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Immobilization of the Proteases of Euphorbia Royleana

Sarfraz Ahmad Nawaz and M. Rafiq Khan
Biochemistry Laboratory, Department of Chemistry,
Governoment College of Science, Wahdat Road, Lahore, Pakistan

Abstract: Immobilization of the protease of *Euphorbia royleana* on DEAE-A50 cellulose was investigated. The percentage of immobilization was found to be 31%. A continuous immobilized enzyme proteolytic system was developed and tested for its hydrolyzing tendency which was found to be significantly effective for continuous proteolysis. The life span of the enzyme immobilized on DEAE-A50 cellulose was 22 days. Thus it is a successful attempt towards the development lit an immobilized enzyme system to preserve enzyme in certain modified forms.

Key words: Euphorbia royleana, DEAE-A50 cellulose, Immobilizaticm, Proteases

Introduction

Enzymes being thermolabile *have* presented the problem of their preservation. Therefore, it is necessary to increase the lite span of biologically important enzymes. The life span of immobilized enzymes increases and thus can be preserved. The enzymes after immobilization undergo certain changes in the spectrum of their activity. Fortunately many enzymes remain significantly active after immobilization. Kumakura and Kaetsu (1984) studied the effect of Polymer matrix on the Thermal Stability of immobilized trypsin. They found that maximum thermal stability of the immobilized enzyme was achieved with a degree at polymer hydration of 0.5 but thermal stability decreased at lower and higher degrees of hydration. Khan and Talib (1986) successfully immobilized the proteases of *calotropis procera* by binding it with Amberlite to constitute a system which can accomplish the proteolysis of casein. Ziegler *et al*. (1992) and Renner *et al*. (1998) found that immobilized DNasel can he used to remove contaminating genomic DNA from RNA samples isolated from single cells for differential display analysis. Ronnenberg *et al*. (1994) found that immobilized trypsin, endoproteinase Glu-C and endoproteinase Lys-C efficiently cleaved insulin B and melittin for HPLC analysis. Rai and Taneja (1998) reported that immobilized d-hydentoinase exhibit 80% enzyme activity and contain 86% protein. Furthermore the immobilization of the enzyme preparation does not change its optimum pH, temperature or affinity constant but increases its shelf life, thermostability and stability in various organic solvents. This immobilized d-hydantoinase can be used for the production of d-amino acids from the corresponding hydentoins and may therefore be of use in the chemical and pharmaceutical industries. Saleemuddin (1999) advocated that bioaffinity based immobilizations are usually reversible facilitating the reuse of support matrix, orient the enzymes favourably and offer the possibility of enzyme immobilization directly from partially pure enzyme preparations or even cell lysates. Enzymes lacking innate ability to bind various affinity supports can be made to bind them by chemically or iaenetically linking the enzymes with appropriate polypeptides domains like cellulose binding domain, histidine rich peptides etc. The present study was undertaken to immobilize the protease of *Euphorbia royleana* on DEAE-A 50 cellulose to develop continuous proteolytic system.

Materials and Methods

The latex of *Euphorbia royleana* was collected and diluted 10 times with phosphate-citrate buffer pH 7.0. To assay the protease activity and for continuous proteolysis casein was used as substrate. It was prepared as follows: One gram casein (BDH Chemicals Ltd) powder was dissolved in 100 ml of 0.2 M Phosphate citrate buffer pH 7.0. For immobilization of protease. DEAE-A50 cellulose (Sigma Chemicals Ltd) anion exchanger was used.

The protease activity was assayed as reported method by Khan *et al.* (1979). One ml of the enzyme sample was incubated with 4 ml of the substrate in a test tube for required interval of time normally one hour at 30°C. The residual protein was precipitated by adding 5 ml of 5% trichloro acetic acid. The precipitates were allowed to settle for 30 minutes. The content of the tube were filtered through Whatman No. 40 filter paper. After filtration I ml aliquot of the filtrate was mixed with 5 ml of alkaline reagent(prepared by mixing 98 nil of 2% sodium carbonate 1 ml of 2.7% sodium potassium tartrate 1 ml of 1% copper sulphate). Then 2 ml of 1N NaOH was added to make the contents of the tube alkaline. After at least 10 minutes 0.5 ml Folin Ciocaiteu reagent was added and the contents were mixed. The blue colour produced was read spectrophotometrically at 660 rim after exactly 30 minutes. The unit of protease activity was defined as the amount of enzyme required to produce an increase in the optical density at 660 nm of 0.1 per hour at 30°C and pH 7.0 under the assay conditions defined.

