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Research Article

Extraction, Purification and Characterization of Protease from Latex of *Plumeria* Sp.

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ABSTRACT:

Protease was isolated from the latex of *Plumeria* sp. using ammonium sulphate precipitation (60% saturation) method and purified by a dialysis followed by DEAE cellulose column chromatography. DEAE Cellulose chromatographic method showed 9.86-fold purification which is about 57.36% yield as compared to crude latex protease. Purified plumerian protease was showing optimum activity at pH 7 and temperature 50^0C. It was activated by 10mM Calcium chloride and betamercaptoethanol, however inhibited by 10mM iodoacetamide, indicated the presence of sulfhydryl as an essential group for its activity. The enzyme kinetic with casein substrate showed k_m and V_{max} of 1.66 mg/ml and 333U/mg, respectively. *Plumeria* sp. showed a single protein band on SDS-PAGE and molecular weight was of 80 kDa. Thus, protease from the latex of *Plumeria* sp. was purified and characterized and it may be explored further to study its impact in medical science as an effective anti-inflammatory agent.

Keywords: Protease, *Plumeria* sp., Sulfhydryl, Purification, Characterization

[I] INTRODUCTION

Protease is one of largest groups of industrial enzymes that catalyzed the hydrolytic reactions by cleaving peptide bonds in protein. Proteases are among the oldest and most diverse families of enzymes known are involved in every aspect of organisms function proteases catalyzed the addition of water across amide (and ester) bonds to cleaving using a reaction involving nucleophite attacks on the carbonyl carbon of the scissile bond. They differ widely in their properties such as substrate specificity, active site and catalytic mechanism and possess different profiles for mechanical stress, chemical environment, pH and temperature for stability and activity. Because of their broad substrate specificity, proteases have a wide range of applications such as in leather processing, detergent formulations, baking, brewing, meat tenderization, peptide synthesis, cheese manufacture, soy sauce production ,protein hydrolysis recovery of silver from waste photographic film, as well as analytical tools in basic research and have high commercial value [1,2]. *Plumeria* flowers are most fragrant at night in order to lure sphinx moths to pollinate them. The flowers yield no nector, however, and simply dupe their pollinators. Most species are deciduous

shrubs or small trees. The species variously are indigenous to Mexico, Central America, the Caribbean, and as far south as Brazil, but are grown as cosmopolitan ornamentals in warm regions. Common names for plants in the genus vary widely according to region, variety, and whim, but variations on that theme are the commonest.

[II] MATERIALS AND METHODS

2.1. Materials and preparation of extract

Plumeria latex was collected from college botanical garden, Nashik and all chemicals used were from Fisher Scientific, Mumbai. Latex was collected into glass tubes containing 1ml of 10% sodium metabisulphite by incision of the flower branches of plants [3]. The crude latex was strained through cotton wool to remove suspended coarse inert materials and then centrifuged at 7000 rpm and 4 ^{0}C for 30mins.The supernatant was collected and used as the crude protease enzyme. Protease assay and protein estimation was done as described below.

2.2. Measurement of protease activity and protein

Protease activity was measured using caseinolytic assay [4]. The culture supernatant (1 mL) was incubated in 4 mL of 0.625% casein at 37^{0}C for 30 min. The reaction was stopped by the addition of 5 mL of trichloroacetic acid (5%) and the casein hydrolysis product was measured by modified Folin–Ciocalteu method, against inactive enzyme. A standard graph was generated using standard tyrosine of 10–50 mg mL^{-1}. One unit (U) of protease activity was defined as the amount of enzyme, which liberated 1 mg tyrosine per min at 37^{0}C. Protein concentration was measured by the method of Biuret method using BSA as the standard [5].

2.3. Purification of protease

Protease from *Plumeria* latex was partially purified by ammonium sulphate precipitation (60% saturation) at 4^{0}C and dialyzed against 50mM phosphate buffer (pH 7) for 24 h. The enzyme preparation was loaded on a DEAE cellulose column (2.5 x 15.0 cm), previously equilibrated with the same buffer.

