

KINETIC AND THERMODYNAMIC STUDIES OF PURIFIED PROTEIN ISOLATED FROM *EUPHORBIA TIRUCALLI* LATEX

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ABSTRACT

The kinetic and thermodynamic parameters of an isolated protein from the latex of *Euphorbia tirucalli* were studied in this work. The isolated protein was named as Euphorbian T and was found to possess proteolytic activity through milk clotting and caseinolytic digestion process. The electrophoresis analysis of purified Euphorbian T was carried out using sodium dodecyl sulfate-polyacrylamide gel. The aminoacids of Euphorbian T were appeared in different bands of 36.5, 39, 44 and 50 Kda. The effect of pH, temperature, and concentration of substrate on proteolytic activity of Euphorbian T was examined. The maximum proteolytic activity of this protein was obtained at pH 6. The temperature $t_{(max)}$ for maximum activity range of the enzyme was found to be 65°C. The Kinetics parameters i.e., K_m and V_{max} of the purified protein with milk and caesin as substrate, were estimated using Lineweaver-Burk plot and were found to be 4 mg/dl, 0.21 U/mg and 2 mg/dl, 1.03 U/mg, respectively. Thermodynamic constants i.e., activation energy (Ea), change in enthalpy (ΔH) and change in entropy (ΔS) and free energy changes (ΔG) of Euphorbian T for milk were calculated using Arrhenius plot and were found to be 1.58 kcal/mol, 1.54 kcal/mol, -10.46 cal mol/deg and 18.87 kcal/mol respectively, whereas with the substrate caesin, the activation energy (Ea), change in enthalpy (ΔH) and change in entropy (ΔS) and free energy changes (ΔG) were calculated as 1.12 kcal/mol, 1.08 kcal/mole, -16.24 cal mol/deg and 16.34 kcal/mol, respectively.

Keywords: *Euphorbia tirucalli*, Euphorbian T, Milk clotting, Caseinolytic.

INTRODUCTION

Proteases are protein digesting biocatalysts, have been playing an important role in the industry since a long time. The proteolytic enzymes are used as milk-clotting agents for the manufacture of cheese. Proteases hydrolyze the peptide bonds of proteins into peptides and eventually into their components, amino acid. They constitute a complex group of enzymes, which are different in their properties such as substrate specificity, active site, optimal pH and temperature, and the stability profile. The specificity of the protease is controlled by the nature of the amino acids, presence of different functional groups close to the peptide bond. Proteases are commonly found in all living organisms and play a significant role in catalysis of various metabolic reactions and industrial processes [1].

It has been found that proteases are commonly responsible for activation of proenzymes, digestion of fibrin clots, defense against plant pathogens, secretory protein processing, germination, senescence, analgesic effects and induction of apoptosis. The proteases are also being used in the food industry, detergent dust preparation, and treatment of industrial effluents, textile industry, leather manufacturing, and pharmaceutical industry, cleaning of surgical and biomedical equipments [2].

Plants belonging to the Euphorbiaceae family usually contain proteolytic enzymes in latex. Latex is a milky fluid with a complex mixture of proteins, vitamins, carbohydrates, lipids, terpenes, alkaloids, and free amino acids. The presence of certain enzymes like chitinase and proteases in latex vacuoles suggest that they may help plants for defense against pathogens, parasites, and herbivores by attacking the invader once the plant cell is lysed [3].

Euphorbia tirucalli linn, family: Euphorbiaceae is a laticiferous tree that grows as an ornamental succulent shrub. The species is commonly known as barki-thohar and grows throughout India. Latex is used as anti-arthritic, neuralgia and tooth ache [4] antimicrobial [5] antioxidant, hepatoprotective [6] immunomodulatory [7] cytotoxic and antiviral activities [8] larvicidal activity [9] anti-inflammatory and analgesic

activity [4]. The latex of *E. tirucalli* are reported to have triterpenes such as euphol, tirucallol, glut-5-en-3-b-ol, cycloephordenol, euphorginol, amyryn, lanosterol, cycloartenol [10].

The stem of *E. tirucalli* is reported to have hentriacontene, hentriacontanol, steroid 4-deoxy phorbol ester, beta-sitosterol, casuarin, corilagin, cycloephordenol, cyclotirucanenol, ellagic acids, euphorbins, euphol, euphorone, ellagic acids, euphorbins, euphol, euphorone, euphorcinol, gallic acids and glucosides [11].

