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IMMOBILIZATION OF THE PROTEASE OF *CALOTROPIS PROCERA*

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Immobilization of the protease of *Calotropis procera* was carried out on amberlite (CG-50). The percentage of immobilization was found to be 23.5. A continuous immobilized enzyme proteolytic system was developed by packing a column with amberlite (CG-50, anion exchanger) using the citrate-phosphate buffer pH 7.0. The system was tested for its hydrolysing tendency by running through the matrix 1% casein solution in citrate-phosphate buffer pH 7.0 as substrate. The column was found to be significantly effective for continuous proteolysis. The life span of the enzyme immobilized on amberlite was about three weeks. A similar proteolytic system was also developed using charcoal as adsorbant matrix.

INTRODUCTION

Different techniques are under intense investigation to immobilize proteolytic enzymes on different materials in order to develop continuous proteolytic systems for the hydrolysis of proteins.

Considerable work has recently been carried out to study the general characteristics of proteases immobilized by different methods [1–3].

The work reported here was carried out to immobilize the protease of *Calotropis procera* (a wild plant found abundantly growing in Pakistan) on amberlite (CG50) resin and charcoal by ion-exchange and physical adsorption techniques respectively to develop continuous proteolytic systems.

MATERIAL AND METHODS

Extraction of Enzyme. The *Calotropis procera* plant was obtained from the Government College, Lahore Oval and processed fresh. To extract the enzyme from *Calotropis procera,* 50 g of plant leaves were homogenised with 100 ml of cold citrate = phosphate buffer pH 7.0 [4], in a blender. The blending was carried out in small intervals to avoid the denaturation of the enzyme due to rise in temperature. The homogenate was then filtered through a filter paper in order to obtain a soluble protease extract. This filtrate was used for the determination of protease activity and afterwards for the immobilization of the enzyme.

Assay of protease activity. The protease activity was assayed by the method of McDonald and Chen [5], modified by Khan *et al* [6]. 1 ml enzyme sample was used and the substrate was 1 g % Casein in 0.2 M citrate = phosphate buffer pH 7.0. The unit of protease activity was defined as the amount of the enzyme required to produce an increase in optical density at 700 nm of 0.1 per hour at 30° and pH 7.0 under the assay conditions defined.

Determination of binding of enzyme with amberlite. 1 g amberlite CG-50 (ROHM and Hass Co., USA) was weighed and placed in a 100-ml conical flask containing 25 ml buffer of pH 7.0. 5.0 ml enzyme extract was subsequently added into the conical flask. The contents were kept at room temperature (30°) for 24 hr with occasional shaking. The material was centrifuged. The protease activity of the supernatent was determined, and compared with the protease activity of the untreated enzyme. The precipitate was washed with a buffer solution of pH 7.0, and the washed precipitate was the immobilized enzyme.

The protease units immobilized on the amberlite were determined by substracting the protease units present in the supernatent from the total protease units present in 5 ml enzyme sample. The result was also checked by the direct determination of the protease activity of the immobilized enzyme.

Development of Continuous Proteolysis System: amberlite system. The stages involved in the development of amberlite system are briefly described below:

Packing of the column. 250 g amberlite (CG-50) was suspended in 400 ml citrate = phosphate buffer at pH 7.0. A glass column of volume 47 cm^3 was then packed and equilibrated with the buffer and allowed to settle overnight with the starting buffer standing on it.

Application of the enzyme sample. 25 ml of the enzyme sample was applied at regular intervals in 5 ml fractions, each time to the pre-packed column. A continuous elution was subsequently carried out and 5 ml fractions were collected. Each of the fraction was assayed for its protease activity. The running was continued till the

activity vanished, indicating that no soluble enzyme was left in the column and the remainder immobilized on amberlite. The protease activity was plotted against fraction number to construct the elution diagram.

Application of the substrate. 1 g of the casein substrate was dissolved in 100 ml citrate = phosphate buffer pH 7.0. It was then passed continuously through the column containing immobilized enzyme. The column swelled up due to the binding of the casein with the amberlite. The elutate was collected in 5 ml fractions for one week for twelve hours every day. The column was switched off during night. The fractions collected were assayed for the soluble products of proteolysis by taking 1 ml of the sample collected in each tube and developing the blue colour with Folin and Ciocalteu phenol reagent and reading the colour in spectrophotometer at 700 nm. The elution diagram was constructed by plotting the optical density corresponding to each tube as a function of fraction number.

After an elution of one week, the column was stopped for one week and the elution was restarted. The products of proteolysis were determined as above. An elution diagram was similarly constructed.

