ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Immobilization and Optimization of Lettuce (*Lactuca sativa*) Alkaline Protease on *in situ* Activated Carrier

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ABSTRACT

The aim of this study was to prepare immobilized protease with high hydrolytic activity for biotechnological applications. Lettuce seeds (*Lactuca sativa*) were used for extraction of alkaline protease using tris-buffer, pH 5 as enzyme solvent. High yield of alkaline protease (35.9 U/g wt. dry seeds) was obtained. The biochemical properties of the prepared enzyme (crude) showed optimum protease activity in alkaline tri-HCl buffer, pH 10 at 60°C. The K_m and V_{max} values were 1.9 mg and 24.3 U/mg at pH 10, respectively, using azocasein in as substrate. It was highly thermostable after heated without substrate at 40°C for 120 min without any loss of activity. It also maintained 100%of its activity after storage at -4°C in tris-HCl buffer, pH 5 for 78 days. The crude enzyme was partially purified with ammonium sulphate at saturation 0-60% saturation (free protease)with 82.48 % yield and specific activity 22.26 U/mg. Free alkaline protease was immobilized on gelatin-alginate glutaraldehyde and alginate-glutaraldehyde beads (*in situ* activated) by covalent binding method. Their activity and immobilization efficiency for casein hydrolysis was investigated. Immobilization of lettuce protease on sodium alginate glutaraldehyde beads showed higher immobilization efficiency than that of gelatin alginate gluteraldehyde one. Immobilization efficiency of the immobilized protease was increased in a linear relationship with increasing gluteraldehyde concentration at optimum pH value 6.5 of gelling solution. The prepared alginate-glutaraldehyde beads were identical and of spherical shape with uniform structure and constant density. Immobilized protease showed good operation stability for 12 recycling run with retained 85.65 % of its initial activity.

Keywords: Alkaline protease, *Lactuca sativa* seeds, Immobilization, Optimization, Covalent binding.

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ISSN: 0975-8585

INTRODUCTION

Proteases are enzymes that catalyzed hydrolytic reactions in which protein molecules are degraded to peptides and amino acids. They accounts for approximately 60% of global market of industrial enzymes [1]. They have been identified and studied from latex and fruits [2-3], from a number of seeds [4], from sunflower [5], from leaves [6] and fromflowers [7]. Proteolytic enzymes from plant sources are better suited for pharmaceutical as well as food industries, as they are active over a wide range of temperature and pH, and possess broad substrate specificity, high stability in extreme conditions [8]. They had been used for the treatment of cancer as antitumorals, for digestion disorders, and for swelling and immune-modulation problems [9-11].

Covalent binding immobilization had been classified as irreversible enzyme immobilization method. The immobilization of proteases by covalent attachment can offer several advantages over the free enzyme including easy handling, recovery from reaction medium and reuse in continuous processing. Stability of enzymes is key important in a variety of commercial and industrial applications due to harsh conditions requirements than in the laboratory assays [1]. Increase in half-life and thermal stability of enzymes have been achieved by covalent coupling with different supports like mesoporous silica, chitosan, etc. [12-13]. Maintaining the structural and functional property of enzymes during immobilization is one of the major roles played by a cross-linking agent. One such agent is glutaraldehyde, popularly used as bifunctional cross-linker, because they are soluble in aqueous solvents and can form stable inter- and intra-subunit covalent bonds.

Support material is a key factor in enzyme immobilization and considerable attention has been paid toward searching for ideal support materials, which may give the best combination of high remaining activity, low cost and friendly to human health and environment. Different natural supports have been used (cellulose, alpha-alumina, gamma-alumina, chitosan and alginates). Cross-linking of alginate with divalent ions (like Ca^{+2}) and glutaraldehyde improves the stability of enzymes [14-15].

In the present work, extraction and partial purification of lettuce protease were performed. Optimization conditions for alginate gluteraldehyed beads were carried out. Physicochemical properties (pH, temperature, kinetic, thermostability, etc….) of the crude protease were studied. The partial purified (free protease) was immobilized successfully onto sodium alginate-gluteraldehyde and gelatin alginate-gluteraldehyde (in situ activated). Optimization conditions for alginate gluteraldehyed beads were carried out. Physical properties of the prepared immobilized beads were investigated. We also examined the reusability of the immobilized protease.

MATERIAS AND METHODS

Materials: Dry lettuce seeds (*Lactuca sativa*) family *Asteracea* were bought from local markets. They were chosen for preparing protease enzyme as they showed previously high amount of alkaline proteolytic activity ([16]. Azocasein, gelatin, sodium alginate, glutaraldehyde and all chemicals were of analytical grade.

Methods:

Preparation of alkaline crude protease: After thorough cleaning and removal of broken seeds and foreign materials, the dry seeds in the rest state (50 g) were ground in a Braun bean mixer and mixed with deionized distilled water with continuous shaking over a period of 12 hours at 5°C [17]. The homogenate was centrifuged at 3,000 r.p.m for 15 min, and the supernatant was used as the crude enzyme. For optimization of extraction methods, the homogenate powder was suspended in 0.01 M Tris-Hcl buffer at variable pH (5.0 and 8.0) to be compared with the extraction with deionized water. Each extract obtained was tested for protease activity. The efficacy of each solvent was estimated by the amount of protease obtained from one gram dry seeds.

