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Immobilization of the Protease of *Carica papaya* on DEAE-cellulose

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ABSTRACT
Proteolytic enzymes can immobilize on different materials to constitute systems for continuous hydrolysis of protein substrates. They are mostly immobilized by binding some matrices filled in chromatographic columns and continuous proteolysis is accomplished by subsequent passing of the buffered substrates through the column bed from top to bottom and collecting of hydrolyzed products eluting out of the column from the bottom. Immobilization of the protease of *Carica Papaya* was carried out on DEAE-cellulose. The percentage of immobilization was found to be 67%.The life span of the enzyme immobilized on DEAE-cellulose was 30 days.

Keywords: DEAE-cellulose, Immobilized protease, *Carica papaya*, Proteolysis

1. INTRODUCTION
Proteases are the enzymes that generally hydrolyze the peptide bonds (-CO-NH-) of the protein molecules or their degradation products such as proteases, peptones, polypeptides, etc. The term is distinguished from "proteinases" that attack intact proteins to produce proteoses, peptones, polypeptides and amino acids.

The proteases are widely distributed in nature. Their major sources are plants and microorganisms. The proteases have been extracted from these sources and put in different uses. These have been, extensively applied in food industry[1-2]. Their major applications in this industry are as glue, modifier in bread making, meat tenderizers in meal preparation, milk coagulant in cheese production and as haze removers in chill proofing of bears. Other applications in industry include bating of leather, degumming of silk, tobacco making, paper making, biological detergents[3- 4] etc.

The proteases have also found extensive applications in medicines as cure of diseases such as stomach upset, fistulas, hyperacidity and cramps. They are being extensively used as anti-inflammatory agents. They digest dead cells of the skins necrotic tissue and wounds selectively without harming the healthy tissue and thus are used as wound cleaners. Similarly; they are being extensively applied as cleaners of contact lenses.

Due to the importance of proteases have been under extensive research in the past and, even, are currently being intensively studies, all over the world[5,6].The immobilized enzymes including protease are also being intensively studied as part of this exercise.

Different techniques are under intensive research to set up systems for continuous hydrolysis by the enzymes. One of such techniques is the immobilization of the enzymes by binding them with some matrices filled in chromatographic columns and subsequently passing through them the buffered substrates and collection of hydrolyzed products eluting out of the column. All that is being done is on the assumption that the enzymes immobilized in this way are equipped with enhanced life and increased stability[7-8].

Considerable work has also been carried in Pakistan to hydrolyze casein by proteases of Calotropis procera[8] immobilized on Amberlite-50 and Euphorbia royleana[9] immobilized on DEAE A-50 and promising results have been obtained. The work being reported here is the extension of the work narrated above, but with the change that protease of *Calotropis procera*[8] was substituted by protease of *Carica papaya* for immobilization on DEAE-cellulose as it exhibited high protease content and increased life span than protease of *Calotropis procera*[8] and Euphorbia royleana[9]. This system will be useful for the preparation of protein products such as amino acid, polypeptides etc. These immobilized enzymes will be better stored without denaturation.

2. MATERIAL AND METHODS
In order to immobilized protease of *carica papaya* following steps as follows:

2.1 *Preparation of sample*
Carica papaya was freeze-dried to preserve the enzymes activity over long period of time Veterinary Research institute, Harikey Road Lahore, provided the freeze- drying facility. The freeze-dried sample was ground to fine powder. The fine powder sample was stored in bottle at -16 ºC in a deep freezer. This sample was used for the determination of protease activity and afterwards for immobilization of the enzyme. After removal from the deep freezer the enzyme sample was kept below -20 ºC to prevent its denaturation.

2.2 *Assay of protease activity*
Assay of protease activity was carried out by the Method of McDonald and Chen[10] modified and applied by Khan *et al*

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in the paper entitled "Continuous proteolysis of casein by the cell bound protease of *Carum copticum*[11]" and "immobilization of *carica papaya* on activated charcoal[12]".

2.3 *Determination of binding of enzyme with DEAE-cellulose*

0.5 g DEAE-cellulose was weighed and placed in a 100 ml conical flask containing 25 ml phosphate citrate buffer pH 7.0 and 5 ml enzyme sample was subsequently added in the conical flask. The contents were kept at room temperature at around 30 ºC for 24 hour with occasional shaking. The material was centrifuged. The protease activity of the supernatant was assayed and compared with the protease activity of the untreated enzyme. The precipitates were washed with phosphate citrate buffer solution of pH 7.0 repeatedly. The washed precipitate was the immobilized enzyme. The protease units immobilized on DEAE-cellulose were determined by subtracting the protease units present in the supernatant from the total protease units present in 5ml enzyme sample. The result was also checked by the direct determination of the activity of the immobilized enzyme.

