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Protease of *Ficus bengalesis*

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The protease contents of the freeze-dried leaves of *Ficus bengalesis* was determined. The extraction of the protease was carried using buffers of different pH. The extraction was maximum with buffer pH 8.2. The extracted protease was characterized by studying on its activity by effect of temperature, pH and substrates such as casein, meat and minced meat. The research activity was further extended to study its thermostability and its pH-stability. The whole plant was found rich in protease activity. Its freeze-dried sample contained 17.6 units of activity/g. The activity of the protease of *Ficus begalinensis* was optimum at 40 ºC and afterward decreased with the increase of temperature. The pH-profile exhibited three pH optima.

Key Words: Protease, *Ficus bengalesis*.

INTRODUCTION

The importance of latex plants widely encountered as wild agricultural species in Pakistan, India, China, *etc*., is well appreciated[1]. A number of latex plants have been investigated for their protease activity and other characteristics particularly in medicinal context[2-5]. Significant research work on these plants has been also carried in Pakistan.

Khan *et al.*[6] investigated the protease of *Calotropis procera*. The same workers in 1983 studied *Ficus bengalenensis*, *Calotropis procera* and *Ficus elastica*, *etc*., for their protease activity and rennet action[7]. Khan and Talib[8] immobilized the protease of *Calotropis procera* on amberlite-50 and thus reported its extent of bonding with the matrix and compared some characteristics of the soluble and immobilized enzyme. Khan and Jilani[9] carried out its partial purification and compared the characteristics of the crude and partially purified protease of *Calotropis procera*. Nawaz and Khan[10] reported results about immobilization of protease of *Euphorbia royleana* by binding it with DEAE-A50.

The work mentioned above was extended to *Ficus bengalenensis* (English name: Banyan tree, Urdu: Bar /Barged and Punjabi: Bohr) that is a wild tree encountered both in Pakistan and India and found abundantly in Bengal and Assam. The importance of its components such as its latex, leaves, bark, root fiber, buds and fruit, *etc*., in medicine has been described in Indian Materia Medica[1]. For instance, the latex of banyan tree has been applied externally to treat the cases of pains, bruises, rheumatism, cracking of the soles of feet, tooth-ache, *etc*. Internally, it is effective in treatment of dysentery and diarrhea. Similarly its bark has been found effective in treatment of leucorrhoea and diabetes.

The work was undertaken on the assumption that the medicinal role of plant may be due to the presence of protease enzymes in banyan tree. Thus, the investigation was carried to assess the protease content of *Ficus bengalinensis* and determine its characteristics to frame an idea about the nature of the enzyme extracted from the tissues of the banyan tree.

EXPERIMENTAL

Collection of sample: In spite of banyan tree being a rare species, we were lucky to see the presence of *Ficus bengalensis* with all its historical importance at our place of work that is Chemistry Department, Government College, Lahore. In spite of its availability in Chemistry Department to take samples daily and process fresh, it was decided to get the sample of tree leaves freeze-dried to preserve its enzymes over prolonged period.

Freeze-drying of sample: As the enzymes deactivate if the sample containing water are kept even in the frozen state for prolonged periods due to hydrolysis, it was thought advisable to freeze-dry the samples so that these could be investigate without deactivation.

The freeze-drying was done in Veterinary Research Institute, Harikay Road, Lahore as they are equipped with this facility to freeze-dry vaccines in bulk for supply as a commer-

cial exercise. The freeze-dried sample was ground to fine powder and stored desiccated in a glass bottle at - 16 ºC in a deep freezer.

Extraction of the enzyme: The extraction of the enzyme was carried using buffer of different pH. For extraction of the protease from the sample, 100 mg of sample powder was shaken with 100 mL each of buffers of different pH. The suspension was filtered to obtain protease as soluble filtrate. One mL of the filtrate was used for the assay of protease activity.