Preparation of free alkaline protease: Partially purified protease was prepared according to the method of Green and Hughes [18]. Adequate volume of the prepared crude enzyme was treated with different concentrations of ammonium sulphate (0-60% and 60-80% saturation) at 5 °C. Each fraction was obtained by centrifugation (13,000 r.p.m, 4°C) for 15 min. The resulting precipitates were dissolved in appropriate amount of distilled water and dialyzed exhaustively against distilled water for 1 day at 4°C to get rid of the excess of ammonium sulphate. Undissolved protein was removed by centrifugation

before enzyme assay. Protease activity and protein contents were determined in each fraction. The most active fraction was used as the free protease.

Immobilization of the free protease by covalent binding with:

Sodium alginate-gluteraldehyde (*in situ* activated). Calcium alginate beads were prepared according to the method of Ortega *et al*. [19], with some modifications. Sodium alginate solution 2% (w/v) was dropped into a solution composed of 200 mM calcium chloride dissolved in 25 mM sodium acetate buffer containing 6.5% gluteraldehyde with a final pH adjusted to 6.5 with continuous stirring. The formed beads were left for 12 hour at 9 °C for complete gel hardening. The beads were washed by distilled water to remove excess gluteraldehyde. Two g of calcium alginate activated beads were mixed with 1.5 ml of the free enzyme followed by 1.5 ml distilled water to ensure full immersion of beads in enzyme solution. The loading process was performed for 1 hour under continuous shaking at 9 °C.

Gelatin alginate-gluteraldehyde (*in situ* activated). The gel beads were prepared according to the method of Tanriseven and Dogän [20], with some modifications. Mixture of 1 g gelatin powder, 1 g sodium alginate and 5ml glycerol were dissolved in 25 mM sodium acetate buffer, pH 4.5 in a water bath at 50 °C under continuous stirring. The prepared mixture was dropped into an iced solution of 4% (v/v) polygluteraldehyde[21] dissolved in 25 mM calcium acetate buffer pH 5.3. The formed beads were left for 12 hour at 9 °C for complete gel hardening. The beads were washed by distilled water to remove excess polygluteraldehyde.

Gelatin-alginate-gluteraldehyde activated beads (2 g) were mixed with 1.5 ml of the free enzyme followed by 1.5 ml distilled water to ensure full immersion of beads in enzyme solution. The loading process was performed for 1 hour under continuous shaking at 9 °C.

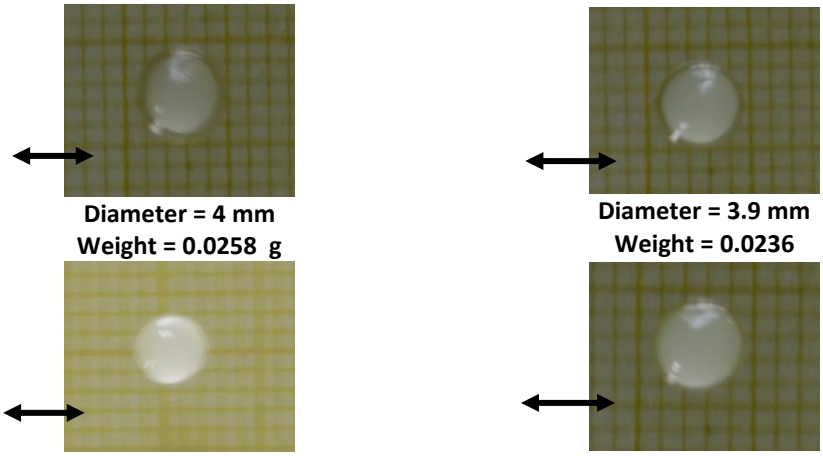
Optimization of sodium alginate-gluteraldehyde beads preparation. Effect of different gluteraldehyde concentrations(varying from 4.8 to 8.5 %) and pH's values of the gelling solution (ranging from 4.5 to 8.5) on immobilization yield and efficiency were investigated. For each immobilization processes, weight, number and beads diameter were determined. Protease activity and protein concentration were measured in wash solution (unbound enzyme). The immobilized protease activity efficiency was determined in the prepared beads.

Physical characterization of the prepared alginate gluteraldahyde beads. Picture (1) showed the weight, number and dimension differences between the alginate and gelatin-alginate (*in situ* activated) beads. Gelatin-alginate-gluteraldahyde beads were of bigger size, weight and lower surface area than the alginate-gluteraldahyde beads. Alginate-gluteraldahyde beads with known weights were subjected to photographing measurement of the beads diameter as shown in photo (2).This photo confirmed the typical spherical shape of the prepared beads and identified the prepared beads.

	Alginate beads	Gelatin-alginate beads
Single bead weight:	**0.023 ± 0.001 g**	**0.032 ± 0.001 g**
Number of beads:	**43.2 ± 4.1/ g gel**	**31.35 ± 2.1/ g gel**
Total surface area:	**18.06 ± 0.78 cm^2/g gel**	**13.96 ± 0.44 cm^2/g gel**

Picture (1): The difference between alginate and gelatin-alginate beads.