Until date, no kinetic study on the isolated protein of the latex of *E. tirucalli* has been carried out. Hence, the present work is undertaken to evaluate the caseinolytic and milk clotting activities of isolated protein of *E. tirucalli* latex using casein and skimmed milk as substrates. We also report herein, the effect of pH, temperature on this enzyme.

METHODS

Plant material

The latex of *E. tirucalli* was collected by surface incisions of stem of healthy plant and allowing the milky latex to drain in clean glass vials, brought to the laboratory and kept in the refrigerator. The plant was identified by Professor S.K. Panda, plant taxonomist, Department of Botany, Orissa University of Agriculture and Technology, Bhubaneswar, where a voucher specimen is deposited for future reference. Diethylaminoethyl cellulose and molecular weight markers were purchased from Sigma-Aldrich, USA. All other reagents used in this study were of analytical grade.

Preparation and purification of crude proteins

The latex was centrifuged at 1000 rpm for 20 minutes, and the supernatant was collected and lyophilized at -40°C. The lyophilized latex was dried to get powder form. The resulting supernatant of latex designated as "crude protein" was used for further purification of proteins. The wet weight of 1 mL of latex of *E. tirucalli* was 1.370 and the dry weight was 0.315 g. Method of purification of proteins from crude protein extract was done using acetone precipitation.

Protein determination

The isolated protein was estimated by Lowry's method [12] using bovine serum albumin as a standard. The protein content of the column eluent was also monitored spectrophotometrically at 660 nm.

Chemical properties of the protein

The chemical properties of the protease isolated by precipitation were studied by qualitative chemical tests such as biuret test for peptides, ninhydrin reaction, xanthoprotic reaction, glyoxylic reaction, millions reaction, nitroprusside test, precipitation by heavy metals and precipitation by acidic reagents.

Caseinolytic activity

Caseinolytic activity was assayed using denatured casein as substrate [13]. 0.4 mL casein (2%) in 0.2 M Tris-HCl buffer, pH 8.5, was incubated separately with different concentrations of protein in a final volume of 1 mL for 2 hrs at 37°C. The reaction was stopped by adding 1.5 mL of 0.44 M trichloroacetic acid, and the mixture was allowed to stand for 30 minutes. The reaction mixture was centrifuged at 1500 g for 15 minutes. An aliquot (1 mL) of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of Folin-Ciocalteu reagent (1:2, v/v). The color developed was read at 660 nm. Activity was expressed as units/hr. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm/h.

Milk clotting activity

The milk clotting activity of protein was determined by the earlier reported method [14]. The substrate (10% skimmed milk) was prepared in distilled water or in 10 mM CaCl₂ in water, and pH was adjusted at 6.5. The milk (2.0 mL) was incubated with different concentrations of protein at 37°C, and curd formation was observed (Berridge, 1952). The end point was recorded when the full separation between whey and curd was observed. One milk-clotting unit was defined as the amount of enzyme that clots 2 mL of the substrate within 180 minutes. Chymosin and 0.15 M NaCl were used as positive and negative controls, respectively. Milk-clotting activity was also determined using skim milk (10% w/v) heated at 30, 50 and 70°C.

Effect of temperature and pH on caseinolytic and milk-clotting activities

Caseinolytic and milk-clotting activities were determined after heating (30 minutes) of protein at 50, 55, 60 and 65°C under the same conditions.

Electroblotting

Purified samples of Euphorbian T was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with Tris-glycine buffer (pH 8.2) in 12% PAGEs [15]. Molecular mass markers (SigmaMarker™ kit from Sigma-Aldrich, USA, containing the standard proteins) were applied on the gel. After running and staining with 0.02% (v/v) coomassie brilliant blue in 10% acetic acid, the gel was dehydrated and scanned. The densitograms was obtained using the software Scion Image Beta 4.02.2 (Scion Corporation, Frederick, MD, USA) and indicated the intensity of polypeptide bands.

Determination of kinetic parameters

The apparent K_m (Michaelis equilibrium constant) and V_{max} (Maximum attainable reaction velocity) values of Euphorbian T were estimated for the substrate (Casein and milk) from the linear portion of line-weaver and Burk double reciprocal plot [16]. The reciprocal of reaction velocity (1/V) was plotted against the reciprocal of corresponding substrate concentration (1/[s]) giving the Lineweaver-Burk plot.

