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Extraction, Purification and Physico-Chemical Properties of a Proteolytic Enzyme From the Latex of *Ficus hispida Linn*

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A proteolytic enzyme has been extracted from the latex of the plant *Ficus hispida* Linn. The optimum temperature and pH for the activity of the enzyme have been determined. Some physico-chemical properties including the stability of the enzyme at different temperature and pH conditions have also been studied. The enzyme was purified by chromatography on DEAE-cellulose and Sephadex G-50 upto 27.34 fold with a yield of 5.23%.

Ficus hispida Linn (Family : Moraceae) is a small tree grown throughout India and in some other tropical countries[1-5]. The plant on injury exudes a milky and sticky latex[4] that changes colour immediately to brown. Almost all parts of the plant are used in the traditional system of medicine and are useful in ulcers, complications of bile secretion, psoriasis, anaemia, piles, jaundice, hemorrhage of the nose and mouth, diseases of blood, and as antidysenteric, purgative and emetic[2,3,5]. Leaves are used for treating boils and wounds[3,4]. Fruits are considered tonic and, are useful in hepatic obstruction, in promoting secretion of milk[3] and in the treatment of vitiligo[6]. The bark of this plant is used as a component of a mixture used in cancer treatment[7]. There are some reports on the usefulness of proteolytic enzymes in the treatment of wounds[8,9]. The use of the latex of some plants of Ficus in rheumatism, and worm infestations has been reported[10]. This is a part of our programme towards investigating the enzyme present in the latex of this plant for any such property that could be useful in modern therapy.

Bark of the plant has been reported to contain a glycosidic principle[4], 10-ketotetracosyl arachidate[7]; leaves contain bergapten, psoralen, β-amyrin, β-sitosterol[8], n-triacontanol acetate, gluanol acetate[12] and oleanolic acid[13]. Three alkaloids, two of them 3,6,7-trimethoxyphenanthroindolizidine and 3,6,7-trimethoxy-14-hydroxyphenanthroindolizidine known earlier and the third, a new alkaloid named as hispidine [6-(3',4'-dimthoxyphenyl)-7-(4-methoxyphenyl)-1,2,3,5,8,8a-hexahydroindolizine] have also been isolated from leaves[14]. But there have been no report of work on the proteolytic enzyme present in the latex of the plant. The present investigation dealt with the extraction and purification of a proteolytic enzyme from the latex of *Ficus hispida* and study of its physico-chemical properties.

MATERIALS AND METHODS

Latex of Ficus hispida was collected by incision of the trunk and branches of the plant, allowing to drain into glass tubes containing 1.0 ml of 10% sodium metabisulphite in each tube of 10 ml capacity as antioxidant. Bovine serum albumin (BSA, fraction V, mol. wt. 66,000), dialysis tubing (cellulose membrane) and diethylaminoethyl cellulose (DEAE-cellulose, capacity : 0.99 meq/g) were obtained from Sigma Chemical Co., USA; Sephadex G-50 (medium) was the product of Pharmacia, Sweden. Casein was purchased from Loba-Chemie, India. The buffer solutions were prepared according to Gomori[15]. All other chemicals were either obtained from qualigens fine Chemicals or E. Merck, India and were of analytical grade.

Table - I : Purification of Protease from *Ficus hispida*

Sl.No	Purification Step	Total Protein (mg)	Total activity (Units)	Sp. activity (Units/mg) X 10^{-2}	Yield(%)	Fold of Purification achieved
1.	Crude extract	6375.00	265.20	4.16	100	1
2.	Ammonium sulphate fractionation (40-60%)	525.00	77.09	14.67	48.45	3.5
3.	DEAE-cellulose chromatography	29.62	24.45	82.55	15.37	19.8
4.	Sephadex G-50 gel filtration	7.315	8.321	113.75	5.23	27.3

Protease assay : The assay of proteolytic activity was performed using casein as substrate at 40°, pH 7.0 by measuring absorbance of the digested product, soluble in trichloroacetic acid, at 280 nm[16]. The unit of activity was defined as the amount of protease which caused an increase of one unit of absorbance at 280 nm per minute of digestion. Specific activity was expressed as the number of units of activity per mg of protein.

Protein estimation : Estimation of protein was carried out according to the method of Lowry et al[17] colorimetrically at 660 nm using bovine serum albumin as the standard protein.

Enzyme extraction : Preliminary clarification of the crude latex (75 ml) was done by straining through cotton wool and then kept overnight in a refrigerator. The floating white gummy material was separated by scraping and the latex was centrifuged at 8,500 rpm for 30 minutes at 3°. The supernatant (50 ml) was used as the starting material (Fraction-1) for purification. The crude extract (Fraction-1) was divided into two parts for extraction of enzyme by two different methods, viz., acetone precipitation and ammonium sulphate fractionation.

Chilled acetone (60 ml) was added slowly to 20 ml of cooled crude extract and the precipitate formed was separated by centrifugation (8000 rpm, 5°, 20 min), washed with acetone and dissolved in minimum volume of water which was reprecipitated by adding chilled acetone. The precipitate was collected by centrifugation and dried (Fraction-2).