ISSN: 0975-8585



Picture (2): Optical microscope photos of some alginate-gluteraldehyde beads with different weights and diameters. Mean weight = 0.019 ± 0.001g and mean diameter = 3.43 ± 0.12.

Since: Weight of the sphere = volume × density and = 4/3πr3 × density and = r3 × (4/3 π density)
A linear relationship was found between beads weight and cubic radius (r^3) with constant slope ((4/3 π density) as shown in Figure (3). Thus, these results confirmed the uniform structure of the prepared beads with constant density.

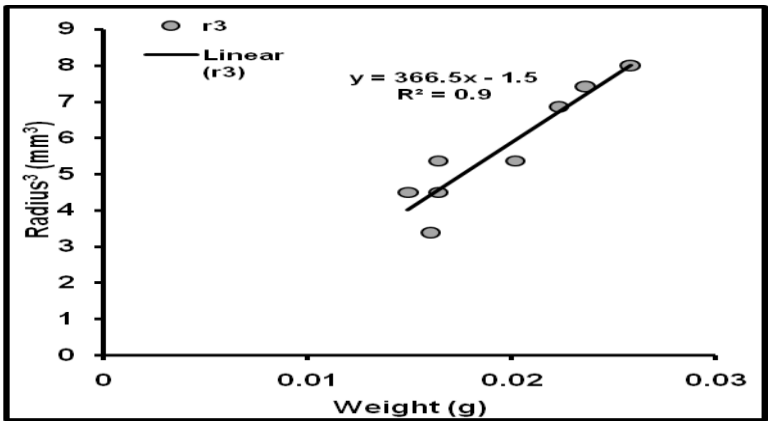


Fig (3): Linear relationship between weight and cubic radius alginate-gluteraldehyde beads.

Proteases activities. The activity of the prepared protease was determined according to the method of Cabral *et al*. [22] using azocasein as a substrate. The standard reaction mixture contained 2 ml of 0.25% (w/v) azocasein dissolved in 50 mM Tris-HCl buffer, pH10 and adequate amount of enzyme (crude, free and immobilized) in a total final volume of 2ml. The reaction mixture was incubated in a water bath at 40 °C for 60 min. The reaction was stopped by adding 0.5 ml 5% (w/v) trichloroacetic acid. The tubes were left to stand for 10 min then centrifuged at 3,500 rpm for 10 min. To 2 ml of the clear supernatant, 1 ml 1N sodium hydroxide solution was added. The absorbance of the samples was measured by LKB Biochrom Nova spec II spectrophotometer at 450 nm. **One unit** of proteolytic activity of the crude and free protease was defined as the amount of enzyme that yielded an absorbance change of 0.1 at 450 nm per one hour in the current experimental conditions using azocasein as substrate. **One unit** of proteolytic activity of the immobilized protease was defined as the amount of enzyme that yielded an absorbance change of 0.1 at 450 nm per one hour for the total surface area of the bead in the current experimental conditions using azocasein as substrate.

Protein determination. The protein concentration was determined by the method of Lowry *et al*. [23], using bovine serum albumin as a standard.

Specific activity. Specific activity of protease was expressed as units per milligram protein.

Relative activity. Relative activity was expressed as a percentage of the maximum activity under the standard assay conditions.

Determination of free bound protease. Free bound protease was defined as the amount of free protease immobilized onto the prepared beads. Bound free protease = Total amount free protease added (U) - Total amount of residual protease activity (U) in calcium solution and in the washing solution of gel beads in the enzyme loading process.

Immobilization efficiency measurements. Immobilization efficiency was defined as both ratio of the immobilized protease activity to the activity of the bound free protease activity (IU/FU)and the ratio of the immobilized protease specific activity to the specific activity of the added free protease (ISA/FSA).

Operation stability of the immobilized protease (Repeatability). Immobilized protease beads (0.045 g) were incubated with 2 ml 0.25% azocasein solution in 50 mM Tris-Hcl buffer, pH 10 at 70°C for 30 min. At the end of the reaction time, the immobilized protease beads were collected and washed with 0.2 mM $CaCl_2$ and re-suspended in new 2 ml of freshly prepared substrate to start a new run. The supernatants were assayed for protease activity. It was repeated for 12 times. The immobilized protease was expressed as a percentage of its residual activity compared to the initial activity.

Surface scanning electron microscope. The alginate-gluteraldehyde plain and loaded beads were sliced into 2 hemispheres then were mounted on SEM stubs with double-sided adhesive tape. Scanning electron micrographs were taken using National Research Center Quanta FEG250. The accelerating voltage and the magnification are shown on the micrograph.

Statistical analysis. Data were expressed as the mean ± standard error from at least three experiments.

RESULTS AND DISCUSSION

Proteases are a unique group of enzymes that have a wide application in pharmaceutical industries. Alkaline protease was extracted from 50 g dry lettuce seeds (*Lactuca sativa*) with different extraction solvents (distilled water, 100mM Tris-HCl buffer, pH5.0 and 8.0).Extraction with 100 mM Tris-HCl buffer, pH 5.0 showed the highest relative protease activity with35.9 U/g dry seeds. This was in agreement to that of germinating legume seeds [17].It was taken as a crude protease.

