35	1.125 ± 0.004	0.950 ± 0.009	0.820 ± 0.020	0.579 ± 0.003	0.368 ± 0.020

The data were represented as mean ± SD.

TABLE-3
STARCH DEGRADATION ACTIVITY OF AGAR-AGAR IMMOBILIZED α-AMYLASE

Temperature (ºC)	Concentration of soluble starch (% w/v)				
	2.4	2.0	1.6	1.2	0.8
	Absorbance				
45	1.123 ± 0.005	1.033 ± 0.011	0.864 ± 0.024	0.616 ± 0.004	0.380 ± 0.015
40	0.934 ± 0.002	0.856 ± 0.021	0.660 ± 0.010	0.463 ± 0.004	0.276 ± 0.011
35	1.077 ± 0.012	0.912 ± 0.001	0.784 ± 0.007	0.550 ± 0.006	0.350 ± 0.009

The data were represented as mean ± SD.

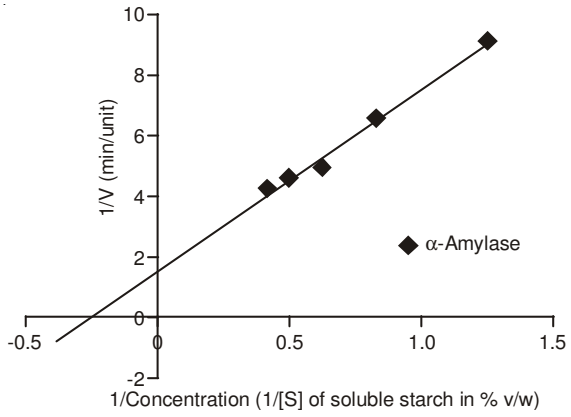

Fig. 3. Lineweaver burk plot of α-amylase (free)

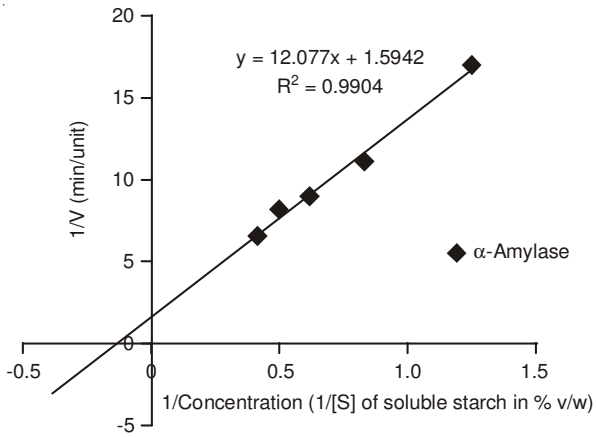

Fig. 5. Lineweaver burk plot of α-amylase immobilized in agar-agar

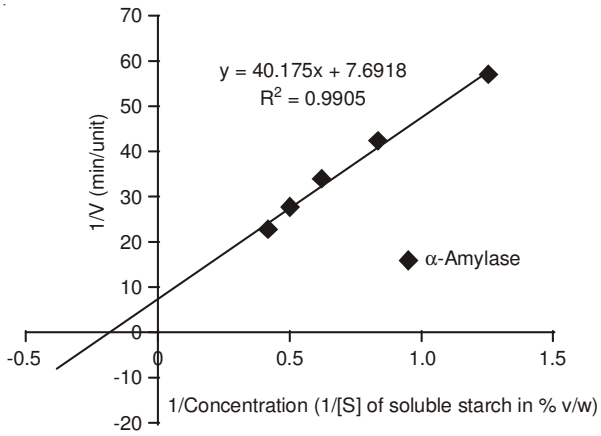

Fig. 4. Lineweaver burk plot of α-amylase immobilized in sodium alginate

$$k = Ae^{-E_a/RT} \quad (1)$$

$$A = \left(\frac{RT}{Nh}\right)e^{\Delta S/R} \quad (2)$$

$$P = e^{\Delta S/R} \quad (3)$$

$$\Delta G^\circ = \Delta H - T\Delta S \quad (4)$$

$$\Delta G^\circ = -RT \ln K \quad (5)$$

$$Z = \frac{RT}{Nh} \quad (6)$$

$$\log k = \log A - \frac{E_a}{2.303}\frac{1}{RT} \quad (7)$$

$$k = \left(\frac{RT}{Nh}\right) e^{\Delta S/R} e^{-\Delta H/RT} \quad (8)$$

$$k = (PZ)e^{-E_a/RT} \quad (9)$$

where, k = the specific reaction rate, R = the gas constant (1.987 calories/deg mole) and T = the absolute temperature. Z = the collision number, N = the Avogadro's number and h = the Planck's constant.

Arrhenius plots for free as well as immobilized α-amylase are presented in Figs. 6-8 and the energy of activation (E_a) was calculated from the slope. Subsequently, the Gibbs free energy (ΔGº), entropy of activation (ΔS), equilibrium constant (K), probability factor (P) and other related values for free and immobilized (both in alginate and agar-agar) α-amylase were computed using above mentioned equation and are presented in Table-4.