Determination of thermodynamic parameters

Thermodynamic parameters of Euphorbian T were evaluated from arrhenius plots (1/T×1000 vs. log of activity) of casein and milk [17]. From the slope of the curve Ea values were calculated and using this (Ea=ΔH) values, other thermodynamic parameters, such as, standard

free energy change (ΔG), entropy change (ΔS), energy of activation (Ea), enthalpy change (ΔH), probability factor or steric factor (P) and collision number (Z) were calculated.

Thermodynamic quantities were computed from the following equations:

- 1) $K=A e^{-E_a/RT}$
- 2) $\log K=\log A-(E_a/2.303RT)$
- 3) $\log K_2/K_1=\Delta H/2.303R (1/T_1-1/T_2)$
- 4) $A=(RT/Nh) e^{\Delta S/R}$
- 5) $\Delta G=\Delta H-T\Delta S$

RESULTS AND DISCUSSION

The electrophoresis analysis of Euphorbian T was performed using SDS-PAGE. The different aminoacids of Euphorbian T were appeared in different bands of 36.5, 39, 44 and 50 Kda (Fig. 1). Maximum proteolytic activity of Euphorbian T was obtained at pH 6. Initial pH of the reaction mixture lower or higher than 6.0 gave relatively less amount of proteolytic activity. Hence, the initial pH value of 6.0 was optimized for proteolytic activity for further studies. The strong acidic or basic pH has an inhibitory effect on the enzyme, thus reducing its maximum enzymatic activity. The active sites of enzymes are affected by a change in pH, which influences the ionization of these amino acids, and are involved in the formation of products by catalyzing the substrate [18].

To study the effect of temperature on milk clotting activity of Euphorbian T, a temperature range from 50 to 65°C was selected. The Fig. 2 shows

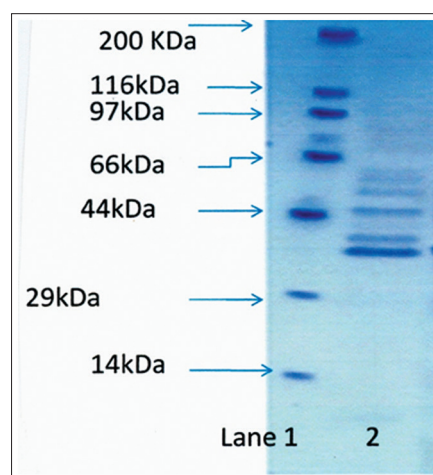


Fig. 1: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of purified Euphorbian T

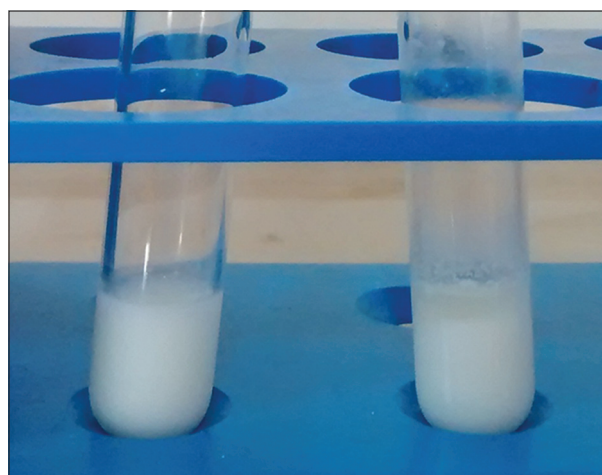


Fig. 2: Milk clotting activity of purified Euphorbian T

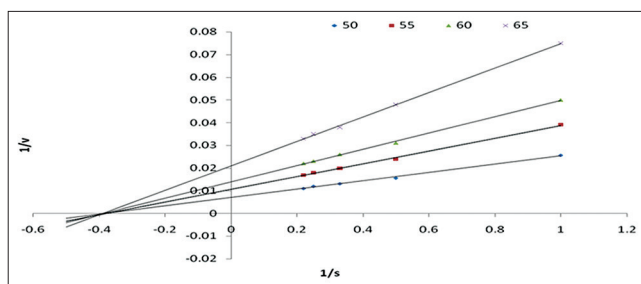


Fig. 3: Lineweaver-Burk plots for Euphorbian T with milk as substrate at different temperature

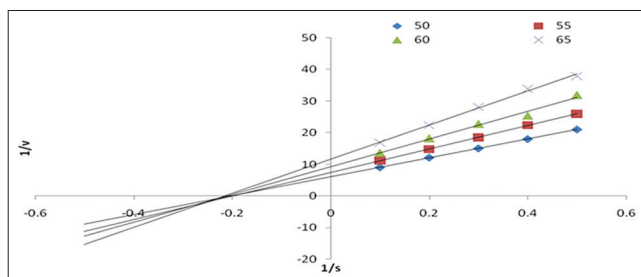


Fig. 4: Lineweaver-Burk plots for Euphorbian T with caesin as substrate at different temperature