Optimum pH of the crude protease was determined by stepwise increasing the pH values from 7 to 10 (Figure 4). The protease activity was higher in alkaline pH 10 than in neutral pH 7 by 70%. This confirmed the presence of alkaline protease as reported previously [16].

The effect of different temperatures on the crude protease activity was shown in figure (5). The optimum temperature for the crude protease activity was at 60^{o}C. The results was lower than that of alkaline protease from *Streblus asper* [3]and was higher than that of *Ficus benghalensis*[8].

The activity of crude protease increased proportionally in a linear relationship with the increase in incubation time from 20 min up to 120 min.

The activity of crude protease increased proportionally in a linear relationship with the increase in protease concentration from 0.04 mg/reaction mixture up to 0.3 mg/ reaction mixture (Figure 6). Protease activity was calculated as azopeptides absorbance per reaction mixture.

Effect of pre-heating temperatures on the crude protease revealed the high stability of the enzyme up to 120 min at 40°C (Figure 7). Proteases from *Streblus asper*, *Crinum asiaticum* and *Euphorbia milii* were stable for 15 min up to 75, 75 and 65°C respectively [3, 24-25].

ISSN: 0975-8585

In our study, increased calcium concentrations caused an increase in crude protease activity from 0.1 to 0.5 mg/ reaction mixture then a steady state effect till 2 mg / reaction mixture. This may be due to the presence of calcium binding sites in the alkaline protease, which contributes to conformational stability of alkaline protease against elevated temperatures and against self autolysis [25-28].

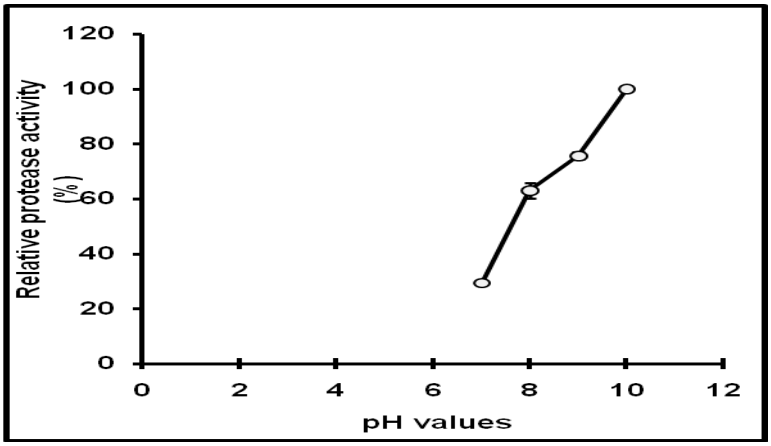


Fig (4): Effect of different pH's on the activity of the crude protease.

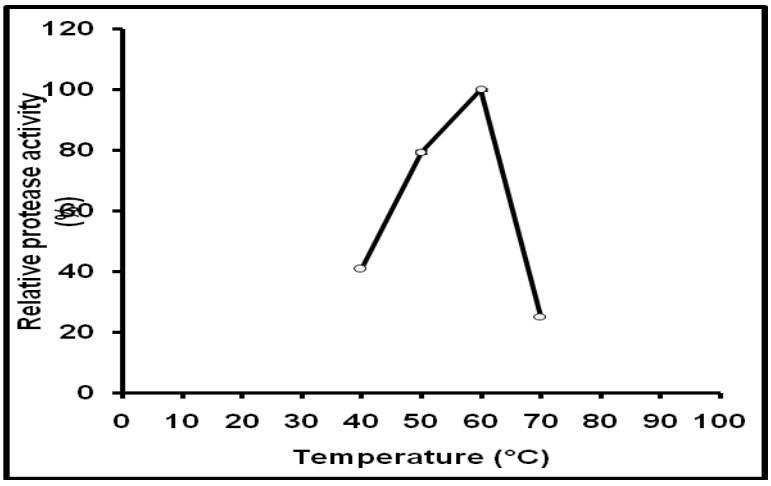


Fig (5): Effect of different temperatures on the activity of the crude protease.

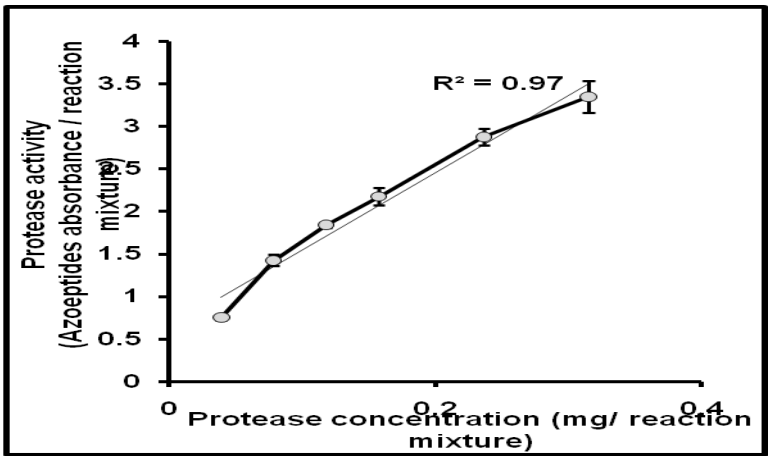


Fig (6): Effect of different protease concentrations on the activity of the crude protease.

