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## Purification and Characterization of a Protease from *Ficus hispida* Linn

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A sulphhydryl plant protease obtained from the latex of *Ficus hispida* Linn was purified by chromatography on Sephadex g-200 upto 36-fold with a yield of 5.22% after initial purification by DEAE-cellulose chromatography. Molecular weight of the protease was estimated by Sephadex G-100 gel filtration and found to be around 23,700 daltons. The Michaelis constant (Km) with substrates haemoglobin and N- $\alpha$ -benzoyl-DL-arginine-p-nitroanilide (BAPNA) was 1.25 mM and 0.83 mM, respectively. The activating and inhibiting effect of some chemical agents on the protease activity of the enzyme was also studied.

*Ficus hispida* Linn (Family: Moraceae) is a small tree grown in shady places and is available in India and some other tropical countries<sup>1-5</sup>. Different parts of the plant are used in the traditional system of medicine<sup>2-7</sup>. The plant on injury exudes a milky and sticky latex<sup>4</sup>. The latex of the plant might also have some useful chemical constituents responsible for the use of the plant in the indicated health problems and as no systematic work has yet been reported on the latex, so the present work was aimed to isolate and purify the proteolytic enzyme present in the latex of the plant and to screen it pharmacologically for any useful therapeutic action in the subsequent studies.

In our earlier paper, the isolation of a protease from the latex by ammonium sulphate fractionation (40-60%), its partial purification by chromatography on DEAE-cellulose and Sephadex G-50 and some of its physico-chemical properties were reported<sup>8</sup>. The optimum temperature and optimum pH for activity of the enzyme was reported to be 40° and pH 7.0, respectively against casein as substrate and showed isoelectric point between pH 4.4 and 4.7.

The present work deals with further purification of the sample of enzyme by chromatography on Sephadex G-200 after initial purification on DEAE-cellulose, estimation of molecular weight, determination of Km and Vmax and effect of some chemical agents on the protease activity of the isolated enzyme.

### MATERIALS AND METHODS

Dialysed (0.02 M phosphate buffer, pH 7.0, 24 h) and lyophilised protein obtained from the crude extract of the latex by 40-60% ammonium sulphate saturation followed by DEAE-cellulose chromatography<sup>8</sup> was the sample of enzyme used for purification and characterization study. Bovine serum albumin (BSA, fraction V, mol. wt. 66,000), dialysis tubing (cellulose membrane), diethyl amino ethyl cellulose (DEAE-cellulose, medium mesh, capacity 0.99 mEq/g), Sephadex G-100 (superfine, 20-50 mesh), Sephadex G-200 (40-120  $\mu$ m), ovalbumin, riboflavine, bovine haemoglobin and N- $\alpha$ -benzoyl-DL-arginine-p-nitroanilide (BAPNA) were products of Sigma Chemical Co., USA. Acrylamide was obtained from Fluka. Casein and N,N-methylene bisacrylamide were purchased from Loba-Chemie Pvt. Ltd., India. All other Chemicals were obtained from either Qualigens Fine Chemicals or E. Merck, India and were of analytical grade. The buffer solutions were prepared as per composition of Gomori<sup>9</sup>.

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### Gel fractionation with Sephadex G-200:

A gel fractionation column (1.25 x 20.0 cm) of Sephadex G-200 was prepared according to the procedure of Railand<sup>10</sup> and was equilibrated with phosphate buffer (0.02 M, pH 7.0). 10.0 mg of enzyme sample obtained from the DEAE-cellulose chromatography was dissolved in 2.0 ml of phosphate buffer (0.02 M, pH 7.0) and the solution was applied on to the column. The enzyme was eluted with same buffer at a flow rate of 30 ml/h in fractions of 3.0 ml at room temperature using Bio-rad econo pump (model EP-1) and fraction collector (model 2110). The absorbance of each fraction at 280 nm was measured. 100  $\mu$ l from each fraction was taken for protease assay<sup>11</sup> with casein as substrate. Fractions possessing protease activity were combined and the total activity and total protein of the combined fraction were determined according to procedure of Kunitz<sup>11</sup> and Lowry *et al.*<sup>12</sup>, respectively.

