

Extraction and immobilization of Proteolytic Enzyme from local Yemeni Bean Seeds (*Dolichos Lablab L.*)

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Abstract

The aim of this study is to prepare immobilized protease with high hydrolytic activity for biotechnological applications. Local Bean Seeds (*Dolichos Lablab L.*) were used for the extraction of acid protease using tris-buffer, pH 4.5 as enzyme solvent. Free acidic protease was immobilized on entrapment in calcium alginate gel (*in situ* activated) by covalent binding method. Their activity and immobilization efficiency for hemoglobin hydrolysis was investigated. Temperature and pH maxima of the immobilized protease showed no changes before and after immobilization. The immobilized protease exhibited good thermal stability and re-usability.

Key words: Acid protease, Yemeni Bean Seeds, Immobilization, Optimization.

Introduction

Enzymes are very efficient catalysts, which serve to accelerate the biochemical reactions of living cells and catalyze a variety of chemical reactions. They speed up biochemical reactions by lowering the energy of activation without themselves appearing in the reaction products [1]. Enzymes may be immobilized by a variety of methods, as shown in Figure 1, which may be broadly classified as physical methods; where weak interactions between enzyme and support material exist and to chemical methods; where covalent bonds are formed between the enzyme and the supporting material [2].

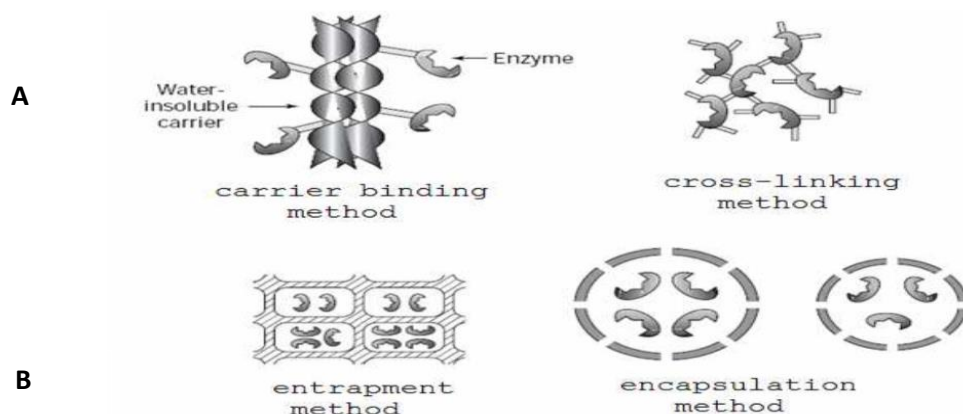


Figure 1: enzyme immobilization methods. A (Chemical method) B (Physical method)

Enzyme immobilization has attracted a wide range of interest from fundamental academic research to many different industrial applications. Immobilized biocatalysts are freely used in the production of medicines, chemicals, food, beverages and wastewater treatments [3]. The basic idea behind enzyme immobilization is to restrict the freedom of the enzyme by fixing it to solid supports or within a semi-permeable support material, which prevents the enzyme from leaving while allowing substrates, products, and co-factors to pass through[4].

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Immobilization on solid carriers is perhaps the most used strategy to improve the operational stability of biocatalysts [5,6]. Seeds of (*Dolichos lablab L.*) are a species of bean in the family Fabaceae. It is native to Africa and is cultivated throughout the tropics for food. It is the only species in the monotypic genus *Lablab*[7]. Yemeni beans (*Dolichos lablab L.*) both in tender green and in mature dry stages is consumed after cooking in Yemen, India and parts of South America. The efficiency of the free enzyme and the immobilized protease will be determined by estimating some physico-chemical properties such as reuse.

Materials and Methods

Plant Material and Chemicals:

The *Dolichos lablab L.* seeds were obtained from local markets in the Republic of Yemen. All chemicals were of analytical or electrophoresis grade and were purchased from Sigma Aldrich, Himedia (Indian company). Deionized (0.5-3 $\mu\text{S}/\text{cm}$) water was used throughout the experiments.