Charcoal enzyme immobilization system. The protease of Calotropis procera was immobilized on activated charcoal and a continuous proteolytic system was developed as described below.

300 g of *Calotropis procera* were blended with 600 ml citrate = phosphate buffer, pH 7.0. The material was filtered to get the enzyme extract. To the enzyme extract were added 30 g charcoal. The mixture was stirred well and kept overnight. Next day the mixture was transferred to a Buckner funnel over a filter paper. The charcoal formed a bed over the surface of the Buckner funnel. The charcoal bed was washed with buffer pH 7.0, for three or four times. 100 ml casein substrate was subsequently applied in fractions and the filtrate was collected for 12 hr. The proteolytic products in the whole volume collected were determined as above. The running was stopped for one week and the process was repeated for three weeks. The extent of proteolysis was compared and an idea was framed about the life span of the enzyme.

RESULTS

Binding of enzyme with amberlite. The details of the follow up of the experiment on the study of the binding of the enzyme with amberlite (CG-50) are given below:

Number of protease activity units present in 5 ml extract	23.5
Number of protease activity units present in the supernatent (soluble enzyme)	18.0
Percentage of immobilized enzyme	23 %

The results indicate that amberlite (CG-50) has a significant tendency to immobilize the proteases of *Calotropics procera.*

Protease activity of the unbound enzyme eluted from the column. The protease activity of the fractions collected after elution with buffer after sample application is shown in Fig. I. From the profile, it is evident that all the soluble

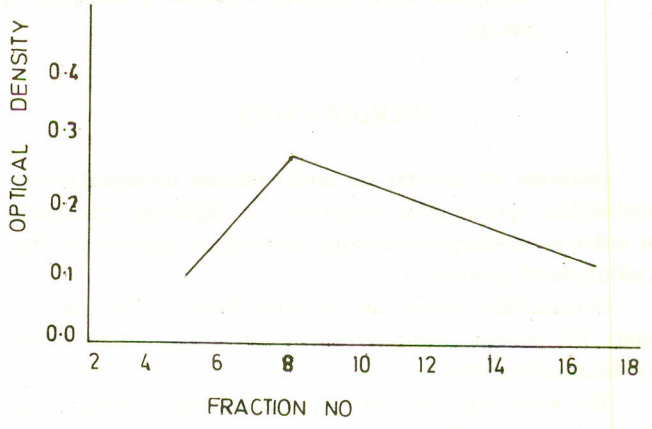

Fig.1 Elution diagram showing the activity of the soluble enzyme washed from the column after sample application.

enzyme was eluted after collection of first 20 fractions, i.e. 100 ml elution volume. The record of the units applied and recovered as soluble enzyme is as follows:

Units of protease activity applied to the column	118
Units of protease activity recovered as soluble enzyme	90

Percentage of immobilization $= \frac{28}{118} \times 100 = 23\%$

Proteolysis by amberlite system. The progress of continuous proteolysis by the protease immobilized on amberlite for one week is shown in Fig. 2. The profile indicates that significant proteolysis occurs when the substrate is passed through the bed of amberlite with protease immobilized upon it. Two points which are remarkable about the diagram are:

1. Existance of peaks at least one in a day.

2. An increase in the height of the peak with the passage of time.

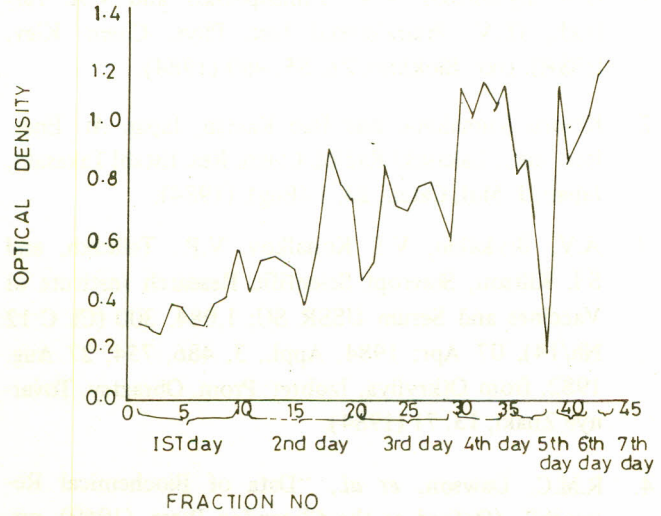

Fig.2. Elution diagram showing the extent of proteolysis by the immobiliozed enzyme after the application of the substrate.