Assay of protease activity: The protease activity assay was carried out by the method of McDonald and Chen[11]. An adequate volume of the test sample usually 1 mL was incubated with buffered substrate. The soluble products formed as a result of protease action were lower proteins, peptides and amino acid mixture. Undigested proteins were precipitated with adequate volume of 5 % trichloro acetic acid (5 mL). The contents were allowed to settle and then filtered. The protein hydrolyzed was measured by developing a blue colour with Folin-Ciocalteau phenol reagent and reading the optical density of the colour at 700 nm in a colorimeter

The unit of protease activity was defined as the amount of the enzyme that caused an increase in optical density of 0.1 under the assay conditions defined.

Assay of protease activity of freeze-dried powdered sample: An adequate amount of the powdered *Ficus bengalenensis* was taken in a test tube along with 1 mL of distilled water or phosphate buffer pH 7.0. The sample was incubated with 4 mL of 1 % casein substrate for 1 h at 30 ºC. The assay was carried out further as narrated above. The activity was reported in units per gram of the sample. The residual protein was precipitated by adding 5 mL of 5 % trichloro-acetic acid. The contents were allowed to settle and then filtered using Whatman No. 40 filter paper. After filtration, 1 mL aliquot of the filtrate was mixed with 5 mL alkaline reaget prepared by mixing 100 mL sodium carbonate (2 %), 1 mL sodium potassium tartarate (2.7 %) and 1 mL copper sulphate (1 %). Then 2 mL of 1 N NaOH was added to make the contents of the tube alkaline. After, at least 10 min, 0.5 mL Folin-Ciocalteu phenol reagent was added and blue colour developed was read in a colorimeter exactly after 0.5 h at 700 nm.

The unit of protease activity was defined as the amount of the enzyme that caused an increase in optical density of 0.1 under the assay conditions defined.

Determination of enzyme characteristics: The effect of different parameters such as substrate concentration, temperature, pH, *etc.*, on protease activity was determined. The temperature stability and the pH-stability were also determined.

Effect on different substrates: 100 mg of freeze-dried powder of *Ficus bengalenensis* in 1 mL of distilled water was incubated with 4 mL of 1 % casein substrate prepared by dissolving 1 g soluble casein in buffer pH 7 and essay of solublized products was carried as above. Alternatively, the same procedure was followed except 4 mL 1 % casein was substituted by 1 g hard piece of beef in 4 mL distilled water or buffer pH 7 or 4 mL of minced beef suspension obtained by homogenization of 1 g minced beef with 100 mL distilled water or phosphate buffer pH 7.

Variation of enzyme activity with amount of sample: To study the effect of amount of sample on enzyme activity different quantities of *Ficus bengalenensis* powder *i.e.*, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg were incubated after addition of 1 mL distilled water in each, with 4 mL 1 % casein substrate for 1 h at 30 ºC. The assay was carried as above. The change in optical density was plotted as a function of amount of sample powder.

Effect of temperature: The variation of reaction velocity with temperature was studied in the range 20-80 ºC. 100 mg sample in 1 mL distilled water was incubated with 4 mL of casein substrate in a test tube for one hour at temperatures in the range 20-80 ºC with difference of 10 ºC. The assay was carried out as usual. The change in optical density was plotted as a function of temperature and the optimum temperature for protease activity was determined.

Determination of thermo-stability: To determine thermo-stability, 100 mg enzyme sample in 1 mL distilled water was subjected to the effect of temperature at 20, 30, 40, 50, 60, 70 and 80 ºC by incubating the enzyme sample in a thermostat for 15 min with occasional shaking. The residual activity of all the samples affected by different temperatures was assayed at 30 ºC using casein as a substrate as usual. The percentage of residual activity was plotted as a function of temperature. Thermostability was examined from the curve.

Effect of pH on enzyme activity: The effect of pH on activity of enzyme sample from *Ficus bengalenensis* towards casein was studied within the pH range 2-11 using citrate-phosphate-borate buffer[12]. To this end 100 mg sample was incubated with buffers of different pH and the activity was determined as above. The activity could not be tested with casein within the whole range as this precipitates down in pH-range 4-6. The optical density was plotted as a function of pH and pH optimum for the protease activity was noted.