### Estimation of molecular weight:

Molecular weight of the enzyme was estimated by gel filtration method<sup>13</sup> using a column of Sephadex G-100 (1.30x50.0 cm) equilibrated with 0.02 M phosphate buffer, pH 7.0 at a flow rate of 9.6 ml/h at room temperature. The void volume ( $V_0$ ) was determined using Blue dextran 2000 (mol. wt.  $2 \times 10^6$ ) by eluting with same buffer at the same flow rate (9.6 ml/h) using Bio-rad econo pump and fraction collector. The column was calibrated with bovine serum albumin (mol. wt. 66,000), ovalbumin (mol. wt. 45,000) and cytochrome C (mol. wt. 12,400). Two and a half milligrams of each protein in 1.0 ml phosphate buffer (0.02 M, pH 7.0) were applied to the column separately and eluted with the same buffer and at the same flow rate (9.6 ml/h). Fractions of 2.7 ml were collected and the peak elution volume ( $V_e$ ) of each of the proteins was determined by measuring absorbance of the fractions at 280 nm.

Purified enzyme solution (1.0 ml) obtained from Sephadex G-200 gel filtration was applied to the column and eluted with the same buffer under identical conditions as mentioned for the calibrating proteins. The peak elution volume ( $V_e$ ) of the enzyme protein was determined by measuring the absorbance of the effluents at 280 nm. A calibration curve was constructed by plotting  $\log$  (mol. wt.) against  $V_e/V_0$  and from the calibration plot, the mol. wt. of the enzyme was determined.

### Polyacrylamide gel electrophoresis:

Disc gel electrophoresis was carried out in tubes according to standard procedure<sup>14</sup>. The enzyme sample from Sephadex G-200 gel chromatography was dialysed against distilled water (1, 1, 12 h). To 1.0 ml of this solution 200 mg of sucrose and 2 drops of 0.05% bromophenol blue solution were added and 50  $\mu$ l of this solution was applied on to top of each tube. Electrophoresis was done by applying a current of 3.0 mA per tube till the dye front came to the bottom of the tubes. The gels were taken out of the tubes and stained with Coomassie Brilliant blue (0.46% in a mixture of methanol and glacial acetic acid) solution for 1 h, and destained for two days with several changes (Methanol, 5% and acetic acid, 7.5%).

### Effect of Substrate Concentration on protease activity:

The velocity of enzyme-substrate reaction at different concentrations of haemoglobin and BAPNA were determined to find out the relative enzyme-substrate affinity. Haemoglobin solution (20.0 mg/ml in 0.1 M acetate buffer, pH 5.0) in volumes of 1.0, 1.5, 2.0, 2.5 and 3.0 ml taken in tubes and diluted to 3.0 ml with the same buffer were incubated with 50  $\mu$ l of enzyme solution in the same buffer (5.5 mg/ml) at 40° for 10 min. The activity of the enzyme in each of the digestion mixture was determined according to the method of Anson<sup>15</sup>.

BAPNA solution (1.0 mg/ml in 0.02 M phosphate buffer, pH 7.0) in volumes of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml were made 5.0 ml with the same buffer and incubated with 50  $\mu$ l of the enzyme solution (5.4 mg/ml in 0.02 M phosphate buffer, pH 7.0) at 40° for 25 min and the activity of the protease in each case was measured according to the procedure of Erlanger *et al.*<sup>16</sup>.

### Effect of Activators and Inhibitors:

The activating and inhibiting effect of different chemical agents on the protease activity of the enzyme was measured by the method of Kunitz using casein as substrate. In a series of tubes, 0.5 ml of enzyme solution (0.54 mg/ml in 0.5 M phosphate buffer, pH 7.0) was mixed with 0.5 ml of aqueous solution of reagents at different concentrations (Table 1) and incubated at room temperature (26°) for 60 min. Then 1.0 ml of casein solution (0.5% w/v in 0.05 M phosphate buffer, pH 7.0) was added to each of

TABLE 1: EFFECT OF ACTIVATORS AND INHIBITORS ON THE ACTIVITY OF PROTEASE FROM *FICUS HISPIDA*

Activator/Inhibitor	Conc. of activator/Inhibitor ( $\mu\text{M}$ )	Percent activity of the control
Control	-	100
cysteine hydrochloride	50	118
Hydrogen sulphide	Sat.	105
p-hydroxy mercuribenzoate	20	12
Cobalt chloride	10	98
Zinc chloride	10	92
Mercuric chloride	10	92
Silver nitrate	10	92
Iodine	1	87
Iodoacetic acid	20	71

the tubes and incubated again at 40° for 10 min. A test solution was prepared which contained 0.5 ml of enzyme solution, 0.5 ml of 0.05 M phosphate buffer, pH 7.0 and 1.0 ml of casein solution. A blank was prepared in similar way containing 1.0 ml of buffer and 1.0 ml of casein solution.