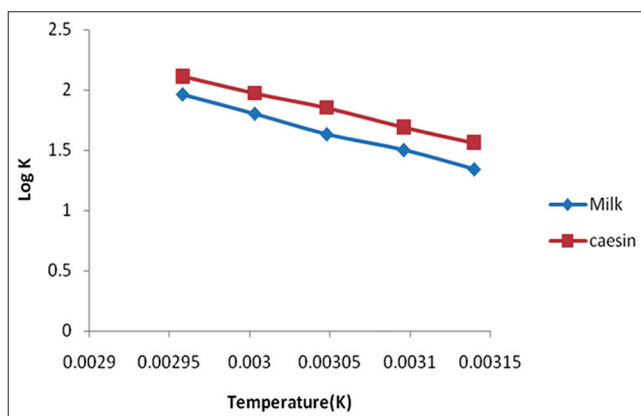


Fig. 5: Arrhenius plot for Euphorbian T with milk and caesin as substrate

that the milk clotting time decreased as temperature increased. The maximum milk clotting activity was obtained at 65°C. Further, increase in temperature resulted in decrease of milk clotting activity. So, reaction temperature of 65°C was optimized for further studies. The milk-clotting activity of Euphorbian T did not interfere from 50 to 55°C, which increased significantly after heating at 60°C. This is supported by milk-clotting activity of isolated milk-clotting enzyme, religiosin B, obtained from *Ficus religiosa* stem latex [19].

Casein was used as substrates to determine the caseinolytic activity of Euphorbian T. The enzyme reaction was measured by absorbance at 366 nm. Caseinolytic activity on casein significantly increased after heating of Euphorbian T at 60°C, while loss of this activity was detected after heating of Euphorbian T at 65°C.

The kinetics constants (K_m and V_{max}) for proteolytic activity extracted from *Euphorbia tirucalli* were determined by incubating fixed amount of enzyme with varied amount of milk (1-4.5 mL) and casein (2%) as substrates. K_m and V_{max} for Euphorbian T with milk were calculated using Lineweaver-Burk double reciprocal plot and were found to be 4 mg/dl and 0.21 U/mg respectively (Fig. 3), whereas with caesin as

Table 1: Kinetic parameters of Euphorbian T for different substrate

Kinetic parameters	Substrate	
	Milk	Casein
K_m (mg/mL)	4	2
V_{max} (unit)	0.21	1.03

Table 2: Thermodynamic parameters of Euphorbian T for different substrates

Thermodynamic parameters	Substrates	
	Milk	Casein
E_a (kcal/mol)	1.58	1.12
ΔH (kcal/mol)	1.54	1.08
ΔS (cal mol/deg)	-10.46	-16.24
ΔG (kcal/mol)	18.87	16.37

substrate were found to be 2 mg/dl and 1.03 U/mg respectively (Fig. 4 and Table 1).

The best substrate for the enzyme depends on two factors that are strong substrate binding or high affinity (low K_m value) and high catalytic efficiency (high V_{max} value) for a fixed enzyme concentration.

The low K_m value and high V_{max} value of the substrates such as milk and caesin for the isolated protein, Euphorbian T are in agreement with the previous report [20].

Activation energy (E_a), enthalpy of activation (ΔH) and entropy of activation (ΔS) and free energy changes (ΔG) were calculated using Arrhenius plot and were found to be 1.58 kcal/mol, 1.54 kcal/mol, -10.46 cal mol/deg and 18.87 kcal/mol respectively with milk as substrate and 1.12 kcal/mol, 1.08 kcal/mol, -16.24 cal mol/deg and 16.34 kcal/mol respectively for casine as substrate.

The logarithm of milk clotting and caesinolytic activity retained by the enzyme, Euphorbian T ($\log k$) plotted against time (Fig. 5), exhibited a linear decrease of activity with time. The linear decrease in activity predicted that Euphorbian T followed first-order kinetic. A number of factors such as conformational bindings, the degree of exposure of the polypeptide backbone, hydrogen and hydrophobic bonds, non-polar residues, etc. determine the activity of enzymes. The values of enthalpy of activation (ΔH) and entropy of activation (ΔS), presented in Table 2, predicted the different kind of interactions with the substrates. The negative values of ΔS (-10.46 and -16.24 cal mol/deg) and ΔH values of (1.54 and 1.08) are the supporting data for the formation of enzyme-substrate and are involved in either ion-dipole or dipole-dipole interactions [21].