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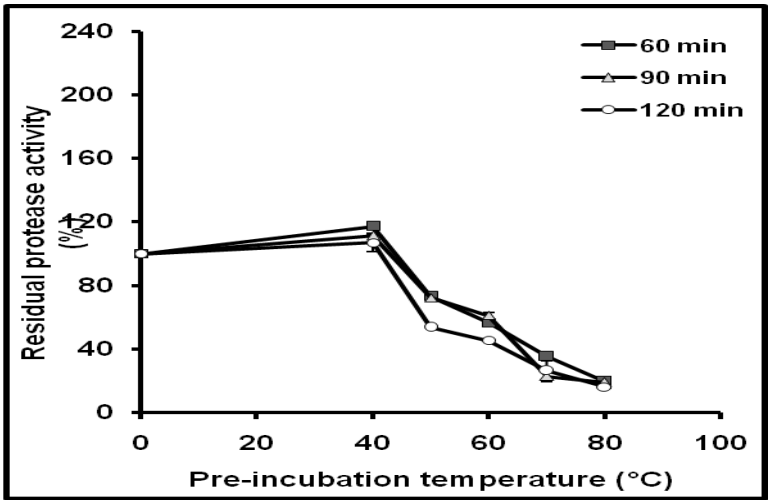

Fig (7): Effect of different pre-incubation temperatures on the activity of the crude protease. Residual activity was expressed as percentage of the initial activity before pre-incubation.

Linear relationship was observed between azocasein and the specific activity of crude protease from 2 to 5 mg. The Michaelis' constant (K_m) and maximum velocity (V_{max}) values for the protease were 1.9 mg and 24.3 U/mg toward azocasein, respectively as shown in figure (8).

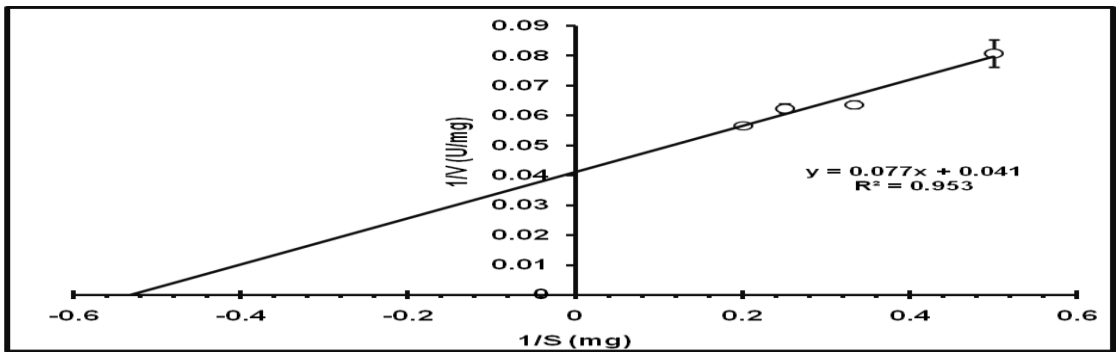

Fig (8): Lineweaver-Burk plot for the crude protease using azocasein as substrate.

The crude protease showed high storage stability when stored in 100 mM Tris-HCl buffer, pH 5 at -4ºC for 78 days. Microbial alkaline protease, from novel bacillus *licheniformis MZK03*,was stable at 4°C for 5 days while maintaining 100% of its activity and 67% loss of activity after 10 days [29]. Owing to the tested protease excellent properties (e.g. thermostable and stable at long storage time periods), so it was subjected to partial purification with ammonium sulphate followed by immobilization. Ammonium sulphate fraction at (0-60%) saturation had high protease activity with specific activity 22.26 ± 0.37 U/mg with high yield 82.48% and 1.81 purification fold than that for the crude one (12.3 ± 0.18 U/mg). It was taken as free protease. Further increase of ammonium concentration up to 80% resulted in loss of protease activity. There had been much interest in preparation of potent immobilized enzymes from plants with promising industrial applications. Enzyme immobilization allows overcoming their application problems in industry: the high operation costs, instability, special storage requirements, sensitivity to microenvironment and difficulty for reusability has limited their industrial wide use [30].

Free protease solution was immobilized onto sodium alginate and gelatin-alginate (*in situ* activated) beads which showed 87.83± 0.31% and 64.71 ± 0.3immobilization yield of added enzyme (Table1). High immobilization efficiency ISA/FSA was found for immobilizing protease onto sodium alginate beads (9.85 ± 0.95) than that for gelatin-alginate (7.69 ± 0.15) one. This means that the alginate-gluteraldahyde beads were able to bind more specific enzyme protein and lower impurities

protein than that for gelatin-alginate-gluteraldahyde beads. From the above results, we chose the immobilized protease onto alginate beads for further study.