2.4 *Continuous proteolysis by enzyme immobilized on DEAE-cellulose*

The stages involved in the development of DEAE-cellulose system are briefly described below:

2.4.1 *Packing of the column*

7 g of DEAE-cellulose was suspended in 400 ml phosphate citrate buffer at pH 7.0. A glass column of volume 50 cm^3 and size 18 × 1 inch (dia) was packed and equilibrated with the buffer and allowed to settle over night with the starting buffer standing on it.

2.4.2 *Application of the enzyme sample*

20 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 fractions 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 remainder immobilized on DEAE-cellulose. The protease activity was plotted against fraction number to construct the elution diagram of eluted soluble protease.

2.4.3 *Application of the substrate*

1 g of Casein substrate was dissolved in 100 ml phosphate citrate buffer pH 7.0 and transferred to the top of the column applying 5 ml at a time after the previous almost disappeared. It was then passed continuously through the column containing immobilized enzyme. The column swelled up due to the binding of casein with the DEAE-cellulose. The eluate was collected in 5 ml fractions for 12 hour every day. The column was kept closed during night. The fractions collected were assayed for the soluble products of proteolysis by precipitating undigested protein, for analysis every alternate fraction was treated with 5 ml, 5 % TCA, filtering and using I ml the filtrate was mixed with 5 ml of alkaline reagent prepared by mixing 98 ml of 2 % sodium carbonate, 1 ml of 2.7 % sodium potassium tatrate, 1 mL copper sulphate, 2 ml 1 N sodium hydroxide was added to make the contents of the tube alkaline and developing blue colour with Folin and Ciocaleu phenol reagent and finally reading the optical density of the colour in a spectrophotometer at 660 nm as done under assay of protease activity. The elution diagram was constructed by plotting the optical density corresponding to each fraction as a function of fraction number. After an elution of 12 days, 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.

3. RESULTS

3.1 *Adsorption of enzyme on DEAE-cellulose*

The results of the experiment on the study of the binding of the enzyme with DEAE-cellulose are given below: Number of protease activity units present in 5 ml extract = 50; Number of protease activity units present in the supernatant = 20; Number of units immobilized: 50 -20 = 30; Percentage of immobilized enzyme: 30/50*100 = 60 %; Percentage of soluble enzyme: 20/50*100 = 40 %.

The results indicate that DEAE-cellulose has a significant tendency to adsorb and immobilize the protease of *Carica papaya*.

3.1.2 *Protease Activity of Unbound Enzyme Eluted from DEAE Column*

The protease activity of the fractions collected after elution with buffer pH 7.0 after sample application is shown in Fig.1

From the profile it is evident that the entire unbound enzyme was eluted after collection of first 12 fractions, i.e. 60ml elution volume.

