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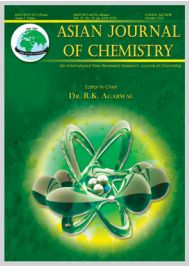

Protease of *Euphorbea pilulifera*

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The content and characteristics of the protease present in the plant tissue of *Euphorbia pilulifera*, a latex plant commonly encountered in Pakistan as Dhokak were studied. The whole plant tissue was found rich in protease activity. It contained 9.6 units/g protease activity in the whole plant tissue. The data on crude state characteristics of the protease of *Euphorbia pilulifera* indicated that its crude extract contained acid, alkaline and neutral proteases. One of the enzyme components was thermo-labile that started deactivating at 40 ºC and another one thermo-stable, which started denaturing at 70 ºC. These indications were supported by the pH-profile of the crude enzyme and also by the pH-stability trends of the enzyme components. These profiles indicated that the dominant protease was neutral. The alkaline protease fell next in order, while acid protease seed to be present in small amount.

Key Words: Protease, *Euphorbia pilulifera*.

INTRODUCTION

Due to a large number of applications of proteases in different fields such as food, agriculture, medicine, industry, *etc.*, an extensive research is being carried on sources, isolation and characterization of proteases[1]. In this connection, a number of latex plants have also been investigated for their protease activity and rennet action[1-3]. An extensive work in this context has also been carried out in Pakistan.

Khan *et al.* studied the protease of *Calotropis procera*[4]. They, studied *Ficus bengalenensis*, *Calotropis procera* and *Ficus elastica*, *etc.*, for protease activity and rennet action[5]. Khan and Talib immobilized the protease of *Calotropis procera* on Amberlite-50 and thus reported its extent of binding with the matrix and compared some characteristics of the soluble and immobilized enzyme[6]. Khan and Jilani carried out its partial purification and compared the characteristics of the crude and partially purified protease of *Calotropis procera*[7]. Nawaz and Khan, reported results about immobilization of protease of *Euphorbia royleana* by binding it[8] with DEAE-A50.

Before describing the work undertaken, it sounds pertinent to highlight the distribution and importance of this *Euphorbia species* in medicinal and social contexts. *Euphorbia pilulifera* (Punjabi name: Dhodak) is a wild latex plant that grows wildly in Pakistan, particularly in Punjab. Its latex contains a strong irritant that causes burning and swelling in humans and animals, even when applied locally to the skin of humans or animals. Its latex has been widely applied as treatment of eczema on skin. After application of latex, the affected skin swells up due to irritation caused by the irritants present in the latex and ultimately ruptures to ooze out *viscous* fluid for a day or so. After this intense reaction, the skin area calms down leaving a scar on the applied area that also *vanishes* after a few days leaving behind healthy skin tissue.

Taking into consideration, its poisonous and irritative tendency and medical applications that were likely to be due to presence of proteases, the work referred above was extended to *Euphorbia pilulifera*. The work schedule included the determination of protease content of its whole plant tissue and study of its kinetic characteristics.

EXPERIMENTAL

Eurphorbia pilulifera was collected from the Botanical Garden of Punjab University (Quaid-e-Azam Campus) Lahore. Fresh plant was collected each time and was processed soon after collection.

Extraction of the Enzyme: The extraction of the protease from the plant was accomplished by blending method. To carry out the extraction, 25 g of plant tissue was blended with 75 mL of pre-cooled phosphate buffer pH 7.0[10]. Homogenization was carried out in small intervals to avoid denaturation with rise of temperature. The suspension was filtered and 1 mL of the filtrate was used for the assay of protease activity.

Assay of protease activity: The protease activity was assayed by method of McDonald and Chen[9]. To carry out the assay, an adequate volume of the test sample (usually 1 mL) was incubated with buffered substrate for 1 h[10]. 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. After filtration, 1 mL aliquot of the filtrate was mixed with 5 mL of alkaline reagent prepared by mixing 100 mL of sodium carbonate (2 %), 1 mL of sodium potassium tartarate (2.7 %) and 1 mL of copper sulphate (1 %). Then 2 mL of 1N-NaOH was added to make the contents of the tube alkaline. After, at least 10 min, 0.5 mL of Folin-Ciocalteau phenol reagent was added and blue colour developed was read in a colourimeter exactly after 0.5 h.