The isolation of protease from the DEAE column was performed by stepwise elution with sodium chloride (NaCl) at various molarities (0.01 – 0.8M) prepared in 20mM phosphate buffer of pH 7.0. The active fractions were pooled and subjected to detect protease activity. All the purification steps were performed at 4^{0}C. Active fraction of purified protease was used for further characterization.

2.4. Characterization of protease

The molecular weight of purified protease was determined by comparing with mobility of standard molecular weight marker proteins of Sigma (St. Louis, MO, USA).

The purified enzyme of *Plumeria* sp. was characterized with different pH values in the range of 6–12 (pH 6, 50 mM acetate buffer; pH 7–8, 50 mM phosphate buffer; pH 9–10, 50 mM Tris–HCl buffer; pH 11–12, glycine–NaOH buffer at 30^{0}C) and temperature range of 30–60^{0}C with 50 mM phosphate buffer (pH 7) conditions. The effect of 10mM concentration of $CaCl_2$, $FeSO_4$, $ZnSO_4$, $MgSO_4$, NaCl, $HgCl_2$, ethylene diaminetetraacetic acid (EDTA), phenylmethanesulphonyl fluoride (PMSF) and β-mercaptoethanol on the enzyme activity was also studied. The substrate specificity of the purified protease was examined against casein, bovine serum albumin, and gelatin. To determine the kinetic parameters, protease enzyme was incubated with various concentration of casein (2 to 20 mg/ml) prepared in 50 mM phosphate buffer (pH 7) and the enzyme kinetic were performed according to Bharathiraja and Jayamuthunagai [6].

2.5. Molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified protease was performed according to the method of Weber and Osborn [7] using 12.5 % polyacrylamide gel.

2.6. Statistical analysis

All data were computed from the mean of at least two independent experiments and expressed as Mean ± S.D. (Standard deviation).

[III] RESULTS

3.1. Purification of protease

Plumeria protease was purified using ammonium sulphate precipitation (60% saturation) followed by dialysis. Total 50 ml dialysate was obtained and showed protease activity of 120.65 U/ml/min with protein concentration of 3.65mg/ml. The specific activity was found to be 33.05 U/mg of protein which showed 1.67 fold purification and 84.02% yield of purified protease.

Total 50 ml dialysate were loaded on DEAE Cellulose column which is pre-equilibrated with 20mM Phosphate buffer (pH 7). The DEAE Cellulose was a affinity chromatography column and the bound protease was eluted using 0.01-0.8M NaCl gradient concentration. Total 24 fraction of 2 ml each were collected and were analyzed for OD at 280nm and protease assay. Total 8 fractions (11-18) showed protease activity and peak at 280nm. Fraction no 14 showed maximum protease activity (Figure 1).

All 8 fractions were pulled together and found protease activity of 256.56 U/ml/min with protein concentration of 1.32.

The calculated specific activity was 195.12 U/mg protein which indicated the 9.86 fold protease purification with yield of 57.39% (Table-1).

[Table-1]. Summary of *Plumeria* Protease purification

Sample	Volume (ml)	Protease activity U/ml/min	Protein content (mg/ml)	Total Protease U/ml/min	Total Protein (mg/ml)	Specific Activity (U/mg protein)	Fold Purification	% Yield
Crude	500	14.36	0.726	7180	363.00	19.78	1	100
Ammonium Sulphate Precipitation	50	120.65	3.65	6032.5	182.50	33.05	1.67	84.02
DEAE Cellulose	16	257.56	1.32	4120.96	21.12	195.12	9.86	57.39