CONCLUSIONS

The effect of pH, temperature, concentration of substrate on the proteolytic activity of Euphorbian T was evaluated in this study. The study showed that maximum milk clotting and caesinolytic activity (0.21 U/mg and 1.03 U/mg respectively) was obtained at pH 6 and 65°C.

REFERENCES

- Sumantha A, Larroche C, Pandey A. Microbiology and industrial biotechnology of food-grade proteases: A perspective. Food Technol Biotechnol 2006;44(2):211-20.
- Liggieri C, Obregon W, Trejo S, Priolo N. Biochemical analysis of a papain-like protease isolated from the latex of *Asclepias curassavica* L. Acta Biochim Biophys Sin (Shanghai)2009;41(2):154-62.
- Vierstra RD. Proteolysis in plants: Mechanisms and functions. Plant Mol Biol 1996;32:275.
- Prabha MN, Ramesh CK, Kuppast II, Mankani KI. Studies on anti-

- inflammatory and analgesic activities of *Euphorbia tirucalli* latex. Int J Chem Sci 2008;6(4):1781-7.
5. Fridous AJ, Islam SN, Faruque AB. Antimicrobial activity of the leaves of *Adhatoda vasica*, *Clatropis gigantean*, *Nerium odorum* and *Ocimum sanctum*. Bangladesh J Bot 1990;227
 6. Jyothi TM, Shankariah MM, Prabhu S, Lakshminarasu S, Srinivasa GM, Setty SR. Hepatoprotective and antioxidant activity of *E. tirucalli*. Iran J Pharmacol Ther 2008;7:25-30.
 7. Valadares MC, Carrucha SG, Accorsi W, Queiroz ML. *Euphorbia tirucalli* L. modulates myelopoiesis and enhances the resistance of tumour-bearing mice. Int Immunopharmacol 2006;6:294-9.
 8. Betancur-Galvis LA, Morales GE, Forero JE, Roldan J. Cytotoxic and antiviral activities of Colombian medicinal plant extracts of the *Euphorbia* genus. Mem Inst Oswaldo Cruz 2002;97(4):541-6.
 9. Yadav R, Srivastava VK, Chandra R, Singh A. Larvicidal activity of latex and stem bark of *Euphorbia tirucalli* plant on the mosquito *Culex quinquefasciatus*. J Commun Dis 2002;34(4):264-9.
 10. Wal A, Wal P, Gupta N, Vishnoi G, Srivastava RS. Medicinal value of *Euphorbia tirucalli*. Int J Pharm Biol Arch 2013;4(1):31-40.
 11. Khan AQ, Malik A. A new macrocyclic diterpene ester from the latex of *Euphorbia tirucalli*. J Nat Prod 1990;53:728-31.
 12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265-75.
 13. Murata J, Satake M, Suzuki T. Studies on snake venom. XII. Distribution of proteinase activities among Japanese and Formosan snake venoms. J Biochem 1963;53:431-43.
 14. Berridge NJ. An improved method of observing the clotting of milk containing rennin. J Dairy Res 1952;9:328-9.
 15. Schägger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368-79.
 16. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc 1934;56:658.
 17. Rodriguez-Correa D, Dahlberg AE. Kinetic and thermodynamic studies of peptidyltransferase in ribosomes from the extreme thermophile *Thermus thermophilus*. RNA 2008;14(11):2314-8.
 18. Khan M, Javed MM, Zahoor S, Ikram-ul-haq. Kinetics and thermodynamic study of urease extracted from soybeans. Biologia 2013;59(1):7-14.
 19. Kumari M, Sharma A, Jagannadham MV, Religiosin B. A milk-clotting serine protease from *Ficus religiosa*. Food Chem 2012;131:1295-303.
 20. Manohan D, Wai WC. Characterization of polyphenol oxidase in sweet potato (*Ipomoea Batatas* (L.)). J Adv Sci Arts 2012;3(1):14-31.
 21. Chanda I, Basu SK, Dutta SK, Das SR. Thermodynamic, kinetic and stability aspects of a purified protease from the latex of *Plumeria rubra* linn. Asian J Pharm Clin Res 2013;6(1):149-51.