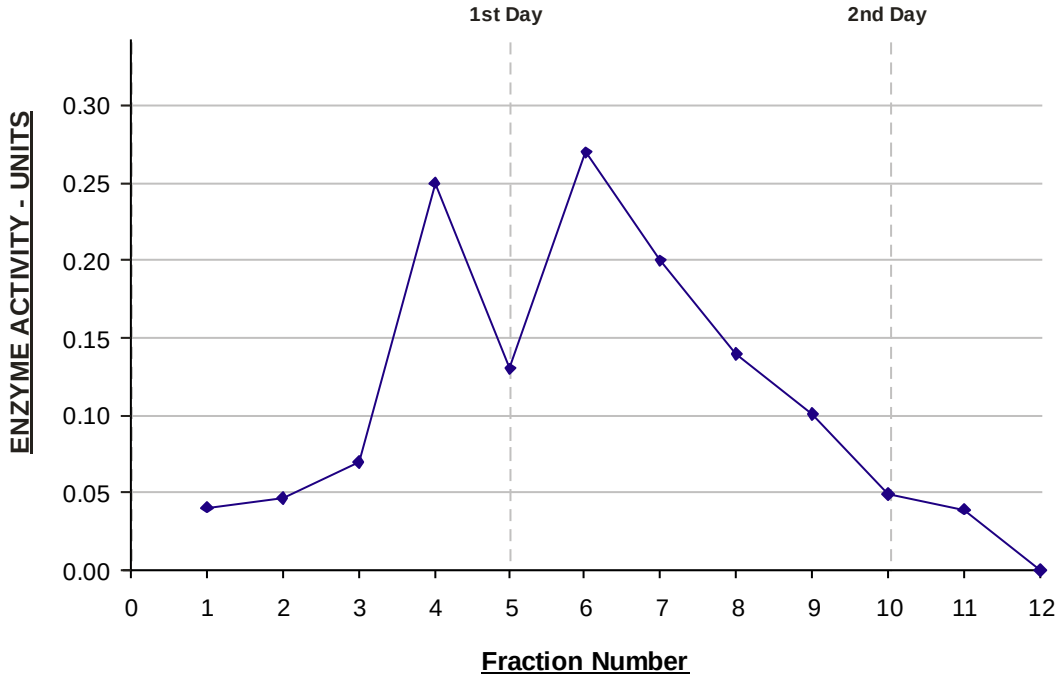

Fig-1: Change in enzyme activity of the unbound protease of *Carica papaya* during elution with 0.2M-citrate phosphate buffer pH 7.0 from DEAE-Cellulose column (Days 1-2)

3.1.3. *Proteolysis by DEAE Cellulose Bound Enzyme*

The progress of continuous proteolysis of casein by the protease immobilized on DEAE Cellulose for 12 days is shown in Fig.2.

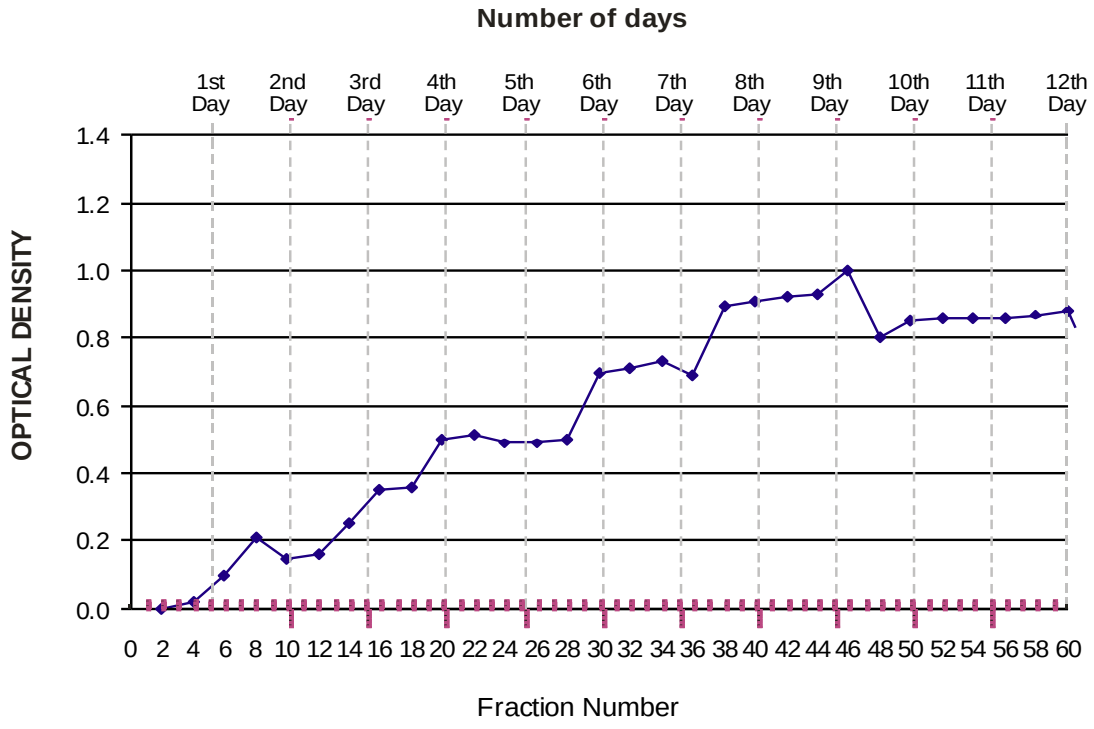

Fig-2: Elution diagram showing the extent of proteolysis by DEAE Cellulose immobilized protease of *Carica papaya* packed in chromatographic column after application of the casein substrate pH 7.0 (Days 1-12).

The profile (Fig-2) indicates that significant proteolysis occurs when the substrate is passed through the bed of DEAE-cellulose with protease immobilized to it. Two points remarkable about the diagram are as under:

1. No clear sharp peaks are obtained.
2. Optical density increases up to 46th fraction having optical density (1.0), with some fall and rise in the profile then decreases and becomes almost constant.