The progress of proteolysis, two weeks after the application of the sample (one week running and one week stoppage) is shown in Fig. 3. The results indicate that the

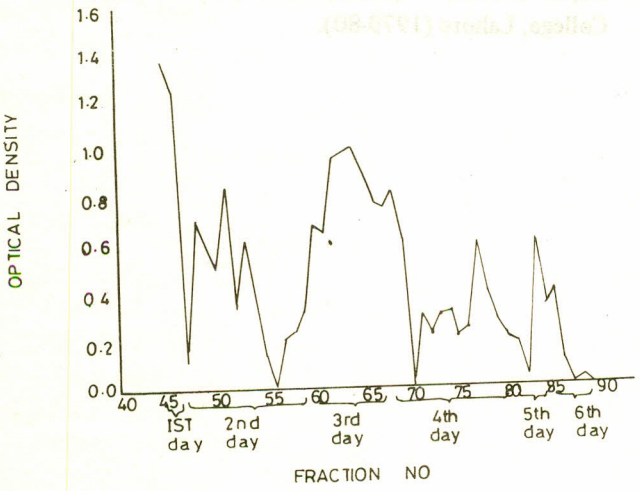

Fig.3. Elution diagram showing the extent of proteolysis by the immobilized enzyme after keeping the column closed for one week subsequent to one week continous elution.

immobilized enzyme is not denatured even after one week stoppage. The life span of immobilized enzyme seems to be at least three weeks.

Proteolysis by charcoal system. In this experiment the fractions obtained per day were bulked to have an idea about the extent of proteolysis per day after keeping the column closed for a week.

The record of proteolysis per day is shown in Table. 1.

Table 1. Record of the proteolysis per week by the protease of *Calotropis procera* immobilized on activated characoal.

Sr. No.	No. of weeks	Optical density per ml
1.	First week	0.26
2.	Second week	0.20
3.	Third week	0.11

DISCUSSION

The results indicate that about 23 % of the proteolytic enzymes are bound with the amberlite (CG-50) and thus are immobilized which is quite a significant percentage of immobilization.

Here an important question to be answered is: Why did the whole enzyme not completely bind with amberlite? A complete binding would have been possible only if the enzyme was just one species and amberlite was in excess. As the *Calotropis* extract was a mixture of a number of proteases differing in their general and binding characteristics [7], the incomplete binding was not unexpected.

After the substrate was applied to the amberlite column, the bed swelled up. This happened due to the binding of casein with the ion exchange matrix which caused a partial choking of the column. That is why the flow rate fell with the passage of time.

The extents of proteolysis are well clear from the Fig. 2 and 3. A number of peaks are present in the elution diagram which show that significant proteolysis was caused by immobilized enzyme. Out of the peaks obtained at least one peak maximum corresponds to a fraction of the eluate collected in 24 hr or one full day. The peak maximum was obtained due to the fact that column was stopped during night; the interval in which the substrate remained in contact with the enzyme for about 12 hr and extensive hydrolysis occurred in the vicinity of the site of the enzyme location on the column.

Another interesting freature of the nature of proteolysis is that the height of the peaks increased in certain cases with the passage of time (Fig. 2). This happened due to partial choking and fall in the flow rate. In the begining about ten tubes were collected on the first day while only two tubes were collected on the seventh. The time of contact between the enzyme and the running buffer increased

severalfold resulting in to extensive proteolysis. Thus the height of the peaks increased in spite of the partial deactivation of the enzyme with the passage of time.

The proteolysis caused by the enzyme was significantly high even in the third week. At the end of third week, no proteolysis was recorded (Fig. 3). The life span of the enzyme thus seems to be at least three weeks.

In the proteolytic system of the enzyme immobilized on charcoal bed formed in Buckner funnel, as the fractions collected after proteolysis were bulked to collect the full day volume of the eluate, the extensive proteolysis caused in the vicinity of the enzyme during night was equally distributed. Table indicates that the bed worked well for three weeks. The life span of the enzyme immobilized on charcoal was even more than three weeks.

From the results reported and discussed above, it is quite clear that the study was a successful attempt towards the development of the immobilized enzyme systems and towards the exploration of techniques to preserve enzymes in certain modified forms. Thus avenues are quite attractive for extending this kind of work to the standardization of conditions for the optimal stability for an increase in the life span of the proteases immobilized on amberlite and charcoal. The work may also be extended to the study of the development of the appropriate systems for the immobilization of enzymes other than proteases for their use in commercial context.

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