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## Research Article

# A Protease Isolated from the Latex of *Plumeria rubra* Linn (Apocynaceae) 1: Purification and Characterization

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## Abstract

**Purpose:** To isolate, purify and characterize protease from the latex of the plant.
**Methods:** Protease was isolated from the latex of Plumeria rubra Linn using acetone precipitation method and purified by a sequence of DEAE cellulose column chromatography, followed by two successive column purification in Sephadex G-50 and Sephadex G-200. The molecular weight of the purified protease was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protease was given a trivial name, Plumerin-R.
**Results:** Plumerin-R showed a single protein band on SDS-PAGE and molecular weight was approximately 81.85 kDa. It remained active over a broad range of temperature but had optimum activity at 55 ℃ and pH 7.0 when casein was used as substrate. Activation of the protease by a thiol-activating agent indicated the presence of sulfhydryl as an essential group for its activity.
**Conclusion:** A protease from the latex of Plumeria rubra Linn was purified to homogeneity by a simple purification procedure and then characterized.

**Keywords:** Protease, Plumerin-R, Sulfhydryl, Purification; Characterization

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## INTRODUCTION

Many proteases from plant latex have been isolated and their properties extensively investigated, e.g., ficin from *Ficus carica*, euphorbains from Euphorbia spp., papain and related proteases from *Carica papaya* [1, 2, 3] and calotropain from *Calotropis gigantea* [4].

Proteases have also been purified and characterized from oat, wheat flag, maize, *Phaseolus vulgaris*, *Onopordum turcicum*, *Spinacia oleracea* and *Petroselinum crispum* leaves [5]. Proteases are important enzymes of plant metabolism and are instrumental in regulating senescence [6]. They are responsible for the degradation of proteins. Proteolytic enzymes are used extensively in industrial and medical applications [7].

*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 [8].

The present study was conducted to isolate, purify and characterize the protease from the stem latex of *Plumeria rubra* Linn.

## EXPERIMENTAL

### Materials

The species of *Plumeria rubra* Linn. was collected in the month of April, authenticated by Forest Research Institute, Dehradun, India and a voucher specimen (no. 1917/136250) was deposited in the herbarium of the Forest Research Institute, Dehradun, for future reference. Latex was collected from the stem of the plant. DEAE cellulose and molecular weight markers were purchased from Sigma Aldrich India. Sephadex G-50 and Sephadex G-200 were products of GE Healthcare, India. All other reagents used were of analytical grade.

### Preparation of the crude extract

Latex was collected into glass tubes containing 1 ml of 10 % sodium metabisulphite by incision of the bark of the trunk and branches of the plant. The crude latex was strained through cotton wool to remove suspended coarse inert materials and then centrifuged at 7000 rpm and 4℃ for 30 mins. The supernatant was collected and used as the crude enzyme extract.

### Salt precipitation

The crude enzyme solution was precipitated by ammonium sulphate (40 - 60% w/v). The solution was kept overnight in cold condition (2 – 8 ℃) and then centrifuged (7000 rpm, 30 min, 4 ºC). The precipitate was dialysed in 0.02M phosphate buffer (pH 7.0). Following dialysis, the fraction obtained was used for protein estimation [9] and enzyme assay [10].

### Solvent precipitation

The crude extract was subjected to protein precipitation by adding 3 volumes of chilled acetone slowly to 1 volume of crude extract and the precipitate formed was separated by centrifugation at 7000 rpm for 30 min. The precipitate was dissolved in a minimum volume of water and the protein re-precipitated by adding chilled acetone. The precipitate was collected by centrifugation (7000 rpm, 30 min) and dried at room temperature. All centrifugation steps were performed at 4 ºC. Protein estimation and enzyme assay were carried out for the acetone precipitated fraction.

### DEAE Cellulose Chromatography

The acetone-precipitated fraction was purified with DEAE cellulose column chromatography. Enzyme solution was applied to DEAE cellulose column which had previously been equilibrated with 0.02M phosphate buffer (pH 7.0). The isolation of protein from the column was performed by stepwise elution with sodium chloride (NaCl) at various molarities

(0.01 – 0.8M) prepared in 0.02M phosphate buffer of pH 7.0. Fractions were collected in different collection tubes at a flow rate of 15 ml/h. Absorbance at 280 nm and protease content of each fraction were determined. The protease-active fraction obtained from DEAE cellulose column was purified further by gel filtration.