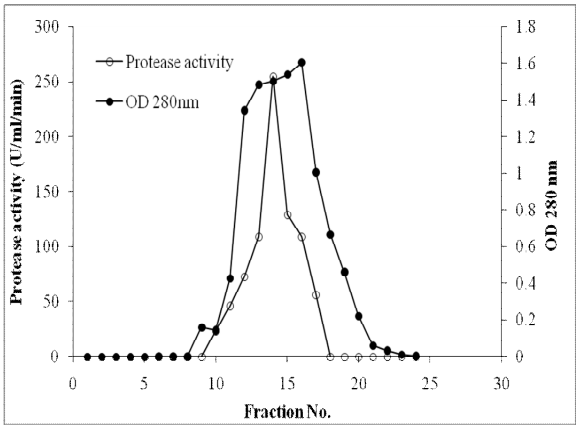

Fig: 1. DEAE Cellulose column chromatographic purification of *Plumeria* purified protease (Solid dot indicate OD at 280nm and hollow circle indicate protease activity).

3.1. Characterization of protease

The purified *Plumeria* protease was found to be highly active in pH range of 6-8 with optimum activity at pH 7 (Figure 2). In acidic pH 4 and 5, it was showing only 36 and 41% activity, respectively. However, in alkaline pH 10, it was completely deactivated. Thus the present study protease is a neutral protease.

The purified *Plumeria* protease was found to be highly active in temperature range of 40-60^0 with optimum activity at temperature 50^0 C (Figure 3).

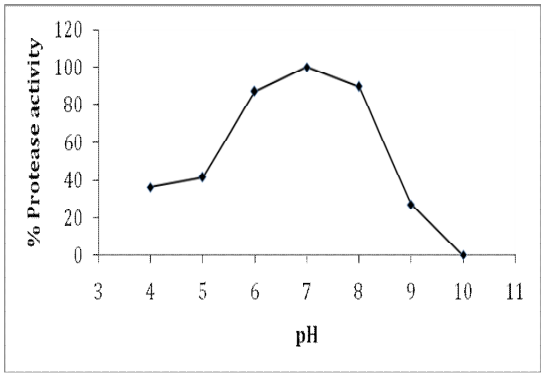

Fig: 2. Effect of pH on *Plumeria* purified protease

In temperature 30 and 35^0 C, it was showing only 26% and 32% activity, respectively. However, in temperature 60^0 C, it was completely deactivated.

Thus the present study protease is slightly thermophilic protease.

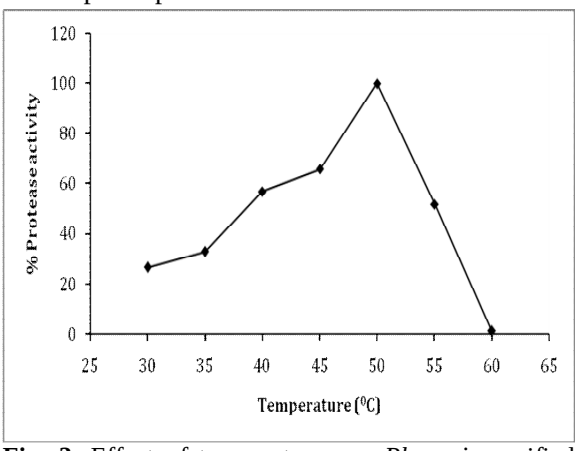

Fig: 3. Effect of temperatures on *Plumeria* purified protease

[Table-2]. Effect of activators and inhibitors on *Plumeria* protease

Activators /Inhibitors (10mM)	Protease activity (U/ml/min ± S.D.)
Control	251.32 ± 9.35
$CaCl_2$	277.78 ± 28.06
$FeSO_4$	165.34 ± 9.35
$ZnSO_4$	257.94 ± 28.06
$MgSO_4$	138.89 ± 18.71
NaCl	208.33 ± 14.03
$HgCl_2$	9.92 ± 4.68
EDTA	205.03 ± 18.71
PMSF	231.48 ± 18.71
Idoacetamide	0
Beta-mercaptoethanol	254.63 ± 4.68

The purified *Plumeria* protease was highly active with 10mM $CaCl_2$ with optimum activity 277.78±28.06 U/ml/min, which is 10 fold higher compared to control. Idoacetamide showed inhibiting effect of purified protease. $ZnSO_4$ also showed slightly improved activity. Slight inhibiting effect on protease was also observed with FeSO4, $MgSO_4$. $HgCl_2$ showed strong inhibition of purified protease with activity of only 9.92 ± 4.68 U/ml/min. EDTA, PMSF and Beta-mercaptoethanol were not inhibiting the protease.

