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Research Article

THERMODYNAMIC, KINETIC AND STABILITY ASPECTS OF A PURIFIED PROTEASE FROM THE LATEX OF *PLUMERIA RUBRA* LINN.

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ABSTRACT

The Present Study was conducted for evalauation of kinetic (K_m & V_{max}), thermodynamic and stability parameters of a proteolytic enzyme (Plumerin-R) which was isolated from the latex of *Plumeria rubra* Linn. Three substrates namely hemoglobin, azoalbumin and BAPNA were used for kinetic and thermodynamic study of the enzyme. K_m values for hemoglobin, azoalbumin and BAPNA were found to be 1.0, 1.25 and 0.031 mg/mL respectively and V_{max} values for the same substrates were found to be 0.83, 1.04 and 0.025 respectively. It was found that the positive (+ve) entropy change (ΔS^o) is the main factor in the unfolding of the enzyme that yields a negative (-ve) ΔG^o in spite of the positive ΔH^o values in cases of all three substrates. It was also found that on the 90th day, Plumerin-R, stored in different storage condition, showed significant change in residual activity and the enzyme is more stable in powder form than that of the solution form.

Keywords: Plumerin-R, Kinetic, Thermodynamic, Stability.

INTRODUCTION

Plumeria rubra Linn. (Apocynaceae) is a laticiferous tree that grows as a spreading shrub or small tree to a height of 7 - 8 m (20 - 25 ft). The species, commonly known as red jasmine, is native to Mexico and grows throughout India[1].

A proteolytic enzyme, "Plumerin-R", has been isolated and purified from the latex of the plant[2]. Molecular weight of Plumerin-R was estimated by SDS-PAGE and was found to be around 81,850 daltons. The optimum pH and temperature for casein digestion activity of Plumerin-R was reported as pH 7.0 and 55°C[2]. The enzyme Plumerin R also reported to have anti-inflammatory and wound healing property[3].

The present study was conducted for further characterization of Plumerin-R by evaluating the kinetic (K_m & V_{max}), thermodynamic and stability parameters.

MATERIALS AND METHODS

Material

Plumerin-R was obtained from the earlier study[2]. Bovine haemoglobin and casein was purchased from Himedia, India and N-α-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) was purchased from Sigma Aldrich India. Azoalbumin was the product of SISCO Research Laboratories Pvt. Ltd, India. Methyl paraben, Propyl paraben were the products of Merck, India.

Determination of kinetic parameters

The apparent K_m (Michaelis equilibrium constant) and V_{max} (Maximum attainable reaction velocity) values of Plumerin-R were estimated for the substrates (hemoglobin, azoalbumin and BAPNA) from the linear portion of Line-weaver and Burk double reciprocal plot[4]. The reciprocal of reaction velocity (1/V) was plotted against the reciprocal of corresponding substrate concentration (1/[s]) giving the Lineweaver-Burk plot (or double reciprocal plot).

Determination of thermodynamic parameters

Thermodynamic parameters[5] of Plumerin-R were evaluated from the Arrhenius plots (1/T ×1000 vs. log of activity) of hemoglobin, azoalbumin and BAPNA. From the slope of the curve E_a values were calculated and using this ($E_a \simeq \Delta H^o$) value, other thermodynamic parameters, such as, standard free energy change (ΔG^o), entropy change (ΔS^o), energy of activation (E_a), enthalpy change (ΔH^o), probability factor or steric factor (P) and collision number (Z) were computed.

Thermodynamic quantities are evaluated from the following equations:

a) $K=A\, e^{-Ea/RT}$
b) $\log K=\log A- (Ea/2.303RT)$
c) $\log K_2/K_1 = \Delta H^o/2.303R\ (1/T_1 - 1/T_2)$
d) $A= (RT/Nh)\, e^{\Delta S^o/R}$
e) $\Delta G^o=\Delta H^o-T\Delta S^o$

Effect of storage conditions and Heat on the Stability of Plumerin-R

The study of storage conditions was carried out as per the guidelines of ICH (Q 1A: R2)[6]. For this study, accelerated storage condition (25°C ± 2°C/60% RH ± 5% RH) for three months was selected and container-closure system was the glass vial with rubber stopper. Three different storage conditions were selected. The frequencies of testing selected were 0 and 3 months.

The residual activity of Plumerin-R powder and solution was measured at respective days, after storage in specified conditions using casein as substrate according to the method of Kunitz[7].

The heat stability of Plumerin-R was studied by keeping the solution of the Plumerin-R at different temperature conditions and measuring the residual activity at predetermined time intervals[8].

STATISTICAL ANALYSIS

All data were derived from at least three independent experiments. Where applicable, results were expressed as mean ± SD and analysed statistically using Student t-test with the aid of Sigma Plot software (version 10).

RESULTS AND DISCUSSION

K_m and V_{max} values for Plumerin-R were obtained from the Lineweaver-Burk plot and tabulated in Table 1, which is evident that the Plumerin-R and substrate affinity is high (small Km) for the substrate BAPNA than the other three natural protein substrates which indicates higher specificity with respect to the hydrolysis of amide bonds in synthetic substrate than the larger molecular weight natural substrates.

Thermodynamic parameters of Plumerin-R those were evaluated from the Arrhenius plots of hemoglobin, azoalbumin and BAPNA are summarised in table 2. When the activated intermediate complex represents a more probable arrangement of molecule than found in the normal reactants (eg. protein – substrate system under study) ΔS^o is +ve and the reaction rate will be greater than normal. The

ΔS^ovalues are found to be +ve (very high values) in all cases. That, the energy of activation, $E_a \cong \Delta H^o$ the standard enthalpy change is a fact, has been established by comparing the respective values of the three substrates as embodied in the table 2.