**Gel filtration on Sephadex G-50 column**

The fraction obtained from DEAE cellulose column chromatography was dialysed against 0.02M phosphate buffer (pH 7.0). The dialysed sample was loaded on to Sephadex G-50 column, pre-equilibrated with 0.02M phosphate buffer (pH 7.0) and eluted with the same buffer at a flow rate of 30 mL/h. Absorbance at 280 nm and protease content of each fraction were determined. Fractions showing high protease activity were pooled and freeze-dried.

**Gel filtration on Sephadex G-200 column**

The purified fraction obtained from Sephadex G-50 was loaded on to Sephadex G-200 column, pre-equilibrated with 0.02M phosphate buffer (pH 7.0) and eluted with the same buffer at a flow rate of 30 mL/h. Absorbance at 280 nm and protease content of each fraction were determined. Fractions showing high protease activity were pooled and lyophilized.

**Protein determination**

The content of protein was determined following the method of Lowry *et al* [9] using bovine serum albumin (BSA) as standard. The protein content of the column eluent was also monitored spectrophotometrically at 280 nm.

**Protease assay**

The assay of proteolytic activity [10] was performed using casein as substrate. Enzyme solution and casein solution were both prepared in 0.02M phosphate buffer of pH 7.0. The enzyme solution (1 ml) was mixed with 1.0 ml of casein solution and allowed to digest at 50 ℃ for 10 min. The reaction was stopped by adding 5.0 ml of 5.0% w/v trichloroacetic acid solution. The precipitate formed was filtered off and the absorbance of the clear filtrate was measured at 280 nm against blank solution. The unit of activity was defined as the amount of protease which caused an increase of one unit of absorbance per minute of digestion. Specific activity was expressed as the number of units of activity per milligram of protein.

**Molecular weight determination**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the lyophilized protease purified by Sephadex G 200 was performed according to the method of Weber and Osborn [11] using 12.5 % polyacrylamide gel. Denatured protein was prepared by incubating the purified protease in boiling water for 3 min in a solution of 0.1 % SDS. The gels were stained with 0.2 % Coomassie Brilliant Blue R-250 in 10 % acetic acid for 1 h at room temperature and destaining was performed by washing the gels in 10 % acetic acid solution. The molecular weight standards used include myosin (199.96 kDa), β-galactosidase (126.37 kDa), bovine serum albumin (82.02 kDa), carbonic anhydrase (38.74 kDa), soybean trypsin inhibitor (32.01 kDa), lysozyme (17.26 kDa) and aprotinin (7.13 kDa).

**Optimum pH and temperature of purified enzyme**

Optimum pH and temperature were determined on casein according to the method of Kunitz ]10]. To study the effect of pH on enzyme activity, the purified enzyme solution was incubated with casein solution of various pH values (4.5 to 8.0) at 50 ℃ for 10 min and activities were measured. In order to determine the optimum temperature, the purified enzyme solution in 0.02M phosphate

buffer (pH 7.0) was incubated with casein solution at various temperatures ranging from 40 to 70 $^0$C for 10 min in a temperature controlled water bath and their activities assayed.

### Assessment of kinetic constants of the purified enzyme

The reciprocal of reaction velocity was plotted against the reciprocal of the corresponding substrate concentration, giving the Lineweaver-Burk plot for the purified protease with the substrate casein. The Michaelis-Menten constant ($K_m$) and maximum reaction velocity ($V_{max}$) of the purified enzyme were determined from the Lineweaver-Burk plot [12].

### Effect of activators and inhibitors on the activity of the purified enzyme

The activating and inhibiting effects of different chemical agents were tested by pre-incubating the pure enzyme with 1.0 mM for each chemical agent at 50 ℃ for 10 min by the method of Kunitz [10] using casein as substrate. The residual activity was measured relative to control. The activity of control was taken as 100 %.

