Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(6):734-739

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Isolation and immobilization of protease isolated from *Glycine max* var. Ogden: A comparison of kinetic properties of free and immobilized enzyme

Umesh Kr. Shandilya*, Ankita Sharma and C. S. Pundir†

Department of Animal Biochemistry, National Dairy Research Institute, Karnal, Haryana, India
†Department of Biochemistry, Maharshi Dayanand University, Rohtak, Haryana, India

---

ABSTRACT

Soyabean (Glycine maxvar. Ogden) protease was immobilized on bristles of plastic brush by covalent linkage. The immobilized enzyme retained 34% of the initial activity of free enzyme. When compared to the free enzyme the optimum pH of enzyme, temperatures for maximum activity and saturation concentration of substrate was increased, while time of incubation was decreased after immobilization. The pH optima of the immobilized alkaline protease was shifted towards the alkaline side by 1.2 unit. The immobilized protease exhibited good storage stability and re-usability.

Keywords: *Glycine max*, protease, immobilized enzyme

INTRODUCTION

Immobilization of enzymes and biological compounds is currently gaining importance due to its wide variety of applications in the food and pharmaceutical industries and also its biomedical applications [1-3]. In general the protein structure can be altered by immobilization thereby changing the properties of theenzyme. Steric hindrance due to immobilization may also interfere with the contact between the enzyme and substrate. The degree of change depends on the method of immobilization, the nature of the support and coupling agent and the specificity of the reactive groups. Hence, immobilization can be achieved in many ways, but it always affects enzyme activity to some extent.

Proteases have been immobilized on natural(organic and inorganic) and synthetic supports [4-9]. Inorganic supports are widely used for immobilization of enzymes mainly due to their good flow through properties, mechanical strength, regeneration and resistance to microbial attack. The commonly usedinorganic supports are controlled porous glass, ceramics and alumina [5, 10]. Small quantities of porous glass or ceramics can immobilize large quantities of enzyme, however these supports are relatively expensive. In the present study, a protease has been partially purified from soyabean seeds and immobilized covalently onto a plastic brush and employed for removal of protein stain from cotton clothes. The advantage of this support is that it is not only cheap but the immobilized enzyme could also be brought in the direct contact of stained cloth by rubbing it over the stain (data not shown here).The aim of the present investigation was to develop an effective method for the immobilization of soyabean protease. The specific objectives were to: (i) immobilize soyabean protease on plastic brush ; (ii) determine the optimum pH, optimum temperature, pH stability and thermalstability of the

immobilized soyabean protease and compare them with the free enzyme; (iii) evaluate the storage stability and re-usability of the immobilized soyabeanprotease.

EXPERIMENTAL SECTION

Chemicals

Trichloroacetic acid (TCA), DEAE-Cellulose, acetone, ninhydrin, sodium dihydrogen phosphate, sodium hydroxide, sodium monohydrogen phosphate, acetic acid, hydrochloric acid, sodium nitrite and potassium hydroxide were from SISCO Research Laboratory Pvt. Ltd., Mumbai. Folin–Ciocalteau reagent was supplied by LobaChemie Mumbai. Plastic washing brush (size 3.5cm × 3cm) made up of polypropylene was purchased from local market. All other chemicals used were of AR grade.

Source of seeds

Seeds of soybean (*Glycine max* var. Ogden) were purchased from local market.

Extraction of protease

Seeds of soybean were ground to powder form in a chilled blender with pauses every 2 minutes. Soybean flour (100g) was mixed in 1.0L of chilled distilled water in a chilled blender. This filled the container to capacity and minimized foam production. The suspension was blended for 6 min with pauses at 2 min intervals to prevent overheating. The resulting suspension was centrifuged at 15,000g for 10 min at 4°C. A thin, white oily layer was skimmed off , both the supernatant and pellet were collected and stored at 4°C. The enzyme activity and protein concentration were measured in supernatant and pellet. The pellet was discarded as it showed very low activity and supernatant was treated as crude enzyme. It was stored at 4˚C until use.

Enzyme assay and protein concentration

The activity of protease was measured by incubating 1ml of diluted solubleenzyme (native or immobilized) with 5ml of 0.65% casein solution in 50mMphosphate buffer, pH 7.6, for exactly 10min at 37°C. The reaction was stoppedby addition of 5ml of 110mMtrichloroacetic acid solution. The precipitate wasremoved by filtration and centrifugation. Then, 2ml of filtrate were mixed with5ml of 500mM Na_2CO_3 solution and 1ml of two-fold diluted Folin's reagent.After vigorous mixing, the colour was allowed to develop for 30min at 37°C.The absorbance due to the amino acids produced was analysed at 660 nm, basedon dl-tyrosine as standard. One unit of activity is defined as the amount ofenzyme that hydrolyses casein to produce equivalent colour to 1 mol of tyrosine per minute at pH 7.6 and 37°C (colour by the Folin&Ciocalteu's reagent).The total protein concentrationwas determined by themodifiedmicro Lowrymethod [11],using bovine serum albumin as standard and Sigma test kit no.P5656.