The progress of proteolysis after stopping running of the column for one week and restarting from 20th day after the application of sample and ending of 30th day is shown in Fig-3.

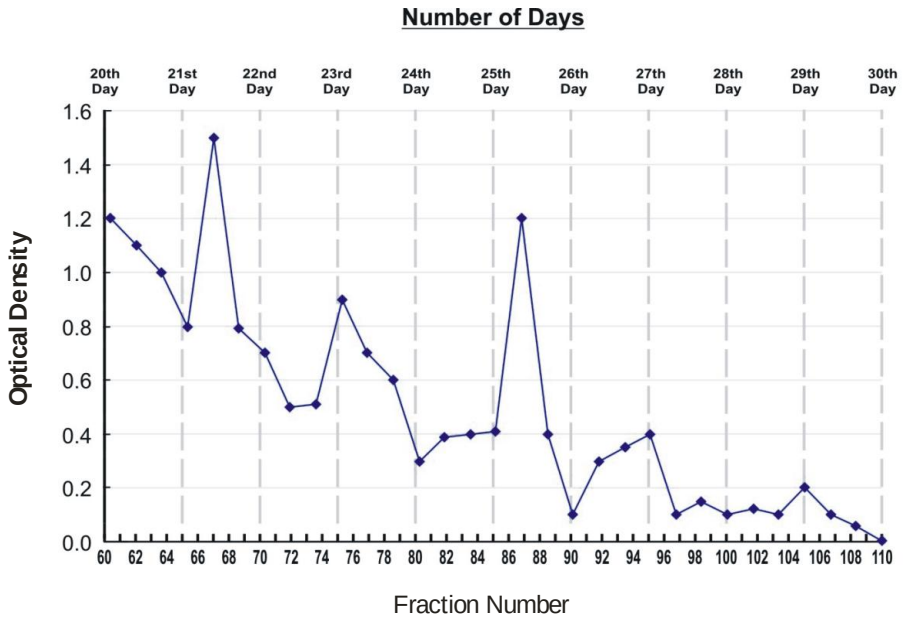

Fig-3: Elution diagram showing the extent of proteolysis by immobilized enzyme at pH 7.0 after keeping the column closed for one-week continuous elution (Days 20-30).

4. DISCUSSION

The results indicate that the percentage of immobilization on DEAE-cellulose 67 % seems significant.

The present work was compared to those using other supports such as the %age of immobilization of the protease of *Calotropis procera*[8] on Amberlite CG-50 that was 23 % and percentage of immobilization of the protease of Euphorbia royleana[9] on DEAE A-50 that was 30%, which is quite less than the percentage of *Carica papaya* immobilization on activated charcoal[12] that is 81 %. Here, an important question to be answered is why the whole enzyme was not completely adsorbed by DEAE-cellulose. The complete adsorption would have been possible only if the enzyme was just one species and DEAE-cellulose was in excess. As the *Carica papaya* might be a mixture of a number of proteases differing in their general and binding characteristics. Thus, the incomplete binding was not unexpected. After the substrate was applied to DEAE-cellulose column, the bed swelled up. This happened due to the binding of casein with DEAE-cellulose, which caused a partial choking of the column. That is why the flow rate fell with the passage of time. The extent of proteolysis is well clear from Fig. 1 and 2. A numbers of peaks are present in the elution diagram, which show that significant proteolysis was caused by immobilized enzyme. Out of those obtained, at least one high peak corresponds to some fraction of the eluate collected in a day. The high peak was obtained due to the fact that column was stopped during night and thus the substrate remained in contact with the enzyme for about 12 h and thus extensive hydrolysis occurred in the vicinity of the site of the enzyme location in the column. Another interesting feature of the nature of proteolysis is that the height of the peak increases in certain cases with the passage of time (Fig. 2). This happened due to partial chocking and fall in the flow rate. Thus, due to reduction in the flow rate the time of contact between the enzyme and the running buffer increased several fold resulting into extensive proteolysis. The column was stopped for 7 d. The proteolysis caused by the enzyme was significantly higher even in the 30 d. No proteolysis was recorded after 30 d. The life span of protease of *Carica papaya* bound on DEAE-cellulose(30 d) is greater than the life span of *Euphorbia royleana*[9] bound to DEAE A-50 that was 20 d and even greater than that of immobilized protease of *Calotropis procera*[8] bound to Amberlite-50 that was 21 d. 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 system and towards the exploration of techniques to preserve enzymes in a modified form. The work may also be extended to the study of development of the appropriate system for immobilization of the enzymes other than proteases for their use in commercial sector.

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