### Statistical analysis

All data were computed from the mean of at least three independent experiments and expressed as mean ± SD. Statistical analysis was carried using Student t-test with the aid of Sigma Plot software (version 10) with significant difference set at $p < 0.05$.

## RESULTS

### Purification of enzyme

The crude extract of the latex contained 83.1 mg/ml of protein with a specific activity 4.65 unit/mg. The fraction of precipitate obtained with acetone showed higher specific activity ($12.73 \times 10^{-2}$ unit/mg) than the ammonium sulphate fraction ($1.78 \times 10^{-2}$ unit/mg). The elution profile of the acetone-precipitated fraction from DEAE-cellulose column is shown in Figure 1; it shows a fraction number ranging from 15 to 21 with good recovery of activity. Sephadex G-50 column elution profile (Figure 2) showed two protein peaks but enzyme activity was present in fractions 20 to 28. On further purification with Sephadex G-200 column, the elution pattern of the protease showed the presence of enzyme activity in fractions 20 to 28 (Figure 3). Table 1 summarizes the results of the purification of protease from 50 ml latex of the plant.

### Molecular weight of protease

The apparent molecular weight of the purified protease was 81.85 kDa on the basis of SDS-PAGE (Figure 4).

**Table 1:** Summary of Plumerin-R purification (mean ± SD, n = 4)

| Purification step | Total protein (mg) | Total activity (unit) | Specific activity (unit/mg)×$10^{-2}$ | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 4155± 2.02 | 193.30±0.43 | 4.65± 0.09 | 100 | 1.0 |
| Acetone fractionation | 421±1.11 | 53.6± 0.55 | 12.73± 0.12 | 46.21 | 2.7 |
| DEAE cellulose chromatography | 32.28±0.90 | 25.61± 0.76 | 79.34± 0.17* | 22.08 | 17.1 |
| Sephadex G-50 gel filtration | 9.62± 1.02 | 11.26± 0.11 | 117.05± 0.07* | 9.71 | 26 |
| Sephadex G-200 gel filtration | 6.02± 0.94 | 9.22± 0.27 | 153.16± 0.08* | 7.95 | 32.9 |

** $p < 0.01$ compared with crude extract.*

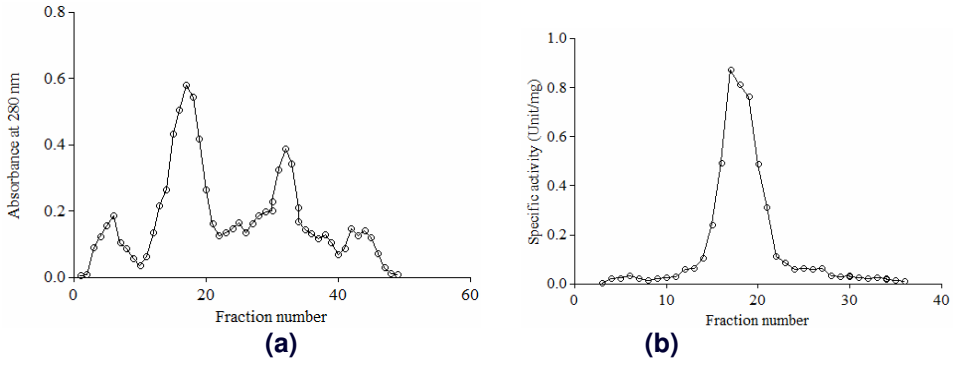


**Figure 1:** Purification of acetone-precipitated fraction by DEAE-celluose column chromatography. ***Note:*** (a) Absorbance and (b) specific activity of different eluted fractions