## RESULTS AND DISCUSSION

The purification of the enzyme present in the latex of *Ficus hispida* by Sephadex G-200 gel chromatography is in continuation of the earlier work done by the authors<sup>8</sup>.

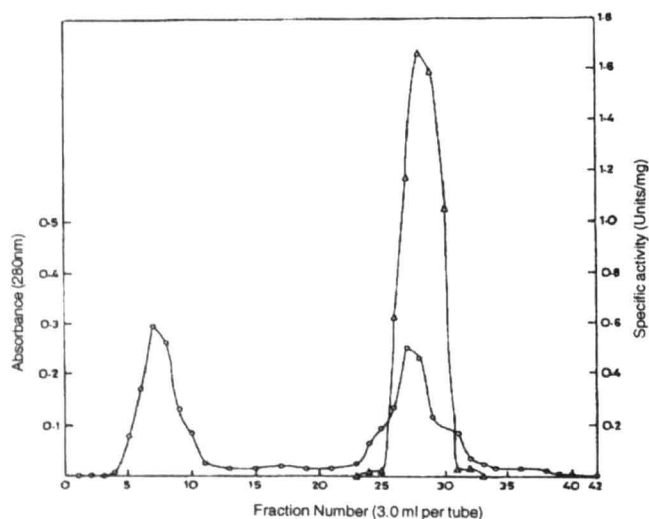


Fig. 1: Sephadex G-200 chromatography of protease from *Ficus hispida*

○—○—Absorbance, -△--△-Specific activity of the protease (Casein as substrate); elution with phosphate buffer (0.02 M, pH 7.0) at a flow rate of 30 ml/h collecting in fractions of 3.0 ml.

The pattern of elution of the enzyme from the gel filtration column of Sephadex G-200 is presented in fig. 1. The plot of absorbance at 280 nm of the fractions against fraction number showed that it contained two major proteins of different molecular sizes eluted in fractions 5 to 10 and 24 to 32. The protease assay of the fractions

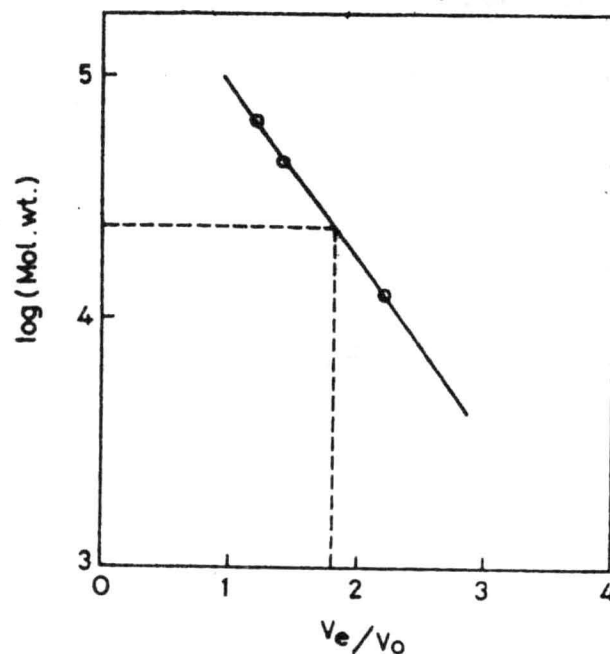


Fig. 2: Estimation of molecular weight of the protease from *Ficus hispida*

Gel filtration of the purified sample on a 1.30 x 50.0 cm column of Sephadex G-100 eluted with phosphate buffer (0.02 M, pH 7.0) at a flow rate of 9.6 ml/h. Calibration of the column was done with BSA (mol. wt. 66,000), ovalbumin (mol. wt. 45,000) and cytochrome C (mol. wt. 12,400)

TABLE 2: PURIFICATION OF PROTEASE FROM *FICUS HISPIDA*

Purification Step	Total protein (mg)	Total activity (units)	Sp. activity (Units/mg) X10 <sup>-2</sup>	Yield (%)	Fold purification
Crude extract	6375.00	265.20	4.16	100.00	1.0
Ammonium Sulphate (40-60%)	525.00	77.09	14.67	48.45	3.5
DEAE-cellulose	29.62	24.45	82.55	15.37	19.8
Sephadex G-200	5.55	8.31	149.73	5.22	35.99

revealed that elution of protease occurred in 24-32 fractions. The active fractions (Fractions 26 to 30) were combined and preserved for characterization study. A total of 5.554 mg of protein was recovered with a total activity of 8.308 units (Specific activity = 1.496 units/mg). The yield was 5.22% with 35.99 folds of purification (Table 2). The purification achieved was much better than the earlier attempt with sephadex G-50 gel filtration (5.23% yield with 27.3 folds of purification<sup>8</sup>). As the purification by gel fractionation with Sephadex G-200 is part of a continuous purification work of the proteolytic enzyme without notable delay between the stages of purification, so the results of isolation and initial purification upto the stage of DEAE-cellulose chromatography reported earlier by the authors<sup>8</sup> have been incorporated in Table 2.

