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Immobilization of the Protease of *Carica papaya* on Activated Charcoal

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Immobilization of the protease of *Carica papaya* was carried out on activated charcoal. A continuous immobilized enzyme proteolytic system was developed by packing a column with activated charcoal using citrate phosphate buffer pH 7.0. The percentage of immobilization was found to be 62.5 %. The column, of course, was partly chocked due to adsorption of casein with activated charcoal, which led to reduction in the flow rate. The life span of the enzyme immobilized on activated charcoal was 37 d.

Key Words: Activated charcoal, Immobilized protease, *Carica papaya*, Proteolysis.

INTRODUCTION

Several techniques have been developed to prepare immobilized enzymes because they have shown several advantages over enzymes in the solution state[1]. For instance, immobilization leads to ease of recoverability and reusability of the enzymes[2]. Proteolytic enzymes (or proteases) refer to the various enzymes that hydrolyze and thus break down proteins to smaller units such as proteoses, peptones, amino acids, *etc*. These enzymes have been used in food industries[3,4] for the preparation of protein hydrolysates with dietary applications[5-12].

Proteolytic enzymes can immobilize on different materials for continuous hydrolyses of protein substrates. Enzymes are usually immobilized 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. Enzymes immobilized in this way may increase the stability of enzymes to thermal treatments and extremes of pH[13-15]. Immobilization of enzymes has been carried out by entrapment, ionic interaction, complex formation with metal, covalent attachment, encapsulation and adsorption to hydrophobic or hydrophilic surfaces[16]. Moreover, enzyme immobilized by adsorption tend to leak from the carriers, owing to relatively weak interaction between the enzyme and

the carrier, which can be destroyed by desorption forces such as high ionic strength, pH, *etc*. Occasionally the enzymes can be strongly adsorbed on suitable carriers[17]. The adsorption of immobilized enzymes has benefit of wide applicability and may provide relatively small perturbation of the enzymes native structure and function, which contributes to maintain sites of enzyme activity[18].

Some research works were reported to study binding, adsorption and desorption of protease of *Calotopis procera* with activated charcoal that lead to quite significant results. Khan, *et al.*[19] isolated the protease of *Calotropis procera* by adsorption and subsequent desorption on charcoal. Khan and Talib[13] developed a system for continuous proteolysis of casein by passing the substrate through protease of *Calotropis procera* on activated charcoal.

The work being reported here aimed at the development of a continuous proteolysis system by passing the protein substrate through immobilized protease of *Carica papaya* on activated charcoal packed in column. This forms a part of our series of attempts made to develop continuous proteolysis systems by binding them or adsorbing to different matrices packed in chromatographic columns. This system, if successfully developed will be useful for the preparation of protein products such as amino acids, polypeptides, *etc*. The immobilized enzymes will also be better stored without denaturation.

EXPERIMENTAL

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.

Assay of protease activity: Assay of protease activity was carried out by the method of McDonald and Chen[20] modified and applied by Khan *et al.*[19].

Determination of binding of enzyme with activated charcoal: 0.5 g Activated charcoal 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 h 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 activated charcoal were determined by substracting the protease units present in the supernatant from the total protease units present in 5 mL enzyme sample. The result was also checked by the direct determination of the activity of the immobilized enzyme.

Continuous proteolysis by enzyme immobilized on activated charcoal: The stages involved in the development of activated charcoal system are briefly described below:

Packing of the column: 7 g Activated charcoal was suspended in 400 mL phosphate citrate buffer at pH 7.0. A glass column of volume 50 mL 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.

Application of the enzyme sample: 20 mL 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 activated charcoal. The protease activity was plotted against fraction number to construct the elution diagram of eluted soluble protease.

Application of the substrate: 1 g 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 activated charcoal. The eluate was collected in 5 mL fractions for 1 week for 12 h 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 of TCA (5 %) and filtered. 1 mL of 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 sodium hydroxide (1 N) 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 14 d, the column was stopped for one week and the elution was restarted. The products of proteolysis were determined.