Enzyme Extraction and Purification:

The seeds, approximately 12 grams, were washed separately with 1% HgCl_2 and wash with distilled water and then soaked in distilled water at room temperature overnight germination. After that, the seeds were ground by electric homogenizer. Then, the homogenates were finely powdered in magneticblander and mixed with 10mM Tris-HCl buffer at pH 7.2 for 3h. The extracted mixtures were centrifugated at 4000 rpm for 30 min below 4°C and collected supernatant. Proteins were precipitated from crude extract using 80% ammonium sulfate solution. Precipitates were centrifugated at 4000 rpm for 20 min below 4°C. The pellets were re-suspended in 10mM Tris-HCl buffer pH 7.2[8] and were dialyzed extensively against the same buffer. The dialyzed ammonium sulfate fraction was loaded on to a DEAE-cellulose column (1.5cm X 15cm), pre-equilibrated and eluted with the same buffer and with a salt concentration gradient of 0.1M NaCl. Absorbance of the fractions was measured at 280 nmby spectrophotometer. The active and catalytically enriched fractions were pooled. Tee fraction collected was placed on the top of sephadex G-70 column, then was eluted with previous buffer and monitored spectrophotometry the same wavelength.

Protein Measurement:

Protein concentration was determined by the method of [9] using bovine serum albumin (BSA) as a standard protein. The amount of the soluble protein was calculated from the standard curve as of protein per ml of test sample.

Enzyme Assay:

Proteolytic activity was assayed by the following the modified method of [10] for soluble enzyme using casein (0.65%) as substrate, 10% trichloroacetica (TCA) for stop reaction, after separation supernatant by centrifuge mix with 500 m M Na_2CO_3 and diluted folinciocatored reagent (FCR). After vigorous mixing, the color was allowed to develop for 30 min and was monitored, using spectrophotometer at 732 nm.

One unit (u) protease was defined as the amount of enzyme that hydrolyzed casein to release 1 μmol of tyrosine per minute at 37°C. The specific activity was expressed in the units of enzyme activity per milligram of protein (u/mg). The enzyme activity computation in (u/ml) were made, using the following equation.

$$U = \frac{\mu\text{mol tyrosin relased X volume of the reaction mixture (ml) X Dilution factor}}{\text{Reaction time (min) X volume of the enzyme used (ml) X volume used for calorimetric estimation}}$$

Electrophoresis:

Purity of enzyme preparation was assessed with sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) system (Adjustable vertical gel system) using slab gel (0.5 mm thick, 10% polyacrylamide) by following the method[11]. The protein in the gel were stained with Coomassie Brilliant blue R-250.

Determination of Molecular Weight by SDS-PAGE:

Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis of purified protease was carried out according to the method [12.].

Immobilization of Protease

Calcium alginate [13] sodium alginate suspension (3%) was prepared by suspending 0.9 g sodium alginate in 30 ml boiling water and autoclaved at 121°C for 15 min. The suspension was cooled to room temperature and 47 µl cell suspensions (equivalent to 0.03 g dry cell weight) was added and mixed for 10 min by stirring with a glass rod. This was taken in a sterile syringe and added drop wise into chilled 0.2 M CaCl solution from 5 cm height with constant stirring. The beads obtained were kept for curing at 4°C for 1 h in refrigerator. The cured beads, so formed, were washed with sterile distilled water and were preserved in 0.9% NaCl solution at 4°C. All the operations were carried out aseptically under a laminar flow hood.

Characteristics of Immobilized Enzyme

a- Effect of pH to Immobilized Enzyme Activity:

Immobilized enzyme reacts to Casein in different pH values within 10 min at 45°C. After reaction, we determine the immobilized enzyme activity in these pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0)

b- Effect of Temperature on Immobilized Protease Activity:

Immobilized enzyme reacts to Casein in different temperature values within 10 minutes, at determined pH. After reaction, we determine immobilized enzyme activity in these temperature values (30, 40, 50, 55, 60,70, 80, 90°C).

Results and Discussion

In the present study, we have purified and characterized protolytic enzyme from seeds of *Dolichos lablab L.* by ammonium sulfate precipitation, DEAE-cellulose and sephadex G-70 column.

Our data showed that the 80% ammonium sulfate saturation fraction correlates with the highest protolytic enzyme and specific activities, compared with the crude protolytic enzyme and other fractions. The purification procedures of the protolytic enzyme, secreted by tested seeds, are summarized in (Table 1). The results showed that the protolytic enzyme was purified 1.074 fold, with a specific activity of 40.82u./mgenzyme, after ammonium sulfate fractionation. The protolytic enzyme was then purified, using DEAE-cellulose column resulting in 1.422 fold purification with a specific activity of 215.4u./mg. The final purification step presented 7 fold enzyme purification with a specific activity of 266.34u./mg and the yield of the enzyme, after this purification, was found to be 54.06%.