Table 1: Kinetic parameters of Plumerin-R for different substrates

Kinetic Parameters	Substrates		
	Hemoglobin	Azoalbumin	BAPNA
K_m (mg/mL)	1.0	1.25	0.031
V_{max} (unit)	0.83	1.04	0.025

Table 2: Thermodynamic parameters of Plumerin-R for different substrates

Thermodynamic Parameters	Substrates		
	Hemoglobin	Azoalbumin	BAPNA
E_a (k cal mole^{-1})	3.29	4.90	11.44
ΔH^o (k cal mole^{-1})	3.24	4.59	11.47
ΔS^o (cal/mole deg)	56.27	53.42	33.89
ΔG^o (k cal mole^{-1})	- 18.46	- 17.526	- 10.96

The table 2 also summarizes the values of ΔH^o and ΔS^o that would be expected depending on the kind of interaction occurring in the intermediate complex. Either ΔH^o and / or ΔS^o is needed to get a –ve (favourable) ΔG^o value. For example, for donor-acceptor and hydrogen bonding interactions bond strength, ΔH^o values (k cal/mole) of 1-7 are indicative of either H-bonding and / or Ion-dipole and / or dipole- dipole interactions[9]. The positive (+ve) entropy change (ΔS^o) is the main factor in the unfolding of proteins that yields a negative (-ve) ΔG^o in spite of the positive (unfavourable) ΔH^o values in cases of all three substrates[10]. An insight into the mechanism of enzyme – $\rightleftharpoons$ ite intermediate complex formation (Enzyme + substrate) $E.S^* \rightarrow E + S$, is thus, obtained.

The results obtained from the study on the effect of storage conditions on the stability of Plumerin-R have been presented in Table 3. It was found that on the 90th day Plumerin-R stored in different storage condition showed significant (A 5% potency loss from the initial assay value) change in residual activity. From the study it is clear that Plumerin-R is more stable in powder form than that of the solution form and should always be stored at below 25°C.

Table 3: Effect of different storage conditions on the activity of Plumerin-R (3 months study)

Batch No.	Conditions of storage (at 25°C ± 2°C/60% RH ± 5% RH)	Time of storage (Months) and corresponding residual activity (Units×10^{-2})		Percentage loss of activity during the whole period (03 months) of storage
		00 (Initial day)	03 months (on 90th day)	
1.	Plumerin-R in powder form	2.46±0.08	2.17±0.05	11.79±0.11
2.	Plumerin-R in solution form	2.46±0.13	1.91±0.05	22.36±0.15
3.	Plumerin-R in solution form with anti-microbial preservative	2.46±0.07	1.89±0.05	23.17±0.17

Values expressed as mean ± SD

Fig. 1 shows the denaturation pattern of Plumerin-R by heat at different temperatures. The logarithm of activity retained by the enzyme (log A) plotted against time (minute), shown in Fig. 2, predicts a linear decrease of activity with time at all the temperatures of study. The enzyme showed a low rate of loss of activity at moderately higher temperature (50° and 60°C) but a sharp decrease was evident at higher temperatures (70° and 80°C). At 80°C, Plumerin-R was inactivated completely after incubation beyond 20 minutes. The linear decrease in activity indicated that thermal degradation reaction of Plumerin-R followed first-order kinetic (Fig. 2). A number of factors determine whether the molecule of an enzyme is native (i.e. active) or of denatured form[11]. The denaturation process of an enzyme molecule might consist of various distinguishable conformation states evoked by random raptures of individual conformational bindings, leading to progressive unfolding of the native tertiary structure to final random coil structure. The extent of denaturation of a protein conformation might be dependent on the degree of exposure of the polypeptide backbone and non-polar residues to the aqueous environment[12]. This supports the observation recorded in this study.

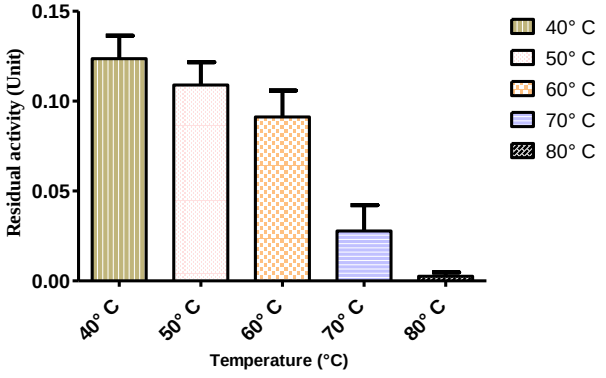

Figure 1: The denaturation pattern of Plumerin-R by heat at different temperatures (in absence of substrate). I t was observed that at 80°C, Plumerin-R was inactivated completely after incubation beyond 20 minutes.

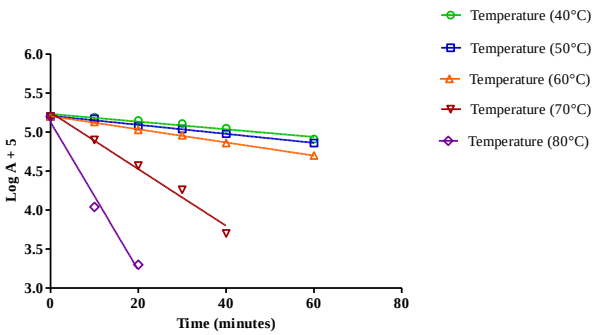

Figure 2: Plot of log of Residual activity versus Time for Plumerin-R at different temperatures. The linear decrease in activity indicated that thermal degradation reaction of Plumerin-R followed first-order kinetic.

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