To 30 ml of crude extract (Second part of Fraction-1) ammonium sulphate (7.29 g) was added slowly with constant stirring to make the solution 40% saturated with the salt[18]. the mixture was kept overnight in a refrigerator (8°) and then centrifuged (8000 rpm, 5°, 30 min). The precipitate collected was dispersed in 10 ml of phosphate buffer (0.02 M, pH 7.0) and dialysed against 2 L of same buffer for 24 h and then lyophilized (Fraction-3).

The supernatant obtained after separating Fraction-3 was brought to 60% saturation with solid ammonium sulphate as before. The precipitate obtained was centrifuged (8000 rpm, 5°, 30 min), dialysed against 2 L of phosphate buffer (0.02 M, pH 7.0) for 24 h and lyophilized (Fraction-4). As the Fraction-4 showed good recovery of activity and a high specific activity, so this fraction was taken for further study.

Purification

Ion exchange chromatography of Fraction-4 : Four ml solution of the lyophilized sample (Fraction-4) in 0.02 M phosphate buffer of pH 7.0 was loaded on to column (1.8x30.0 cm) of DEAE-cellulose[19] previously equilibrated with 0.02 M phosphate buffer (pH 7.0) and eluted stepwise with 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8 M sodium chloride solution in the same buffer. Fractions of 5 ml at a flow rate of 15 ml/h were collected. The active fractions were pooled (45 ml) and dialysed against 2 L of 0.02 M phosphate buffer (pH 7.0) for 12h and then lyophilized (Fraction-5). The total protein and total activity of Fraction-5 was determined.

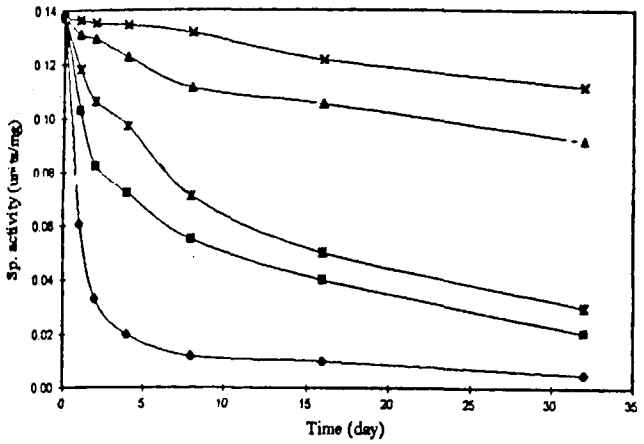

Fig.1 Effect of pH on the stability of protease from *Ficus hispida*.
pH 4.0 (♦-♦)), 5.0 (□ -□), pH 6.0 (▲-▲), PH 7.0 (x-x), pH 8.0 (*-*).

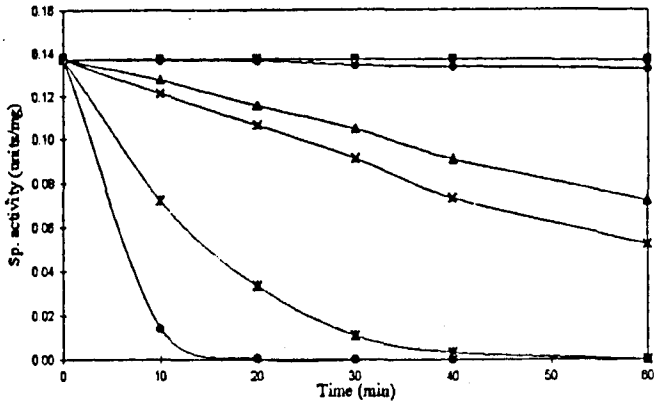

Fig.2 Effect of temperature on the stability of *Ficus hispida* protease.
30° (♦-♦)), 40° (□ -□), 50° (▲-▲), 60°(x-x), 70° (*-*), 80° (O-O).

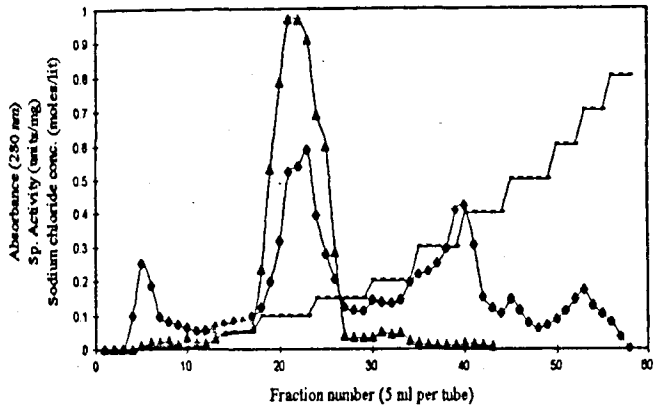

Fig.3 DEAE-cellulose column chromatography of protease preparation from Ficus hispida.
Absorbance (♦-♦), Specific activity (▲-▲), Sodium chloride concentration (--).