Table (1): Immobilization yield and efficiency of immobilized protease by covalent binding on different carrier.

Gel type	Free protease (F)				Immobilized protease (I)		
	Added	Unbound	Bound				
	Activity (U)	Activity (U)	Activity (U)	Activity (%)	*Relative activity (%)	Immobilization efficiency (IU/FU)	Immobilization efficiency (ISA/ FSA)
Alginate	67.3 ± 1.2	8.19 ± 0. 2	59.11 ± 0.2	87.83 ± 0.31	100.0 ± 9.62	6.58 ± 0.63	9.85 ± 0.95
Gelatin-alginate	67.3 ± 1.2	23.75 ± 0. 2	43.55 ± 0.2	64.71 ± 0.3	89.3 ± 1.78	7.97 ± 0.16	7.69 ± 0.15

*Relative immobilized protease activity (%) was expressed as percentage of immobilized protease related to maximum one.

Immobilization of free protease by covalent binding onto calcium alginate beads at different concentrations of glutaraldahyde (GA) ranging from 4.5 to 8.5 % were investigated. The immobilization efficiency and immobilized protease activity (U) increased in a linear relationship with increasing gluteraldahyde concentration up to 8.5 % (Figure 9a&b). The weight and surface area of the prepared beads increased with increasing gluteraldahyde concentrations (Figure 10). Glutaraldehyde concentration was an important factor for successful immobilization. Ferreira *et al*[31], Chae et al. [32]and Ortega *et al.* [19] reported that the optimum gluteraldehyde concentration was found at 1.0, 0.5 and 6.2 %, respectively. This could be attributed to the increased weight and surface area of the resulted beads so no overlapping or crowded attachment points for the enzyme. pH of the gelling solution was essential for the special binding of our targeted enzyme on the surface of the beads.Dropping solution at pH 6.5 showed the highest immobilization efficiency (figures 11 a&b). It may directly influences the special binding of alkaline protease Flavourzyme. Chae *et al.*[32] suggested that pH adjustment turns the conditions more favorable for the surface binding of protease. The immobilized protease showed successful operational stability for 12 recycling run with retained 85.65 % of its activity (Figure 12).

As shown in photo (13), surface scanning electron microscope showed a surface modification at different magnification power when beads were loaded with free protease.

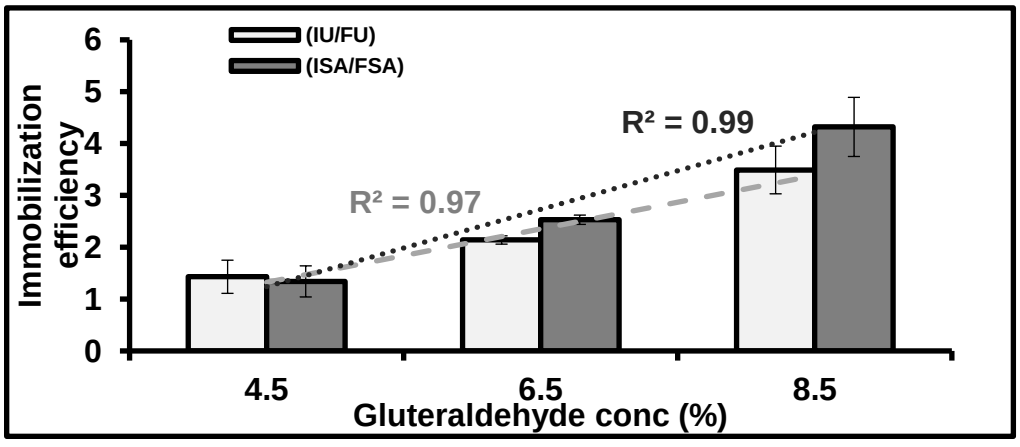

Fig. (9a): Immobilization efficiency of immobilized protease at different gluteraldehyde concentrations.

ISSN: 0975-8585

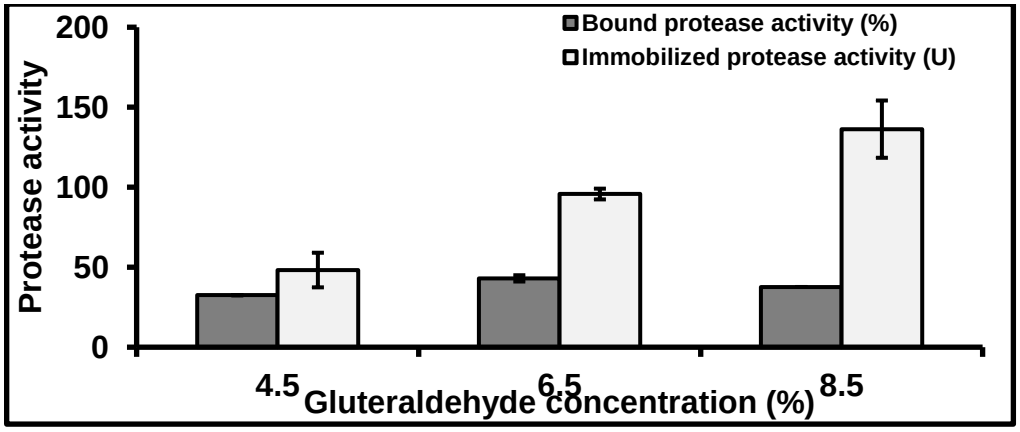

Fig. (9 b): Free bound protease (%) and immobilized protease activity (U) at different gluteraldehyde concentrations.