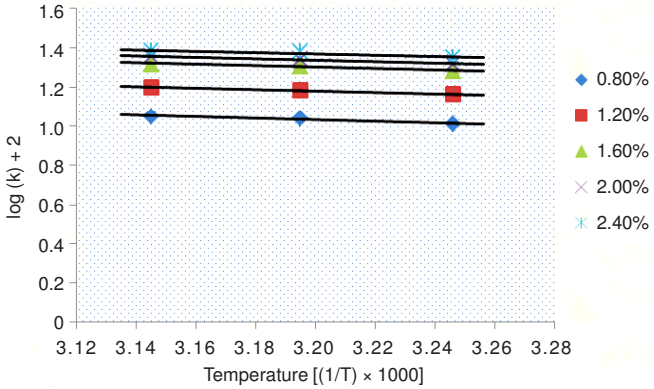

Fig. 6. Arrhenius plot of free enzyme (α-amylase)

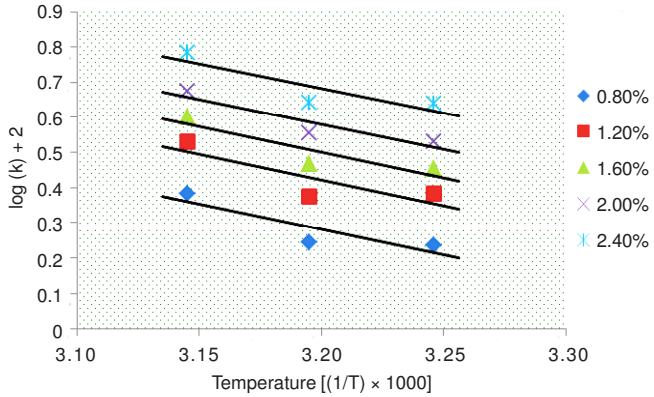

Fig. 7. Arrhenius plot of α-amylase immobilized in sodium alginate

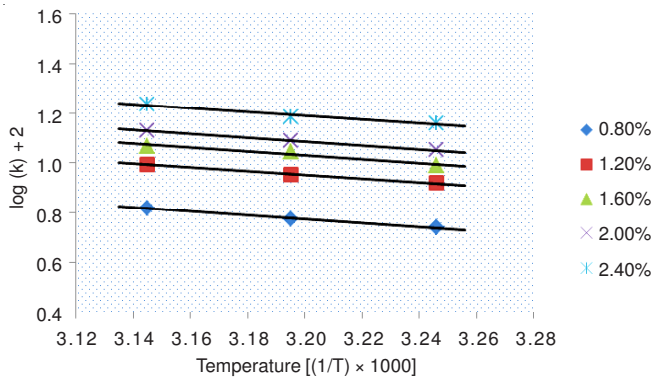

Fig. 8. Arrhenius plot of α-amylase immobilized in agar-agar matrix

Lower the entropy values of the enzyme, more efficient are the formation of transition state or activated complex between enzyme-substrate[18]. The present study showed the lower entropy value for the free enzyme but higher entropy value for the immobilized enzyme indicating the good efficiency in the formation of transition state for free enzyme in comparison to immobilized enzyme. Computed ΔS value for free enzyme (-47.21 cal/mol-deg) is more negative than the alginate immobilized beads (-33.59 cal/mol-deg) or agar immobilized beads (-42.33 cal/mol-deg).

The reaction rate is proportional to formation of transition state complex by lowering the Gibb's free energy of activation (ΔGº). The increasing value of ΔGº in immobilized enzyme (17009 cal/mol for alginate immobilized and 16791 cal/mol for agar-agar immobilized) than in free form (16386 cal/mol) indicates that the enzyme substrate reaction is slower during immobilization.

Starch degradation activities of immobilized enzyme after different time of storage are presented in Table-5. It is evident from the table that the immobilized enzyme retained is activity even after 12 days. However the beads developed small fissure or softened or partially broken in the reaction mixture after more than 12 days storage.

Immobilized enzymes are generally more stable compared to free enzymes, due to the curtailment of their degrees of freedom (of rotation). Entrapped enzymes are unable to rotate freely. Prevention of the unfolding of its structure could be preserving its function[13]. The present work demonstrated a promising application potential of the alginate and agar-agar beads for enzyme immobilization.

TABLE-4
COMPUTED DATA

Enzyme	E_a /ΔH (cal/mol)	A (s^{-1})	ΔS (cal/mol)	ΔGº	K	K_m (% w/v)	P
Free α-amylase	1609	0.315×10^3	-47.21	16386	0.361×10^{-13}	0.40	0.048×10^{-9}
Alginate immobilized amylase	6495	2.97×10^5	-+33.59	17009	1.326×10^{-12}	0.52	4.55×10^{-8}
Agar immobilized amylase	3542	3.66×10^3	-42.33	16791	1.883×10^{-12}	0.76	0.561×10^{-9}

TABLE-5
STARCH DEGRADATION ACTIVITIES OF IMMOBILIZED α-AMYLASE AFTER DIFFERENT TIME OF STORAGE

	Absorbance on 1st day	Absorbance on 6st day	Absorbance on 12st day
Alginate immobilized enzyme	0.491 ± 0.019	0.494 ± 0.001	0.496 ± 0.015
Agar-agar immobilized enzyme	0.463 ± 0.004	0.473 ± 0.001	0.480 ± 0.015

The data were represented as mean ± SD.

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