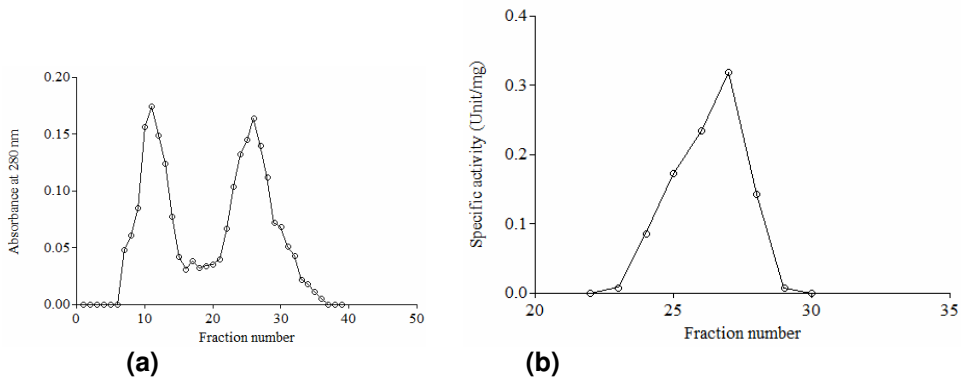


**Figure 2:** Purification of fraction showing protease activity, and obtained from DEAE cellulose column by gel filtration on Sephadex G-50 column. ***Note:*** (a) Absorbance and (b) specific activity of different eluted fractions

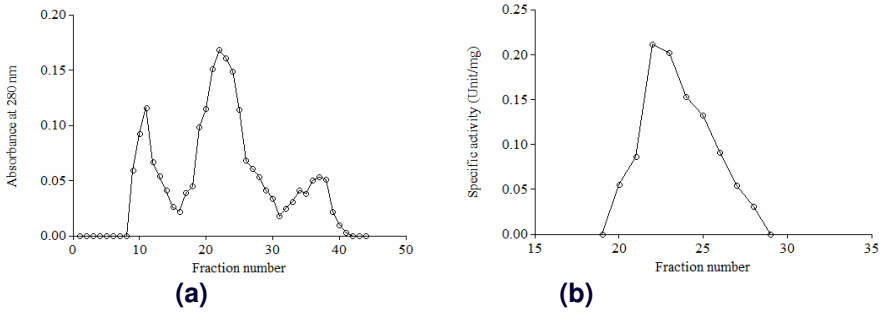


**Figure 3:** Purification of fraction obtained from Sephadex G-50 by gel filtration on Sephadex G-200 column. ***Note:*** (a) Absorbance and (b) specific activity of different eluted fractions

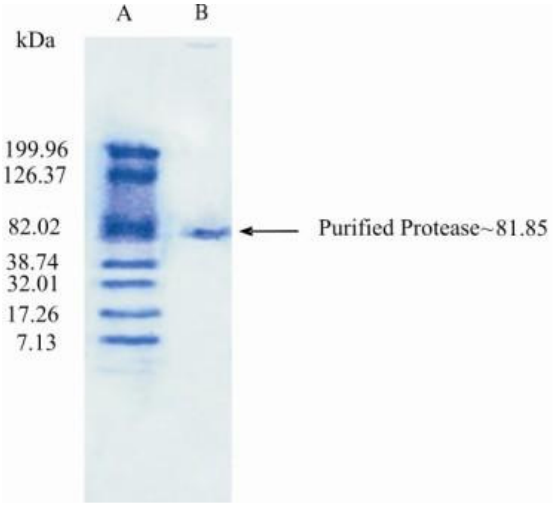


**Figure 4:** SDS-PAGE of the purified protease (A = molecular weight standards; B = purified protease)

### Optimum pH and temperature of purified enzyme

Plumerin-R showed a pH optimum at pH 7.0 and maximum activity at 55 ℃ with casein as substrate. In the presence of the substrate, Plumerin-R was active at temperatures ranging from 40 to 65 ℃; above the optimum temperature, its activity decreased to approximately 51 % at 70 ℃.

### Kinetic constants of the purified enzyme

FrBased on Lineweaver-Burk plot of the purified protease and casein, $K_m$ and $V_{max}$ values were 0.625 mg $ml^{-1}$ and 0.047 mmoles $min^{-1}mg^{-1}$, respectively.

### Effect of activators and inhibitors

The purified protease was activated by agents as follows: cysteine (122.0 %), hydrogen sulphide (109.5 %) and 2-mercaptoethanol (119.6 %). It was inactivated by iodine (89.3 %), iodoacetic acid (64.3 %), cobalt chloride (97.0 %), zinc chloride (88.1 %), silver nitrate (92.9 %), mercuric chloride (94.6 %) and p-hydroxy mercuribenzoate (18.5 %). These results are consistent with the enzyme being a sulfhydryl protease.