The molecular weight of the enzyme was estimated to be 23,700 daltons from the calibration plot (fig. 2) of log (mol. wt.) against the ratio of void volume ( $v_o$ ) to elution volume ( $v_e$ ) for the calibrating standard proteins BSA, ovalbumin and cytochrome C. The molecular weight of Ficin, a protease isolated from *Ficus glabrata* was reported to be 25,000±700 determined by equilibrium ultracentrifugation and 23,800±700 from the amino acid composition<sup>17</sup> is quite comparable with the molecular weight of this enzyme.

The homogeneity of the purified enzyme sample was established by disc electrophoresis study on polyacrylamide gel which showed a single band of enzyme protein (fig. 3).

The kinetic constants,  $K_m$  and  $V_{max}$  for the enzyme with the substrates haemoglobin and BAPNA were obtained from the Lineweaver-Burk plots given in fig. 4 and fig. 5, respectively. The  $K_m$  and  $V_{max}$  values with hae-

moglobin were 1.25 mM and 0.4 units, respectively whereas the corresponding values with BAPNA were 0.83 mM and 4.0x10<sup>-4</sup> units. It is evident that protease-substrate affinity is high (low  $K_m$ ) for the substrate BAPNA indicating higher specificity with respect to the hydrolysis of amide bonds in synthetic substrate than the larger molecular weight natural substrates.

The enzyme is activated by reducing agents like cysteine and hydrogen sulphide which resembles the finding of Greenberg and Winnick<sup>18</sup> on bromelin, asclepain-m and asclepain-s. This activation is indicative of the presence of sulphhydryl as an essential group for the activity of the protease. This is also supported by strong inhibition of

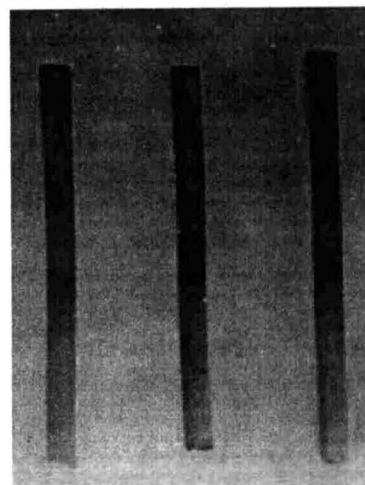


Fig. 3: Disc electrophoresis of the protease sample on polyacrylamide gel.

Electrophoresis of sample (50 µl) obtained from Sephadex G-200 gel fractionation using 3.0 mA current per tube; Stained with Coomassie Brilliant blue (0.46% in a mixture of methanol and glacial acetic acid) and destained by solution containing 5.0% methanol and 7.5% acetic acid.

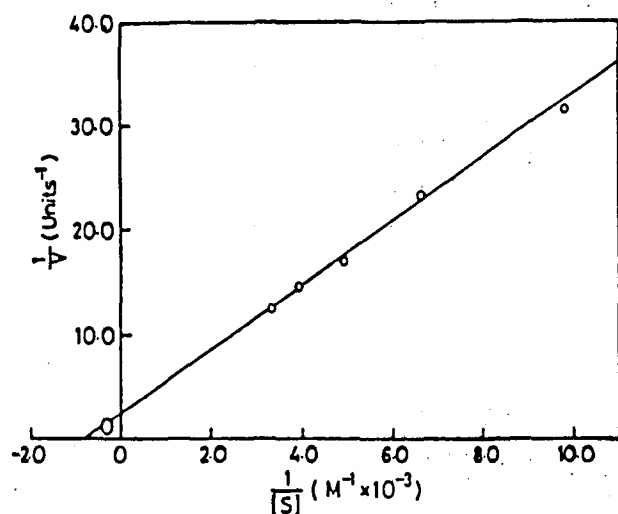


Fig. 4: Lineweaver-Burk plot for the protease from *Ficus hispida*  
Haemoglobin as substrate in 0.1 M acetate buffer, pH 5.0;  
digestion at 40° for 10 min.