To determine the binding of enzyme with DEAE-A50 cellulose 0.5 gm DEAE-A50 cellulose was transferred to a 100 ml conical flask containing 25 ml buffer pH 7,0 and 5 ml of latex enzyme sample was subsequently added The contents were kept at room temperature (30°C) for 24 hours 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 buffer pH 7.0 repeatedly. The washed precipitate was the immobilized enzyme. The protease units immobilized on DEAE-A50 cellulose were determined by subtracting the protease units present in 5 ml enzyme sample. The result was also checked by direct determination of the protease activity of immobilized enzyme. The stages involved in the development of DEAE-A50 cellulose enzyme proteolytic system are briefly described here.

7.0 gm DEAE-50 cellulose was suspended in 400 ml citrate phosphate buffer at pH 7.0. A glass column of volume 150 cm^3 was mounted vertically on a stand and packed with DEAE-A50 cellulose suspended and equilibrated with buffer pH 7.0. The column was washed with buffer and allowed to settle over night with buffer standing on it.

20 ml of the enzyme sample was applied at regular intervals

to the packed column with the help of 5 ml syringe fitted with a plastic tube on its needle. Continuous elution was carried out with buffer pH 7.0. and 5 ml fractions of the elute were collected in test tubes. Each of the fraction was assayed for its imatease activity as usual. The running was continued till the activity vanished indicating that no enzyme was left in the column. The protease activity was plotted against fraction number to construct the elution diagram.

1 gm of the casein substrate was dissolved in 100 ml citrate phosphate buffer pH 7.0. It was then applied to the column containing immobilized enzyme in 5 ml fractions each time. The column swelled up due to the binding of the casein with DEAE -A50 cellulose. The elute was collected in 5 ml fractions for seven days for seven hours every day. The column was closed during night. The fractions collected were assayed for the soluble products of proteolysis by taking 1 ml of the sample collected in each tube precipitating the extra protein with TCA and developing the blue colour of the filtrate with Folin and Ciocalteu reagent as done in case of the assay of protease activity and reading the optical density of the colour in spectrophotometer at 660 nm. The elution diagram was constructed by plotting the optical density corresponding to each tube as function of fraction number. The column was closed for seven days and elution was restarted after seven days. The products of proteolysis were determined as above and elution diagram was similarly constructed.

Results

The result of binding of enzyme with DEAE-A50 cellulose are given as:

Number of protease activity units present in 5 ml Latex = 45

Number of protease activity units present in the supernatant (soluble enzyme) = 14

Thus the percentage of immobilized enzyme = $14/45 \times 100$ = 31%

The percentage of soluble enzyme = $31/45 \times 100$ = 69%

The result indicate that DEAE-A50 cellulose has a significant tendency to immobilize the proteases of *Euphorbia royleana.*

The protease activity of the fractions collected after elution with buffer pH 7,0 after sample loading (Fig. 1).

From the profile it is evident that all the soluble enzyme was eluted after the collection of 14 fractions 1.e 70 ml elution volume.

Enzyme activity al units applied and recovered as soluble enzyme are as follows:

Units of protease activity applied to the column = 180

Units of protease activity recovered as soluble enzyme = 126

Percentage of soluble enzyme = $126/180 \times 100$ = 70%

Percentage of immobilization = $54/180 \times 100$ = 30%

The progress of the continuous proteolysis of casein by the protease immobilized on DEAE-A50 cellulose for seven days (Fig. 2). The profile indicates that significant proteolysis occurs when substrate is passed through the bed of DEAE-A50 cellulose with protease immobilized upon it. Points which are remarkable about the diagram are existence of peaks, at least one in a day, an increase in the peak height with the passage of time Fall in the extent of proteolysis at the end of the day The progress of the proteolysis of the casein after the application of the sample seven days stoppage and seven days running (Fig. 3).