RESULTS AND DISCUSSION

Adsorption of enzyme on activated charcoal: The results of the experiment on the study of the binding of the enzyme with activated charcoal are given below: Number of protease activity units present in 5 mL extract = 52; Number of protease activity units present in the supernatant = 18; Number of units immobilized: 52 -18 = 34; Percentage of immobilized enzyme: 34/52*100 = 65.3 %; Percentage of soluble enzyme: 18/52*100 = 34.6 %.

The results indicate that activated charcoal has a significant tendency to adsorb and immobilize the protease of *Carica papaya*.

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. 1.

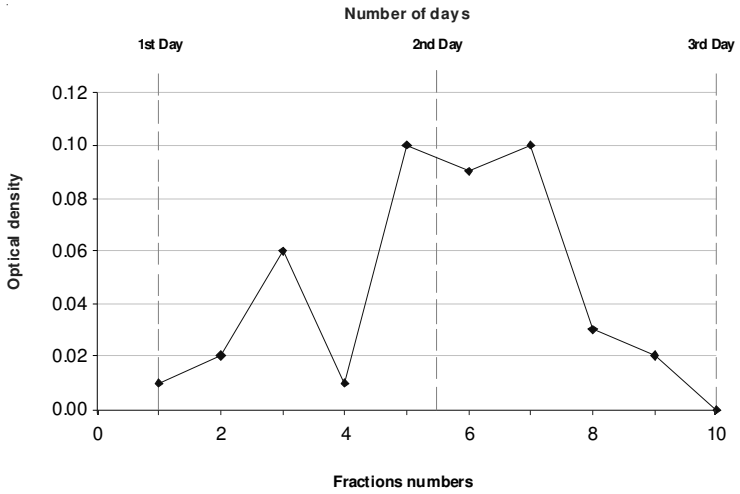

Fig. 1. Change in the optical density of the soluble substances of the activated charchol during elution with buffer pH 7.0 (days 1-3)

It is evident that the entire soluble enzyme was eluted after collection of first 10 fractions, *i.e.* 50 mL elution volume. The record of the units applied and recovered as soluble enzyme is given below: Units of protease activity applied to the column: 118; Units of protease activity recovered as soluble enzyme: 44.2; Percentage of soluble enzyme: 44.2/118*100 = 37.4 %; Number of units immobilized: 118-44.2 = 73.8; Percentage of immobilization: 73.8/118*100 = 62.5 %.

Proteolysis by enzyme immobilized on activated charcoal: The progress of continuous proteolysis of casein by the protease immobilized on activated charcoal for 14 d is shown in Fig. 2.

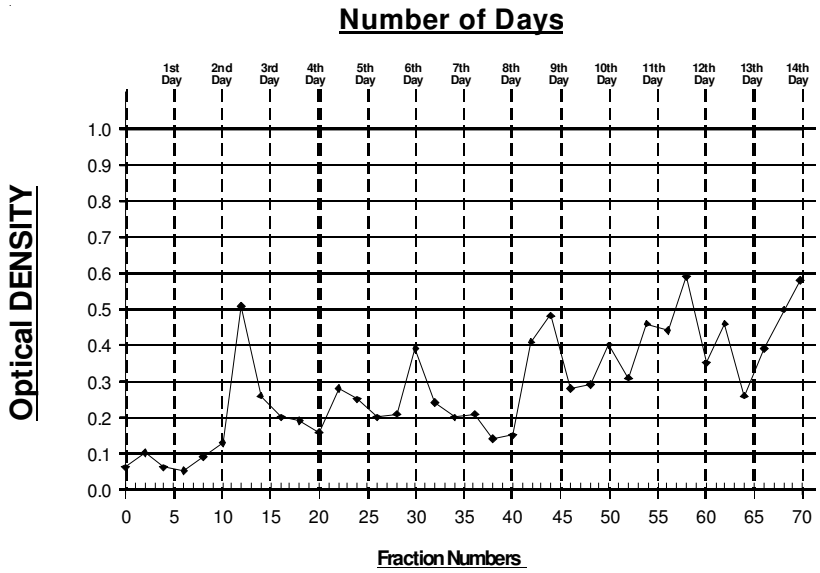

Fig. 2. Elution diagram showing the extent of proteolysis immobilized enzyme at pH 7.0 after the application of the casein substrate (days 1-14)