## DISCUSSION

The proteolytic enzyme present in the latex could be precipitated by both ammonium sulphate and acetone, but the acetone precipitated fraction showed the highest specific activity. The low specific activity observed in ammonium sulphate fraction indicates a considerable loss of activity during the precipitation of the enzyme by the salt.

The protease was finally purified 32.9 fold through different purification steps and a single protein band was observed by SDS–PAGE, indicating that the protease had been purified to homogeneity. Following the common practice, the trivial name “Plumerin-R” has been given to the protease. The molecular weight of Plumerin-R is comparable with that of artocarpin, a protease isolated from jackfruit latex which was reported to be around 79.5 KD [13].

The optimum pH for activity of plumerin-R suggests that it can be classified as a neutral protease, according to the terminology of Hartley [14]. The enzyme was activated by reducing agents - cysteine and hydrogen sulphide. This is in agreement with the finding of Greenberg and Winnick [15] on bromelain, asclepain-m and asclepain-s. Plumerin-R was also activated by thiol activating agent MSH. These activations are indicative of the presence of sulphydryl as an essential group for the activity of the protease. This is also supported by the strong inhibition of protease activity by p-hydroxy mercuribenzoate. The difference in the degree of activation by cysteine and hydrogen sulphide is an indication that the oxidized sulfhydryl groups of the enzyme are not readily reducible by sulphides. There was considerable inactivation shown by iodine which suggests that aromatic groups might be iodinated in these reactions. Iodoacetic acid reacts vigorously with –SH group-containing compounds which is evident from the

significant inactivation of the enzyme after treatment with iodoacetic acid. Inactivation of the purified protease by heavy metal ions such as $CO^{2+}$, $Zn^{2+}$, $Hg^{+}$ and $Ag^{+}$ was due to the formation of mercaptides [16].

## CONCLUSION

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

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## REFERENCES

1. *Arnon R. Papain. Methods Enzymol 1970; 19: 226-244.*
2. *Liener IE, Friedensen B. Ficin. Methods Enzymol 1970; 19: 261-273.*
3. *Pal G, Sinha NK. Isolation, crystallization, and properties of calotropins DI and DII from Calotropis gigantea. Arch Biochem Biophys 1980; 202: 321-329.*
4. *Abraham KJ, Joshi PN. Studies on Proteinases from Calotropis gigantea Latex. I. Purification and some properties of two proteinases containing carbohydrate. Biochim Biophys Acta 1979; 568: 111-119.*
5. *Jiang WB, Lers A, Lomaniec E, Aharoni N. Senescence related serine protease in parsley. Phytochem 1999; 50: 377-382.*
6. *Lauriere C. Enzymes and leaf senescence. Physiol Veg 1983; 21: 1159-1177.*
7. *Ward O P. Proteolytic Enzymes. In: Comprehensive Biotechnology, Vol. 3, Moo-Young M, editors., Oxford: Pergamon Press; 1985; pp 789–818.*
8. *Kritikar KR, Basu BD. Indian Medicinal Plants. Dehradun: International Book Distributors; 2006; p 1561*
9. *Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265-275.*
10. *Kunitz MI. Crystalline soybean trypsin inhibitor. J Gen Physiol 1947; 30: 291-310.*
11. *Weber K, Osborn M. The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. J Biol Chem 1969; 244: 4406-4412.*
12. *Lineweaver H, Burk D. Determination of enzyme dissociation constants. J Amer Chem Soc, 1934; 56: 658-666.*
13. *Renuka PKM, Virupaksha KT. Purification and characterization of a protease from jackfruit latex. Phytochemistry 1990; 29: 1763-1766.*
14. *Hartley BS. Proteolytic enzymes. Ann Rev Biochem 1960; 29: 45-72.*
15. *Greenberg DM, Winnick T. Plant Proteases: I. Activation-inhibition reactions. J Biol Chem 1940; 135: 761-773.*
16. *Hellerman L. Reversible inactivations of certain hydrolytic enzymes. Physiol Rev 1937; 17:454-484.*