Table 1: Purification of protolytic enzyme from 12 g seeds

Step	Volume (ml)	Total protein (mg)	Total activity (u)	Enzyme activity (u/ml)	Specific activity (u/mg)	Purification fold	Yield (%)
Crude	102	26	1010	9.9	38.85	-	100
(NH ₄) ₂ SO ₄ 80%	80	23.52	960	12	40.85	1.074	95.05
DEAE- chromatography	90	3.25	700	7.8	215.38	1.422	69.31
Sephadex G-70	30	2.05	546	18.2	266.34	7	54.06

SDS-PAGE analysis of the proteins at each step of purification revealed a monomer band with a molecular weight of 50 KDa (Figure 2).

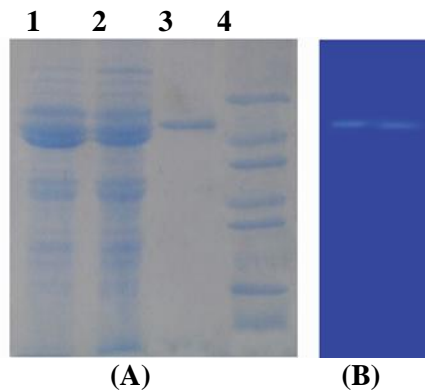


Figure 2: Electrophoretic analysis of protease. (A) Lane 1 is crude, lane, 2 is crude after ammonium sulfate purification, lane 3 is enzyme purified by chromatography and lane 4 is standard protein. (B) Protein activity on zymogram

Immobilization of Acid Protease

Purified protease enzyme after immobilization is shown in Figure 3.

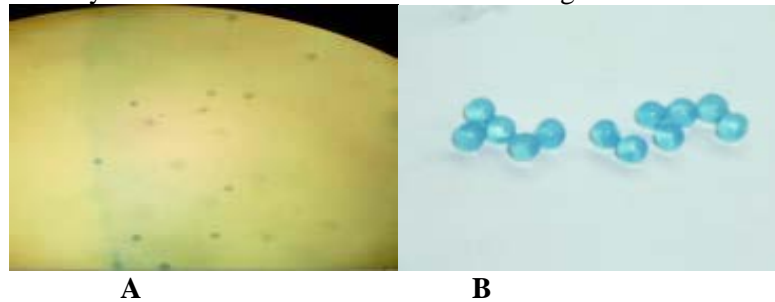


Figure 3: (A) Enzyme inside alginate bad & (B) Beads dyed bradford

Characterization of Immobilized Enzyme

Effect of pH to Immobilized Enzyme Activity

In this experiment, immobilized enzyme with Casein solution was done in pH ranges (4-7), within 10 minutes, 45°C. We have also performed the control test using free enzyme. Results of immobilized enzyme activity on Casein at different pH values are shown in Figure 4.

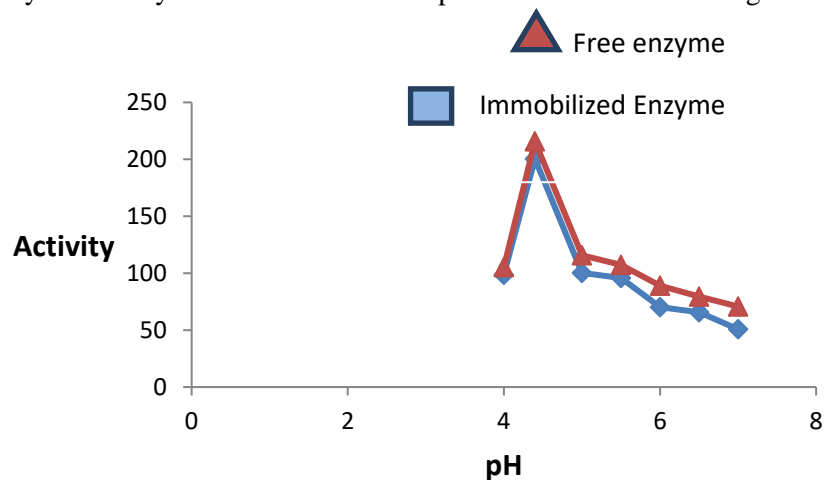


Figure 4: Immobilized and free enzyme activity at different pH values

Effect of Temperature on Immobilized Enzyme Activity:

In this experiment, immobilized enzyme with Casein was done in the temperature ranging from 10 to 90°C, in 10 min, at pH 4.5. We have also conducted the control tests with free enzyme. Results of immobilized enzyme activity on Casein at different temperature values are shown in Figure 5.