As seen from Fig. 2, the significant proteolysis occurs when the substrate is passed through the bed of activated charcoal with protease immobilized on it. Two points which are notable about the diagram are: (1) Existence of peaks, at least one in a day. (2) An increase in the peak height with the passage of time.

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

The results indicate that the immobilized enzyme is not denatured after one-week stoppage. The life span of immobilize enzyme seems to be 37 d.

The activated charcoal adsorbs significantly the protease of *Carica papaya*. The percentage of immobilization on activated charcoal 62.5 % seems highly significant.

Considering that no report about the immobilization of *Carica papaya* was found in literature, on activated charcoal, the data obtained in the present work were compared to other reports such as the percentage of immobilization of the protease of *Calotropis procera*[13] on Amberlite-50 was 23 % and percentage of immobilization of the protease of Euphorbia royleana[14] on DEAE A-50 was 30 %, which is quite less than the percentage of *Carica papaya* immobilization on activated charcoal, which is 62.5 %. Here, an important question to be answered, why the whole enzyme was not completely adsorbed by activated charcoal? The complete adsorption would have been possible only if the enzyme was just one species and activated charcoal

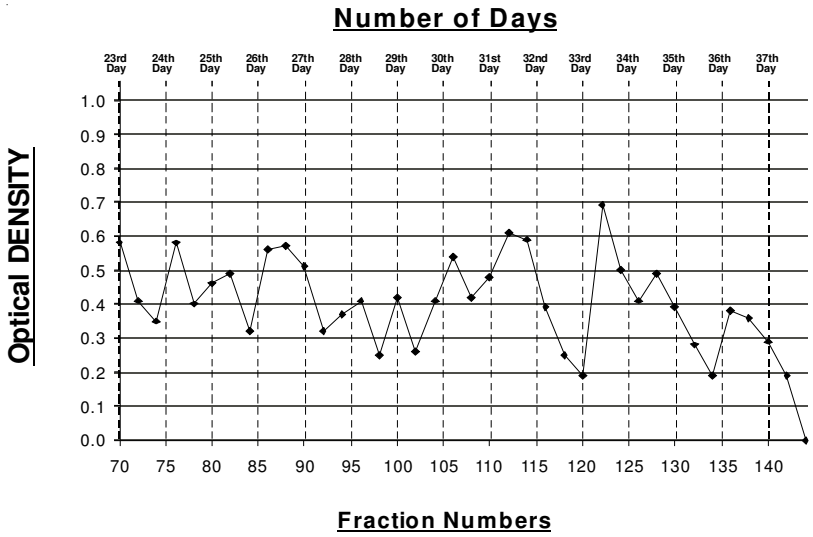

Fig. 3. Elution diagram showing the extent of proteolysis by the immobilized enzyme after keeping the column closed for 1 week continuous elution (days 23-37)

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 activated charcoal column, the bed swelled up. This happened due to the binding of casein with activated charcoal, 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 Figs. 1 and 2. A numbers of peaks are present in the elution diagram 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 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 after running of 7 d. The proteolysis caused by the enzyme was significantly higher even in the 37 d. No proteolysis was recorded after 37 d. The life span of protease of *Carica papaya* bound on activated charcoal (37 d) is greater than the life

span of *Euphorbia royleana*[14] bound to DEAE A-50 (20 d) and even greater than that of immobilized protease of *Calotropis procera*[13] bound to Amberlite-50 (21 d).

Conclusion

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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