Effect of indirect heating on protease activity: The effect of indirect heating the change in enzyme activity of *Ficus bengalenensis* was studied by heating 100 mg enzyme samples in 2 mL distilled water on a boiling water bath for 30, 60, 90, 120, 150 and 160 min. The residual activity was assayed as narrated above.

Effect of direct heating on protease activity: The effect of direct heating on protease activity was examined by boiling 100 mg powder of *Ficus bengalenensis* on Bunsen's flame. To each sample tube was added excess volume of water such that it remained as 1.0 mL after heating for different intervals of time. The rest of the procedure was the same. The residual activity was plotted as a function of time of direct heating.

Effect of enzyme on meat: The effect of enzyme on meat was studied to check its tenderizing power. 100 g meat suspended in an adequate volume of water was boiled with 1 g freeze-dried sample. 2 mL of liquid sample was withdrawn after 10, 20, 30, 40, 50, 60, 70, 80 and 90 min. Each withdrawn sample was mixed with 4.0 mL alkaline reagent used above for protease assay. After at least 10 min, 0.5 mL Folin and Ciocalteu reagent was added and the contents were mixed. The blue colour developed was read for absorbance exactly after 0.5 h. The optical density was plotted as a function of time.

RESULTS AND DISCUSSION

The units of protease activity present per gram of freeze-dried *Ficus bengalenensis* using 1 % casein as substrate were 17.6. The extent of proteolysis of other substrates is compared with that of casein in Table-1.

TABLE-1
COMPARISON OF THE EXTANT OF PROTEOLYSIS OF 1 % CASEIN, 1 g/100 mL WATER SUSPENSION OF MINCED MEAT AND 1 g SOLID MEAT PIECES IN 4 mL WATER UNDER THE ASSAY CONDITIONS DEFINED

Substrate	Optical density (4 times dilution)
1 % Casein	0.44
Meat (1 g piece in 4 mL water)	0.20
1 % minced meat suspension in water	0.15

The results indicate that *Ficus bengalenensis* leaves are rich source in protease activity. The comparison of extant of proteolysis of different substrates indicates that casein is a better substrate than meat and minced meat. This is due to the reason that casein is a soluble substrate and thus it is more prone to hydrolysis than meat and minced meat. A piece of meat intact with enzyme is slightly better substrate a compared to minced meat. This may be due to the reason that minced meat stands at par with solid meat as both consist of insoluble protein. Minced being in 1 g/100 mL water suspension offers less surface protein area and thus is an inferior substrate.

The variation of reaction velocity with increase in amount of sample powder is shown in Fig. 1.

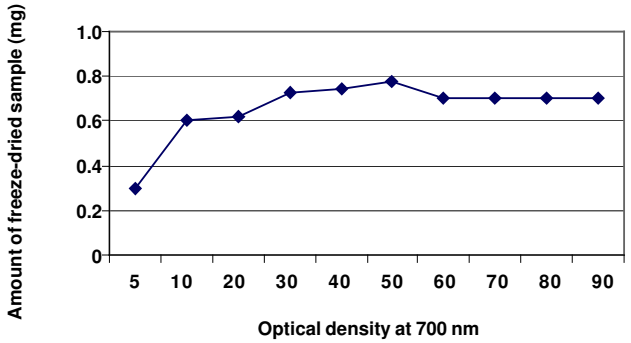

Fig. 1. Variation of the reaction velocity with the amount of the enzyme powder

Variation of protease activity with the pH of the extraction buffer is shown in Fig. 2.

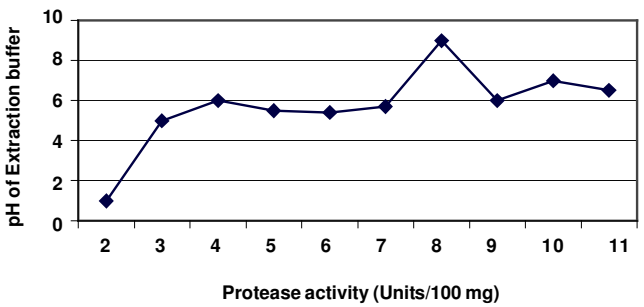

Fig. 2. Variation of the protease activity with the pH of extraction buffer

The temperature profile of the protease of *Ficus bengalenensis* is shown in Fig. 3.

