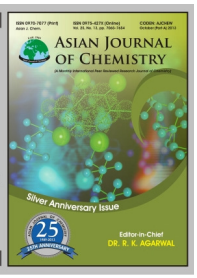
Asian Journal of Chemistry; Vol. 25, No. 13 (2013), 7186-7188

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.14505



Immobilization of the Protease of *Carica papaya* on Amberlite CG-400

H. BOKHARI[1,*] and M.R. KHAN[2]

[1]Departrment of Chemistry, Government College University, Lahore, Pakistan
[2]Center of Policy and Environment, Lahore School of Economics, 19-Km Burki Road, Lahore, Pakistan

*Corresponding author: E-mail: a1hamim@yahoo.com

(*Received*: 1 September 2012; *Accepted*: 19 June 2013) AJC-13669

Immobilization of the protease of *Carica papaya* was carried out on amberlite CG-400. The percentage of immobilization was found to be 54 %. A continuous immobilized enzyme proteolytic system was developed by packing a column with Amberlite CG-400 using citrate phosphate buffer pH 7. The column was partly chocked due to adsorption of casein with Amberlite CG-400, which led to reduction in the flow rate. The products of proteolysis collected in the form of 5 mL fractions were assayed by Lowry's method. The system operated successfully for proteolysis on continuous basis. The life span of the enzyme immobilized on Amberlite CG-400 was 27 d.

Key Words: Amberlite CG-400, Immobilized protease, *Carica papaya*, Proteolysis.

INTRODUCTION

Enzymes are biological catalysts produced by living cells to catalyze cellular-reactions vital to the organism to survive in life. The enzymes being thermo-labile have presented the problem of their preservation as they are denatured both by heat and action of atmospheric chemical reagents. That is the main reason that the scientists all over the world are in search of suitable methods to increase life spans of these biologically important compounds. One of the method being studied involves their immobilization by trapping them in certain active materials such as activated charcoal or by binding them with polymeric matrices equipped with the tendency to bind with these species through ionic or covalent binding forces. The enzyme in the immobilized state are less subject to denaturation and thus can be preserved for prolonged period of time; some even for the years. Several techniques have been developed to prepare immobilized enzymes because they have shown many advantages over enzymes in solution state[1]. For instance, immobilization leads to the ease of recoverability and reusability of the enzymes[2]. Immobilized enzymes have been used in food industries[3,4] for the preparation of protein hydrolyzates with dietary applications[5,6].

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. 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*[9] immobilized on Amberlite-50 and *Euphorbia royleana*[10] immobilized on DEAE A-50 and promising results have been obtained. The work being reported here is the extension of the work stated above, but with the change that protease of *Calotropis procera*[9] was substituted by protease of *Carica papaya* for immobilization on Amberlite CG-400 as it exhibited high protease content and increased life span than protease of *Euphorbia royleana*[10]. This system will be useful for the preparation of protein products such as amino acid acids, polypeptides *etc*. These immobilized enzymes will be better stored with out denaturation.

EXPERIMENTAL

In order to immobilized protease of *carica papaya* following steps as follows:

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.

Assay of protease activity: Assay of protease activity was carried out by the method of McDonald and Chen[11] modified and applied by Khan *et al*.[12] in the paper entitled continuous proteolysis of casein by the cell bound protease of *Carum copticum* and immobilization of *Carica papaya* on activated charcoal[13].

Determination of binding of enzyme with Amberlite CG-400: 0.5 g Amberlite CG-400 was weighed and placed in a 100 mL conical flask containing 25 mL phosphate citrate buffer pH 7 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 repeatedly. The washed precipitate was the immobilized enzyme. The protease units immobilized on Amberlite CG-400 were determined by subtracting 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 Amberlite CG-400: The stages involved in the development of Amberlite CG-400 system are briefly described below:

Packing of the column: 7 g of Amberlite CG-400 was suspended in 400 mL phosphate citrate buffer at pH 7. 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.

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 Amberlite CG-400. The protease activity was plotted against fraction number to construct the elution diagram of eluted soluble protease.

Application of the substrate: 1 g of Casein substrate was dissolved in 100 mL phosphate citrate buffer pH 7 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 Amberlite CG-400. The eluate was collected in 5 mL fractions 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 (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 10 d, the column was stopped for 1 week and the elution was restarted. The products of proteolysis were determined as above. An elution diagram was similarly constructed.