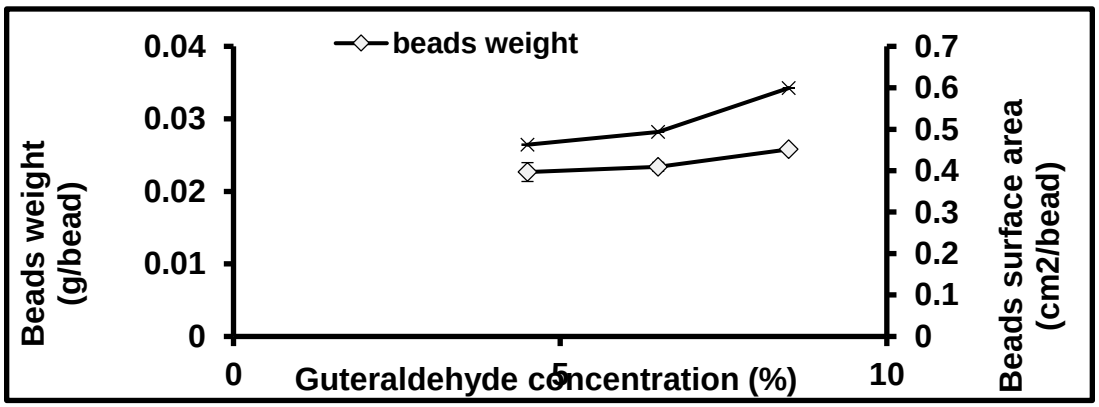

Fig. (10): Effect of different gluteraldehyde concentrations on the weight of ca alginate gel beads.

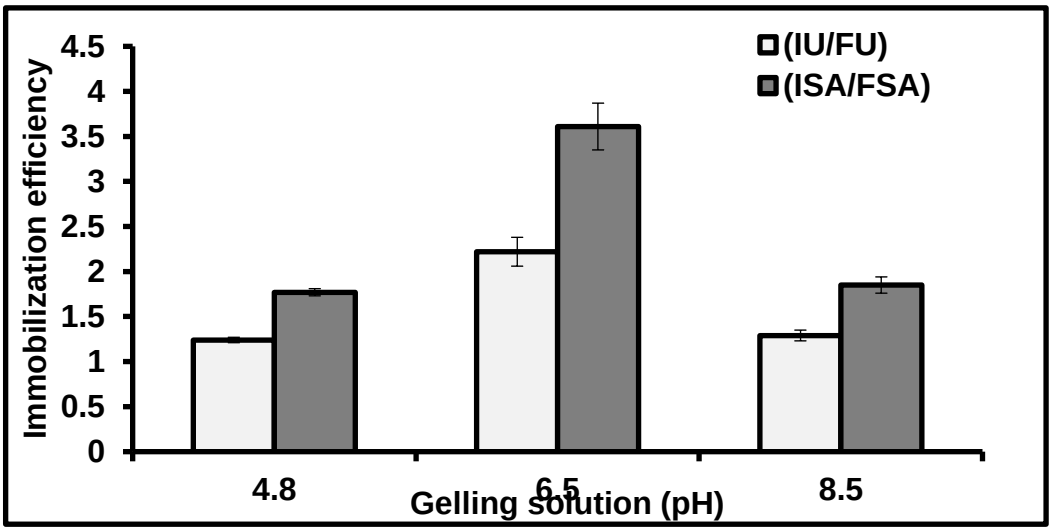

Fig. (11a): Immobilization efficiency of immobilized protease at different gelling solution pH's.