Extent of proteolysis as on 7th day before closing is approximately same as that on 1st day elution after seven days closing of the column, compare Fig. 2 and 3. Pattern

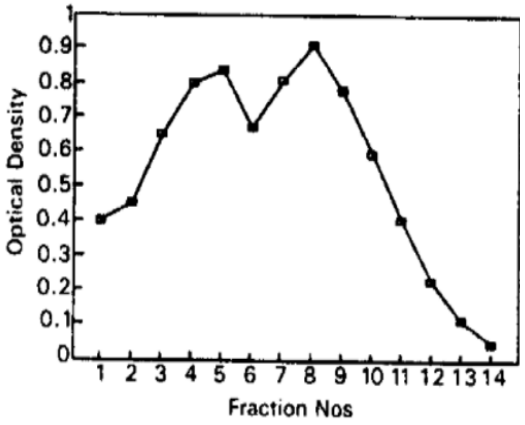

Fig. 1: Elution diagram showing the activity of the soulble enzyme washed from the column after the column atter sample application

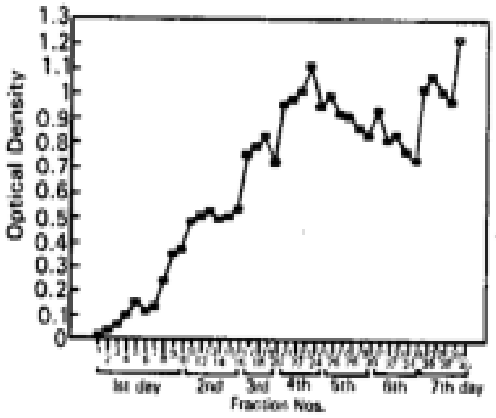

Fig. 2: Elution diagram showing the extent of proteolysis by the immobilizing enzyme atter the application of the substrate

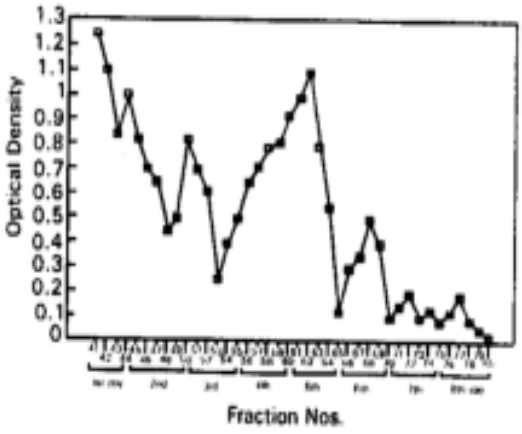

Fig. 3: Elution diagram showing the extent of proteolysis by the immobilizing enzyme after keeping the column closed for one week subsequent to one week continuous elution

of the peak after closing is similar to that of before closing of the column. The result indicate that immobilized enzyme is not denatured even after seven days stoppage of the

column. The profile shows that the activity of the enzyme in the column does not exist after 22 days. Thus the life span of the Immobilized enzyme seems to be 22 days.

Discussion

The results indicate that DEAE-A50 cellulose significantly binds the protease of *Euphorbra royleana.* The percentage of immobilization on DEAE-A50 cellulose was 31%. Here an important question to be answered is why the whole enzyme was not completely bound by DEAE-A50 celluloses. The complete binding would have been possible only if the enzyme was just one species and DEAE-A50 cellulose was In excess. As the *Euphorbra royleana* latex is *a* mixture of a number of proteases differing in their general and binding characteristics reported by Khan *et al*. (1980). The incomplete binding was riot unexpected. After the substrate was applied to DEAE-A50 cellulose column, the bed swelled up. This happened due to the binding of casein with the ion exchange matrix. The flow rate fell with the passage of time due to partial choking of the column as a result of the binding of excess casein with the bed of material. The extents of proteolysis are well clear from the Fig. 2 and 3. A number of peaks are present in the elution diagrams which show that significant proteolysis was caused by immobilized proteases. The correspondence of at least, one high peak to some fraction of the elute collected in a day may be due to the fact that the column was stopped during the night. During night substrate remained in contact with the enzyme for about 12 hours, thus extensive proteolysis occurred in the vicinity of the enzyme location in the column. Another interesting feature of the nature of the proteolysis is that the height of the peaks increases in certain cases with the passage of time (Fig. 2). This happened due to the partial choking 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 was increased manifold which resulted into the extensive proteolysis. The fall in the extent of proteolysis at the end of the day was encountered as the products of proteolysis were washed out through out the day. The column was stopped for seven days after running of the seven days. The proteolysis caused by the enzyme was significantly high even after 20 days. No proteolysis was recorded after 22 days.

Thus the life span of the protease of *Euphorbia royleana* bound to DEAE-A50 cellulose is 22 days. The result is in quite agreement with the result of Khan and Talib (1986).

From the result reported and discussed above it is quite clear that the study was a successful attempt towards the development of an immobilized enzyme system and to preserve enzyme in certain modified forms.

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