RESULTS AND DISCUSSION

Adsorption of enzyme on Amberlite CG-400: The results of the experiment on the study of the binding of the enzyme with Amberlite CG-400 are given below:

Number of protease activity units present in 5 mL extract = 40; number of protease activity units present in the supernatant = 28; number of units immobilized: 40-28 = 12; percentage of immobilized enzyme: 12/40 × 100 = 30 %; percentage of soluble enzyme: 28/40 × 100 = 70 %.

The results indicate that Amberlite CG-400 has a significant tendency to adsorb and immobilize the protease of *Carica papaya*.

Protease activity of the Amberlite (CG-400) unbound enzyme eluted from the column: The protease activity of the fractions collected after elution with buffer pH 7 after sample application is shown in Fig. 1.

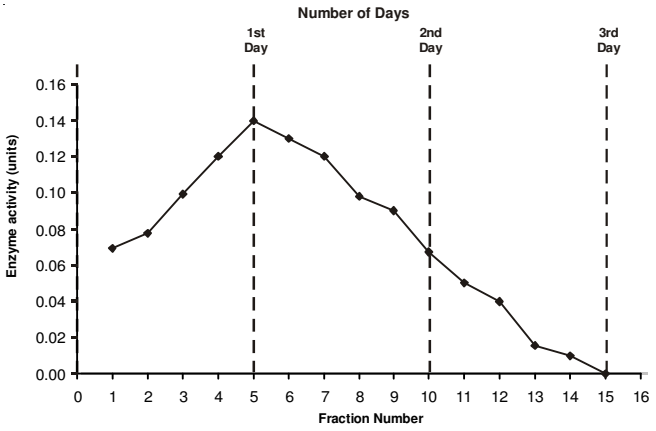


Fig. 1. Change in enzyme activity of the unbound protease of *Carica papaya* during elution with 0.2 M-citrate phosphate buffer pH 7.0 from Amberlite (CG-400) column (Days 1-3)

From the profile it is evident that the entire soluble enzyme was eluted after collection of first 15 fractions, *i.e.* 75 mL elution volume. The record of the units applied and recovered as soluble enzyme is displayed is given below:

Units of protease activity applied to the column; 120 units of protease activity recovered as soluble enzyme: 56; Percentage of soluble enzyme: 56/120 × 100 = 46 %; Number of the units immobilized: 120-56 = 64: Percentage of immobilization: 64/120 × 100 = 54 %.

Proteolysis by Amberlite (CG-400) anion exchanger bound enzyme: The progress of continuous proteolysis of casein by the protease immobilized on Amberlite (CG-400) for 10 days is shown in Fig. 2.

The profile (Fig. 2) indicates that significant proteolysis occurs when the substrate is passed through the bed of Amberlite (CG-400) with protease immobilized to it. Points that are significant about the diagram are as under:

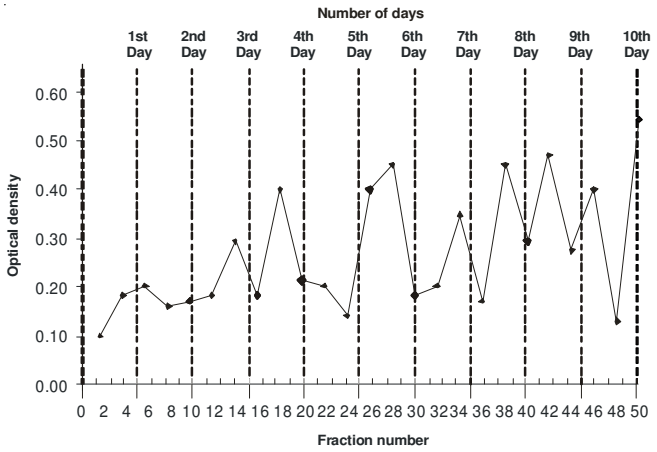


Fig. 2. Elution diagram showing the extent of proteolysis by immobilized enzyme at pH 7.0 after application of the casein substrate to the Amberlite (CG-400) immobilized enzyme column (days 1-10)

1-Along with sharp peaks few small peaks also exist.