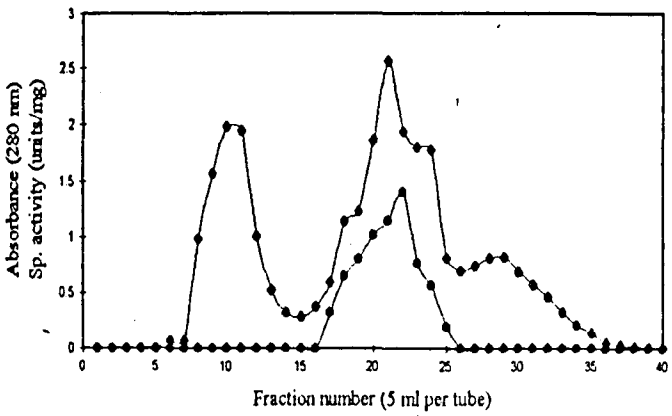

Fig.4 Sephadex G-50 chromatography of protease from ficus hispida.
Absorbance (♦-♦), Specific activity (O-O).

Gel Chromatography of Fraction-5 on Sephadex G-50: Four ml solution of fraction-5 in 0.02 M phosphate buffer (pH 7.0) was applied to the Sephadex G-50 gel column[20] (2.0x43.0 cm) previously equilibrated with phosphate buffer (0.02 M, pH 7.0). Elution was done with the same buffer and fractions of 5 ml at room temperature were collected at a flow rate of 30 ml/h. The fractions showing protease activity were combined (45 ml) to give a partially purified enzyme (Fraction-6).

RESULTS AND DISCUSSION

The crude extract of the latex (Fraction-1) contained 127.5 mg/ml of protein with specific activity 4.16×10^{-2} units mg. The pH of this extract was found to be 4.83. The proteolytic enzyme present in the latex could be precipitated by both chilled acetone and ammonium sulphate, but the fraction of precipitate obtained with 40-60% ammonium sulphate saturation showed high specific activity

(14.67×10^{-2} units/mg) and was used for purification as well as characterization. A low specific activity (2.17×10^{-2} units/mg) observed in acetone precipitated fraction revealed a considerable loss of activity during the precipitation of enzyme with this solvent.

The enzyme solution in water showed positive biuret, ninhydrin, xanthoproteic, glyoxalic reaction, Pauly's test and Millon's reaction. It was precipitated by salts of heavy metals, and acids such as picric acid, 20% trichloroacetic acid. However, nitroprusside test was found negative. But the proteolytic enzyme was activated by cysteine and inactivated by p-hydroxymercuribenzoate, suggesting that it was a sulphydryl proteinase.

The optimum temperature and optimum pH for activity of the enzyme were found to be 40° and 7.0 respectively. The isoelectric point was recorded to be between pH 4.4-4.7 as determined by the method of Malhotra[21] which was near to the isoelectric point of ficin isolated from the latex of Ficus carica[22].

The plot of specific activity at different pH conditions vs time shows the pH stability pattern of the enzyme (Fig.1). Activity of the enzyme in buffer solutions (5 mg/10 ml) of pH 4,5 (Acetate buffer, 0.02 M), 6, 7 and 8 (Phosphate buffer, 0.02 M) which also contained methyl and propyl paraben in 0.1% w/v and 0.025% w/v concentrations, respectively as antimicrobial preservatives was measured after interval of 1, 2, 4, 8, 16 and 32 days of storage at room temperature (30° ± 2). The enzyme showed maximum stability at and around pH 7.0 and considerable loss of activity was observed towards either acidic or basic pH conditions.

The stability of the enzyme at different temperature conditions was studied by incubating enzyme solutions (5 mg/ml) in phosphate buffer (pH 7.0, 0.02 M) at temperatures varying from 30° to 80° with an interval of 10° and measuring activity at 10, 20, 30, 40 and 60 minute intervals. The plot of specific activity at different temperature conditions vs time shows the stability pattern of the enzyme at different temperature conditions (Fig.2). The enzyme showed a linear decrease in activity with time at moderately high temperature, but a sharp decrease at higher temperature. At 80°, the enzyme was inactivated completely after incubation beyond 20 minutes.

The chromatographic separation of the enzyme on DEAE-cellulose column is given in Fig.3. Enzyme was eluted between fractions 18 to 26 at 0.10-0.15 M sodium chloride. The elution pattern of the enzyme on gel chromatography on Sephadex G-50 is presented in Fig.4. The protein peaks in the elution diagram reveals that the enzyme sample contained two proteins of different molecular sizes, but the enzyme activity was found to be in fractions 17-25.

Total protein and total protease activity of the fractions at each purification step was determined and specific activity in each case was also calculated. The overall purification achieved was 27.34 fold with a yield of 5.23%. A summary of the purification steps is presented in Table-1. The above study revealed that the latex of *Ficus hispida* contained an enzyme possessing appreciable protease activity.

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