protease activity by p-hydroxy mercuribenzoate. The difference in the degree of activation by cysteine and hydrogen sulphide gives the indication that the oxidised sulphhydryl groups of the enzyme are not readily reducible by sulphides<sup>18</sup>. There was a considerable inactivation shown by iodine suggesting that aromatic groups might be iodinated in these reactions<sup>18</sup>. 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 enzyme by heavy metal ions such as Co<sup>2+</sup>, Zn<sup>2+</sup>, Hg<sup>2+</sup>, Ag<sup>+</sup> was observed which might be because of mercaptides with the sulphhydryl group of the protease<sup>18</sup>.

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#### REFERENCES

1. Kanjilal, U.N., Kanjilal, P.C., Das, A. and De, R.N., In; Flora of Assam, Vol. 4, Periodical Expert Book Agency, Delhi, 1982, 252.
2. Kirtikar, K.R. and Basu, B.D., In; Indian Medicinal Plants, 2nd Edn., Vol. III, M/S Bishen Singh Mohendra Pal Singh, Dehradun, 1975, 2322.
3. Nadkarni, K.M., Eds., In; Indian Materia Medica, 3rd Edn., Vol. I, Bombay Popular Prakashan, Bombay, 1982, 550.

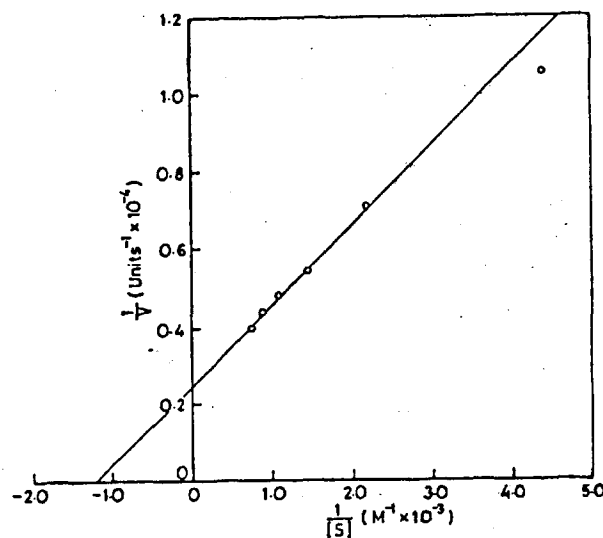


Fig. 5: Lineweaver-Burk plot for the protease from *Ficus hispida*  
BAPNA as substrate in 0.02 M phosphate buffer, pH 7.0;  
digestion at 40° for 10 min.

4. Sastri, B.N., Eds., In; The wealth of India (Raw materials), Vol. IV, Council of Scientific and Industrial Research, New Delhi, 1956, 36.
5. Chopra, R.N., Nayar, S.L. and Chopra, I.C., In; Glossary of Indian Medicinal Plants, CSIR, New Delhi, 1956, 119.
6. Wang, S. and Coviello, D.A., *Tetradedron*, 1975, 31, 929.
7. Trivedi, V.P. and Ansari, Z., *J. Res. Ayur. Sidd.*, 1981, 4, 34.
8. Chetia, D., Nath, L.K. and Dutta, S.K., *Indian J. Pharm. Sci.*, 1999, 61, 29.
9. Gomori, G., In; Colowick, S.P. and Kaplan, N.O., Eds; *Methods in Enzymology*; Vol. I, Academic press, New York, 1955, 138.
10. Railand, J., In; Jakoby, W.B., Eds., *Methods in Enzymology* Vol. XXII, Academic press, New York, 1971, 265.
11. Kunitz, M.I., *J. Gen. Physiol.*, 1947, 30, 291.
12. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., *J. Biol. Chem.*, 1951, 193, 265.
13. Andrews, P., *Biochem. J.*, 1964, 91, 222.
14. Gabriel, P., In; Jakoby, W.B., Eds., *Methods in Enzymology*, Vol. XXII, Academic press, New York, 1971, 565.
15. Anson, M.L., *J. Gen. Physiol.*, 1938, 22, 79.
16. Erlanger, B.F., Kokowsky, N. and Cohen, W., *Arch Biochem. Biophys.*, 1961, 95, 271.
17. Englund, P.T., King, T.P., Craig, L.C. and Walti, A., *Biochemistry*, 1968, 7, 163.
18. Greenberg, D.M. and Winnick, T., *J. Biol. Chem.*, 1940, 135, 761.