ISSN: 0975-8585

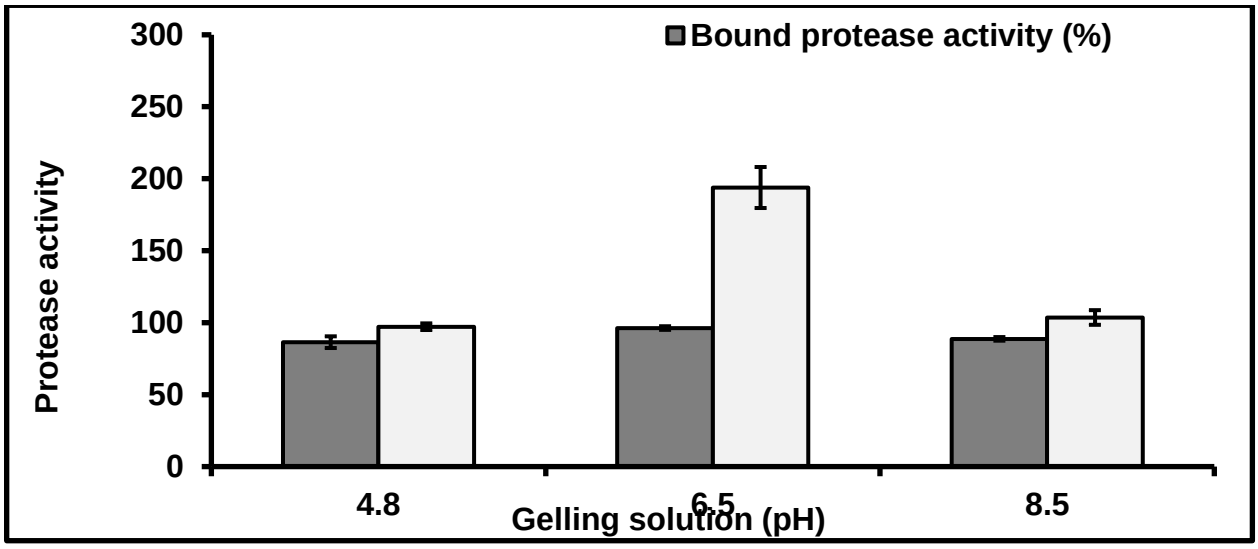

Fig. (11b): Free bound protease (%) and immobilized protease activity (U) at different gelling solution pH's.

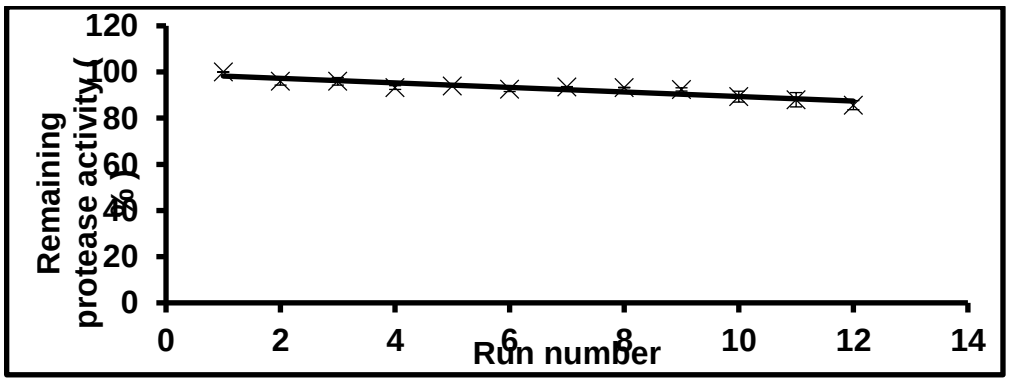

Fig. (12): Operational stability of the immobilized protease during 15 batches (each batch of incubation time 40 min, pH 10 and at 70 °C with 0.2 M $CaCl_2$.

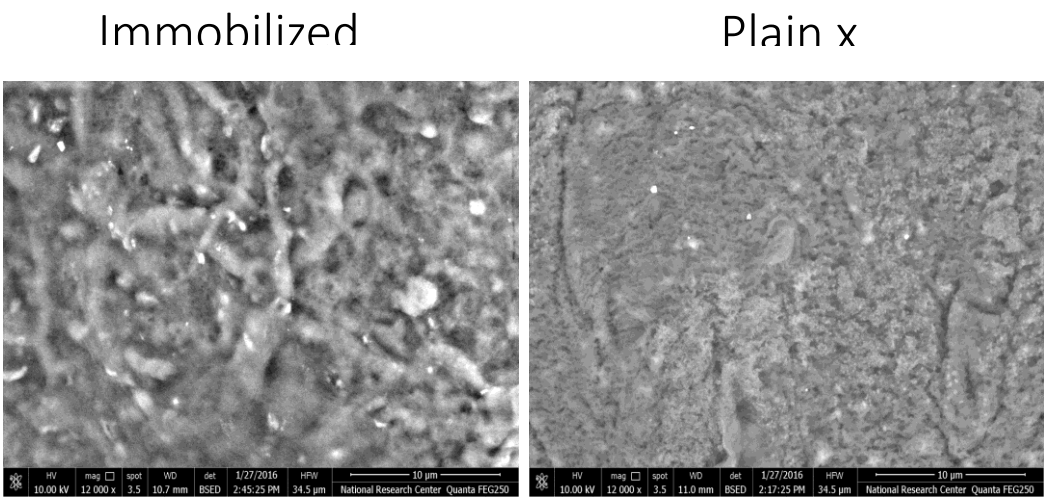

Fig. (13): SEM analysis of alginate-gluteraldehyde beads immobilized with protease enzyme from lettuce seeds (left) and plain (right) at different magnification power.

CONLUSIONS

Lettuce are considered as economic source for alkaline protease preparation (with yield 35.9 % U /g dry seeds). Covalent binding method of the free protease with alginate gluteraldehyde beads was simple and inexpensive. The prepared immobilized beads showed high specific activity (221.5 U/mg) and immobilization efficiency at pH 10.0 and 70°C. Immobilized protease had significant thermal and storage stability and high operational stability. Thus, the prepared immobilized protease may be suitable for industrial application. More research is needed to fully explore this new immobilized enzyme and its potential application in bioactive peptides preparation.

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