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

The progress of proteolysis, after 1 week stoppage and restart on 19th day after the application of sample and end of 27th day is shown in Fig. 3.

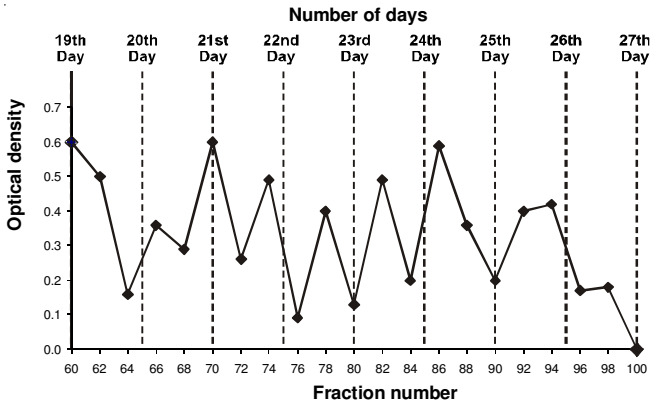


Fig. 3. Elution diagram showing the extent of proteolysis by immobilized enzyme after keeping the Amberlite (CG-400) column closed for one-week restart (days 19-27)

The results (Fig. 3) indicate that the immobilized enzyme is not denatured after 1 week stoppage. The life span of immobilized enzyme seems to be 27 days.

The results indicate that the percentage of immobilization on Amberlite CG-400 54 % seems significant.

The present work was compared to those using other supports such as the % age of immobilization of the protease of *Calotropis procera*[9] on Amberlite CG-50 that was 23 % and percentage of immobilization of the protease of *Euphorbia royleana*[10] on DEAE A-50 that was 30 %, which is quite less than the percentage of *Carica papaya* immobilization on activated charcoal[13] that is 81 %. Here, an important question to be answered is why the whole enzyme was not completely adsorbed by Amberlite CG-400. The complete adsorption would have been possible only if the enzyme was just one species and Amberlite CG-400 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 Amberlite CG-400 column, the bed swelled up. This happened due to the binding of casein with Amberlite CG-400, 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, 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 27 d. No proteolysis was recorded after 27 d. The life span of protease of *Carica papaya* bound on Amberlite CG-400 (27 d) is greater than the life span of *Euphorbia royleana*[10] bound to DEAE A-50 that was 20 d and even greater than that of immobilized protease of *Calotropis procera*[9] 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.

REFERENCES

1. S. Afaq and J. Iqbal, *Electr. J. Biotechnol.*, **4**, 120 (2001).
2. P. Zhuang and D.A. Butterfield, *J. Membr. Sci.*, **66**, 247 (1992).
3. J.R. Whithaker, Principles of Enzymology for the Food Science, Marcel Dekker (1994).
4. X.L. Huang, G.L. Catignani and H.E. Swaisgood, *J. Agric. Food Chem.*, **43**, 895 (1995).
5. J.J. Boza, O. Martinez-Augustin and A. Gill, *J. Agric. Food Chem.*, **43**, 872 (1995).
6. L.R. Soares, E.A.R. Biasutti, M. Capobiango, C.R. Vieira, V.D.M. Silva, J.N. Januario, M.J.B. Aguiar and M.P.C. Silvestre, *Acta Farmac. Bonear*, **25**, 325 (2006).
7. European Federation of Biotechnology, Guidelines for the Characterization of Immobilization Biocatalysts, *Enzyme Microbial Technol.*, **5**, 304 (1983).
8. J.F. Kennedy and J.M.S. Cabral, Enzyme Immobilization in Biotechnology, Enzyme Technology, Weinheim: VCH Verlagsgesellschaft mbH, vol. 7, pp. 347-404 (1987).
9. M.R. Khan and A. Talib, *Pak. J. Sci. Ind. Res.*, **29**, 4 (1983).
10. S.A. Nawaz and M.R. Khan, *Pak. J. Biol. Sci.*, **3**, 2210 (2000).
11. E. McDonald and I.L. Chen, *Anal. Biochem.*, **10**, 175 (1965).
12. M.R. Khan, M.I. Raja and H. Bokhari, *Asian J. Chem.*, **20**, 5729 (2008).
13. M.R. Khan and H. Bokhari, *Asian. J. Chem.*, **20**, 6582 (2008).