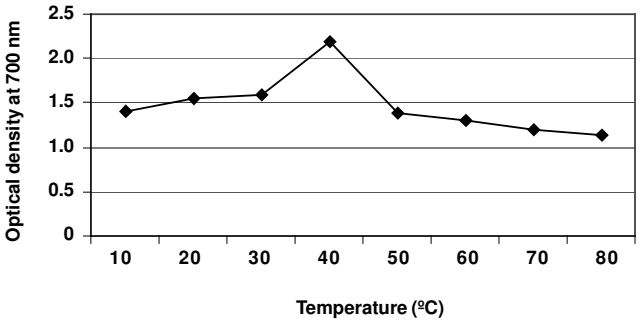

Fig. 3. Temperature profile of the protease of *Ficus bengalenensis*

The profile indicates that the reaction velocity increases with the increase in temperature up to 40 ºC, after which it starts decreasing due to enzyme denaturation. The temperature optimum is 40 ºC. In spite of the temperature optimum being very low, significant activity persists even at 80 ºC.

Thermo-stability of protease of *Ficus bengalenensis* is demonstrated in Fig. 4.

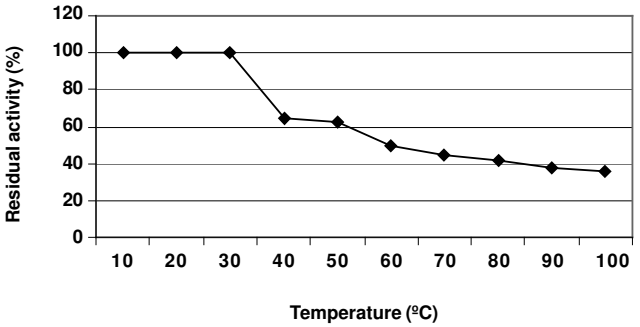

Fig. 4. Thermo-stability of protease of *Ficus bengalenensis*

The profile indicates that the enzyme is stable up to 30 ºC. The shape of the profile reveals the presence of two proteases: one thermo-labile and other thermo-stable. The denaturation of thermo-labile protease starts at 30 ºC while of thermostable protease starts at 50 ºC and continues with further increase of temperature. About one third of activity persists at 100 ºC and that seems to be due to the presence of the thermo-stable component.

The variation of reaction velocity of protease of with increasing pH is shown in Fig. 5.

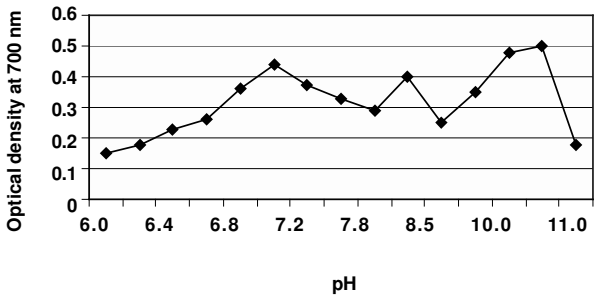

Fig. 5. pH-profile of the protease of *Ficus bengalenensis*

The profile indicates that the protease activity of the sample is optimal at pH values 7.0, 8.2 and 10 to 10.5. Thus, the protease of *Ficus bengalenensis* seems to be a mixture of neutral and alkaline proteases. The activity being more on the alkaline side, the alkaline components of protease dominate the neutral components.

The major point that may be highlighted about the crude protease from *Ficus bengalenensis* is that in contrast to the proteases of many other plants reported our predecessors[6-9] that were dominated by the neutral components, it is dominated by the alkaline proteases. Thus the plant may be investigated further as source of biological detergents.

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