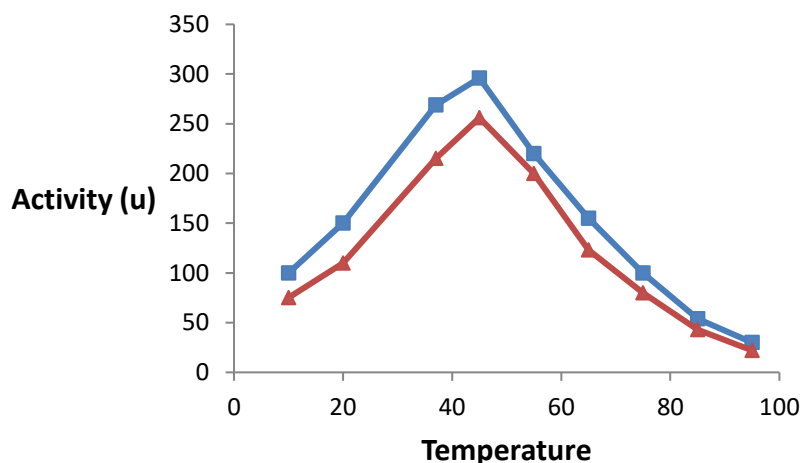


Figure 5: Immobilized enzyme activity at different temperature values

Enzyme immobilization on solid supports represents an efficient approach to improve enzyme stability as well as offering unique merits over the soluble biocatalyst such as operational stability, reusability of the enzyme, bioprocess control, and simplifying product separation [14,15].

Among different immobilization techniques, entrapment in sodium alginate gel offers many advantages due to its simplicity and nontoxic character[16].

The catalytic behavior of the immobilized enzyme was examined on hemoglobin in terms of enzyme activity, stability and reuse in an aqueous medium at variable pH and temperatures.

Temperature and pH maxima of the immobilized enzyme showed no changes before and after immobilization. This result agrees with the study of [17]. The domination of processed food in the modern human diet results in a lack of active exogenous enzymes and as a consequence—a rapid depletion of their metabolic reserves.

Since enzymes found in food are an important part of maintaining a healthy diet, the food rich in active enzymes, especially proteases, is applied as an important therapeutic and anti-ageing diet component[18].

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Conclusion

The immobilized enzymes on calcium alginate gel are prepared for the purpose of repeated use and the possibilities of continuous reaction system. One of the most important properties is the stability of proteins when they are used in some medical and industrial applications. The immobilization of the enzymes improves this property as well as many other properties. In this study, protease was purified and immobilized on calcium alginate gel. Protease was used in this study for its biological and industrial applications, such as in paper textile, pharmaceutical applications, food, and detergent industries. The free and immobilized enzymes are the same in the relative activity.

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استخراج وتجميد إنزيم البروتياز من بذور الفاصوليا اليمنية المحلية

(*Dolichos Lablab L.*)

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المخلص

الهدف من هذه الدراسة هو تحضير البروتياز المقيد بالنشاط المائي العالي لتطبيقات التكنولوجيا الحيوية. تم استخدام بذور الفاصوليا المحلية (*Dolichos Lablab L.*) لاستخلاص البروتياز الحمضي باستخدام محلول منظم ترايس-حمض الهيدروكلوريك، الرقم الهيدروجيني 4.5 كمذيب للإنزيم. تم تجميد البروتياز الحمضي بطريقة فيزيائية عند حبس جل الجينات الكالسيوم (في الموقع المنشط) بواسطة طريقة الارتباط التساهمية. تم قياس نشاط وكفاءه الإنزيم البروتياز في وجود المادة الهدف (الهيموغلوبين). وأظهرت درجة الحرارة ودرجة الحموضة المثلى لإنزيم البروتياز لا تغيير قبل وبعد الجمود. كما أظهر الإنزيم المجمد استقرار حراري جيد وإعادة الاستخدام.

الكلمات المفتاحية: البروتياز الحمضي، بذور الفاصوليا اليمنية، التجميد، التحسين.