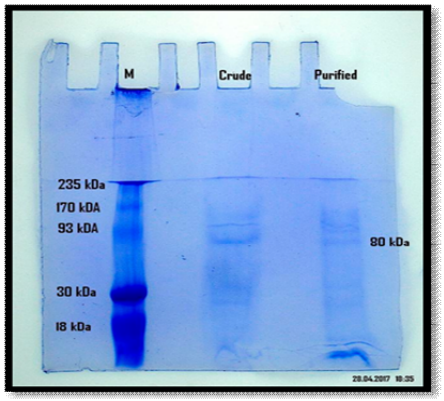

Fig: 4. SDS_PAGE of *Plumeria* purified protease

The molecular weight of the purified protease was 80 kDa on the basis of SDS-PAGE (Figure 4).

[IV] DISCUSSION

The proteolytic enzyme present in the latex of *Plumeria* sp. was precipitated by ammonium sulphate precipitation at 60% saturation. Similar *Plumeria* protease was precipitated using two ways such as ammonium sulphate and acetone but acetone precipitated fraction showed the highest specific activity [3]. But the specific activity of present protease showed highest 33.02 U/mg protein and reported specific activity of Chanda et al.[3] was less due to loss of activity during the precipitation of the enzyme by the salt as well as acetone.

The protease was finally purified 9.86 fold through different purification steps with recovery yield of 57.39%. However, Chanda et al., [3] showed 32.9 fold purification through DEAE Cellulose followed by Sepahadex G50 and 200gel filtration column chromatography with final yield of 7.95%. Thus our results were showed better yields.

The purified protease characterization showed in pH range of 6-8 with optimum activity at pH of 7 and temperature 50^0C. Thus the present study protease is a neutral thermophilic protease.

Chanda et al., [3] also showed optimum pH 7 but it was slightly thermophilic in nature.

The enzyme was activated by $CaCl_2$ indicating a calcium requirement during enzyme folding or activation. Inhibition of protease using iodoacetamide indicated the presence of sulphahydryl group in the active site of enzyme. Iodoacetic acid reacts vigorously with –SH group-containing compounds which is evident from the significant inactivation of the enzyme after treatment with iodoacetic acid [8]. Inactivation of the purified protease by heavy metal ions such as Fe^{2+} and Hg+ was due to the formation of mercaptides [8].

Kinetic studies showed higher substrate specificity against casein followed by BSA and gelatin. Hence different concentration of casein substrate were studied for kinetic studies. It was found that the Km and Vmax of purified protease were 1.66U/mg and 333 mg/ml, respectively. The most matched results of Chanda et al. [3] showed kinetic results with casein substrate with Km and Vmax of 0.625 mg/ml and 0.047 mmoles/min/mg, respectively. Lower the Km higher will be the substrate specificity casein as compared to the reported one.

Purification of protease was checked with a single protein band was observed by SDS–PAGE, indicating that the protease had been purified to homogeneity. The molecular weight 80 kDa of Plumerin sp. is comparable with that of Plumerin-R protease isolated from Plumeriarudra Linn. latex which was reported to be around 81.85 kDa [3] which is very close to the present one. Similaly, Renula et al. [9] reported a protease isolated from jackfruit latex which was reported to be around 79.5 kDa.

[V] CONCLUSION

A protease from the latex of *Plumeria* sp. has been successfully purified to homogeneity by a simple purification procedure and has also been characterized.

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