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 the protease activity of the plant tissue: 1 mL of the filtrate obtained after processing 25 g of plant tissue was incubated with 4 mL of 1 % casein substrate for 1 h at 30 ºC and the assay was carried out further as narrated above. The activity was reported in units per gram of the sample.

Determination of enzyme characteristics: The effect of parameters such as temperature, pH, *etc.*, on protease activity was studied. The temperature stability and the pH-stability were also determined. Methods applied are described below.

Effect of temperature: The variation of reaction velocity with temperature was studied in the range 20 to 80 ºC. 1 mL enzyme sample was incubated with 4 mL of casein substrate in a test tube for 1 h at 20 to 80ºC. 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, 1 mL of enzyme sample was subjected to the temperature shock 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 shock of different temperatures was assayed at 30 ºC using casein as a substrate[9]. The percentage of residual activity was plotted as a function of temperature. Thermo-stability was judged from the curve.

Effect of pH on enzyme activity: The effect of pH on activity of enzyme sample from *Euphorbia pilulifera* towards casein was studied within the pH range 3 to 10 using citrate-phosphate buffer. The activity could not be tested with casein within the whole range as this precipitates down in pH-range 4 to 6. The optical density was plotted as a function of pH and pH optimum for the protease activity was noted.

Determination of pH stability: 10 mL of the filtrate prepared as described above was transferred to each of ten test tubes. The pH of the tubes was adjusted in the range of 3 to 10 by adding acid and alkali respectively. The contents of tubes were allowed to stand for *ca.* 4 h and then pH was pulled up or down to 7. 1 mL from each sample was incubated with casein substrate of pH 7 at 30 ºC for 1 h as usual and protease activity was determined as above. The percentage of residual protease activity was plotted as function of pH.

RESULTS AND DISCUSSION

The units of protease activity present per gram of *Euphorbia pilulifera* plant tissue are reported in Table-1.

TABLE-1
PROTEASE ACTIVITY OF *Euphorbia pilulifera*

Sample	Protease activity in Units/g
Plant Tissue	9.6

The results indicate that *Euphorbia pilulifera* is a rich source of protease. The variation of reaction velocity with increase in temperature is shown in Fig. 1.

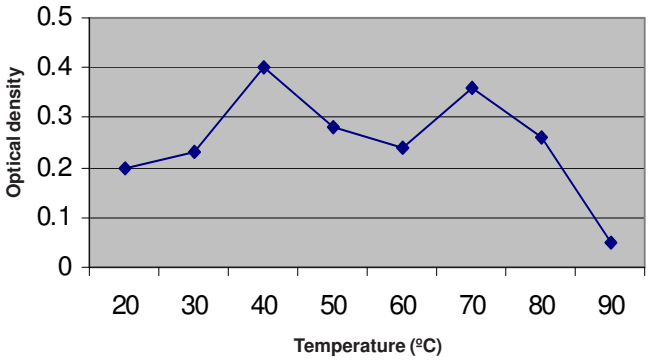

Fig. 1. Variation of reaction velocity of protease of *Euphorbia pilulifera* with temperature

The temperature profile (Fig. 1) shows that the reaction velocity increases with temperature up to 40 ºC, then decreases due to enzyme denaturation to 60 ºC. It increases again up to 70 ºC and starts falling again. At 90 ºC, a little activity persists. It can be said that the complete deactivation of protease will occur above 90 ºC. The temperature profile, thus, indicates two temperature optima; one at 40 ºC and other at 70 ºC. The overall picture demonstrates that the protease of *Euphorbia pilulifera* is a mixture of thermo-stable and thermo-labile components.

The percentage fall in the residual activity of *Euphorbia piluliera* after exposure shock at different temperatures is shown in Fig. 2.

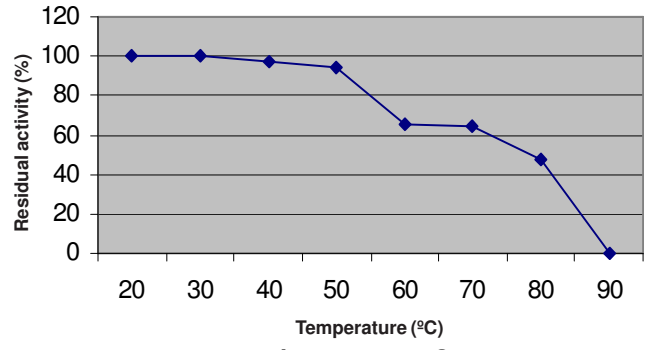

Fig. 2. Fall of percentage residual activity of *Europhobia pilulifera* after subjection to temperature shocks

The profile indicates that the enzyme is stable up to 50 ºC. It seems that the denaturation of thermo-labile protease components starts at 50 ºC. The profile from 70 to 90 ºC indicates the

presence of a thermo-stable component, which is responsible for the persistence of activity in high temperature region of the profile.

The variation of reaction velocity with protease of *Euphorbia pilulifera* with increasing pH is shown in Fig. 3.

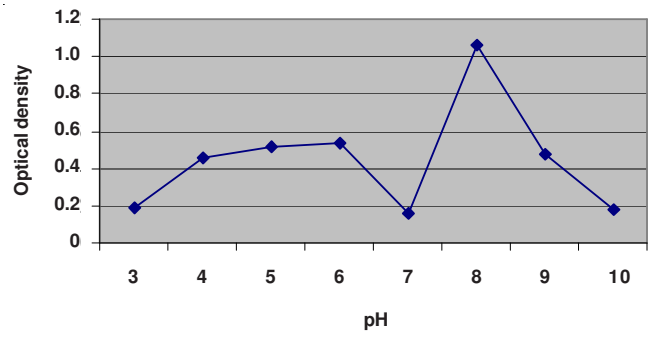

Fig. 3. pH-profile of the protease of *Euphorbia pilulifera*

The profile (Fig. 3) indicates that the activity of the enzyme is higher near neutrality on the alkaline side than that in the acidic and alkaline media. There are two pH optima (one at pH 5 to 6 and other at 7.5) in the pH profile, which indicate the presence of two proteases, one neutral and other alkaline. The crude enzyme seems to be dominated by the neutral protease.

The pH-stability of the protease of *Euphorbia pilulifera* is shown in Fig. 4.

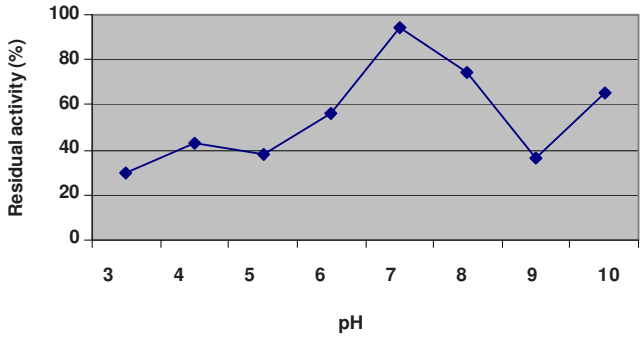

Fig. 4. pH-stability of the protease of *Euphorbia pilulifera*

The profile shows that the protease components of the enzyme are stable in different regions. The dominant ones seem be neutral and alkaline.

The central theme of the work being reported was the determination of the protease content of *Euphorbia pilulifera* and nature of the proteases present in it and subsequent comparison of the content and characteristics of this plant with the proteases of other plants studied by other workers.

The protease content of *Euphorbia pilulifera* was significantly high. It was found to be 9.6 units/g of whole plant. It is greater than that of *Euphorbia roylena* (6.0 units/g) as reported by previous workers.

The temperature profile (Fig.1) contains two temperature optima (40 and 70 ºC) that mean presence of two or more proteases, one thermo-labile that starts denaturing at 70 ºC. These results compared very well with those of Toor[11] on protease of *Euphorbia roylena*.

The results on thermo-stability of the protease of *Euphorbia pilulifera* indicate that the enzyme is stable up to 50 ºC after which it starts deactivating due to the denaturation of the thermo-labile component. The presence of protease is significantly high even at 80 ºC, which fortifies the presence of the thermo-stable component. The results on thermo-stability of protease of *Euphorbia pilulifera* compare with those of *Euphorbia roylena*. The extract of *Euphorbia pilulifera* seems to contain more thermo-labile component.

The pH-profile (Fig. 4) confirms the presence of acid alkaline and neutral proteases. The dominant component seems to be the neutral protease.

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