Immobilization of soyabean protease on plastic brush

It was carried out as described by Pundir*etal*., with modification [12]. The commercially available Polypropylene plastic brush was first incubated with nitrating mixture (mixture of nitric acid and sulfuric acid in 5:1 ratio) for 10 h, to cleave vinyl groups of polymer into small chain polymers having protruding end towards the surface.

$$\underset{\text{Polypropylene Brush}}{[- CH_2 - CH(CH_3) - CH_2 - CH(CH_3) -]} \xrightarrow{HNO_3} \underset{\text{Propylene}}{(-CH = CH_2) + (CH_2 = CH - CH_3)}$$

Activation of bristles of brush:The bristles were activated by glutaraldehyde by putting the brush into 2.5% (w/v)glutaraldehyde in 0.05 M sodium phosphate buffer pH 7 for about 8 h at 30 ºC. The excess of glutaraldehyde was discarded and the brush was repeatedly washed in 0.05 M sodium phosphate buffer (pH 7.0) until the pH of washing discard was 7.0.

$$\underset{\text{Glutaraldehyde}}{CH_3 - CH = CH_2 + OHC - (CH_2)_3 - CHO} \longrightarrow \underset{\text{Activated Bristles of brush}}{CH_3 - CH = C = HC - (CH_2)_3 - CHO}$$

Immobilization of protease onto activated bristles: The glutaraldehyde activated brush was kept into enzyme (protease) solution at 4°C for 24 hrs. After that, the brush was taken off and the protein concentration was estimated in the unbound enzyme by Lowry method.

$CH_3 – CH = C = HC – (CH_2)_3 – CHO + NH_2 – E \longrightarrow CH_3 – CH = C = HC – (CH_2)_3 – HC = N - E$

Activated Bristles Free enzyme Immobilized enzyme

Propertiesof free- and immobilized protease

The following kinetic properties of immobilized protease were studied and compared with those of free enzyme.

Effect of pH: -In order to determine the optimum pH of immobilized enzyme, following reaction buffers in the pH range of 4-8 were used each at a concentration of 0.1 M sodium phosphate buffer in the pH range of 6.5-8.0 and the sodium acetate buffer in the pH range of 4-6. The activity of immobilized protease was determined by assaying with different buffers of these pH values.

Effect of incubation temperature:-To determine the effect of incubation temperature the maximum activity of immobilized enzyme, the reaction mixture was incubated at 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C for 90 min. The activity of immobilized protease was determined as described above.

Effect of time of incubation:- To determine the incubation time for maximum activity of immobilized enzyme, the reaction mixture was incubated for 15 min., 30 min, 45 min, 60 min, 75min and 90 min the activity of immobilized protease was determined as described above.

Effect of substrate concentration: -To determine the effect of protein conc. on the immobilized protease, the following casein conc. was used: 0.2%, 0.4%, 0.6%, 0.8% and 1.0%.

Storage stability and Re-usability:-The plastic brush containing immobilized protease was stored in sodium phosphate buffer (pH 7.0) at 4ºC and 25ºC for 60 days. The activity was measured every 10 days. The immobilized protease was repeatedly used to hydrolyse casein. After each run the immobilized enzyme was washed with distilled water followed by sodium phosphate buffer and stored in the same buffer.

RESULTS AND DISCUSSION

The method of immobilization of protease on plastic brush using glutaraldehyde involves covalent attachment of the amino groups to available aldehyde groups present in the glutaraldehyde-activated support.The amount of protease immobilized on bristles of plastic brush with glutaraldehyde was found to be 0.21mg/g of support and the specific activity retained was 33.87% when assayed under standard conditions using a high molecular weight substrate, casein.The amount of protein coupled onto the matrix and the activity retained by the immobilized enzyme is dependent on the enzyme specificity, support, substrate and the coupling method involved [13]. In earlier report[6], when neutral protease was immobilized on vermiculite by two different methods, it was found that the amount of protein bound was 9-4 and 10-2 mg/g of vermiculite and the activities retained were 72.05 and 64.54% respectively.

Table 1-A comparison of kinetic parameters of free soyabean protease and Enzyme bound to plastic brush

Parameters	Free protease	Protease bound to plastic brush
Optimum *p*H	6.3	7.5
Optimum temperature	50˚C	55˚C
Time of incubation	90 min.	60 min.

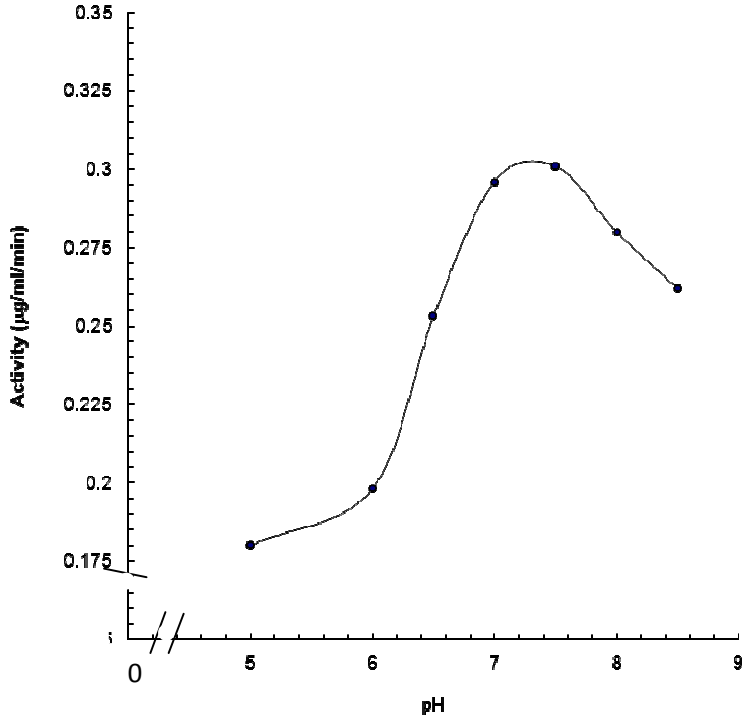

Fig. 1-Effect of pH on activity of immobilized protease

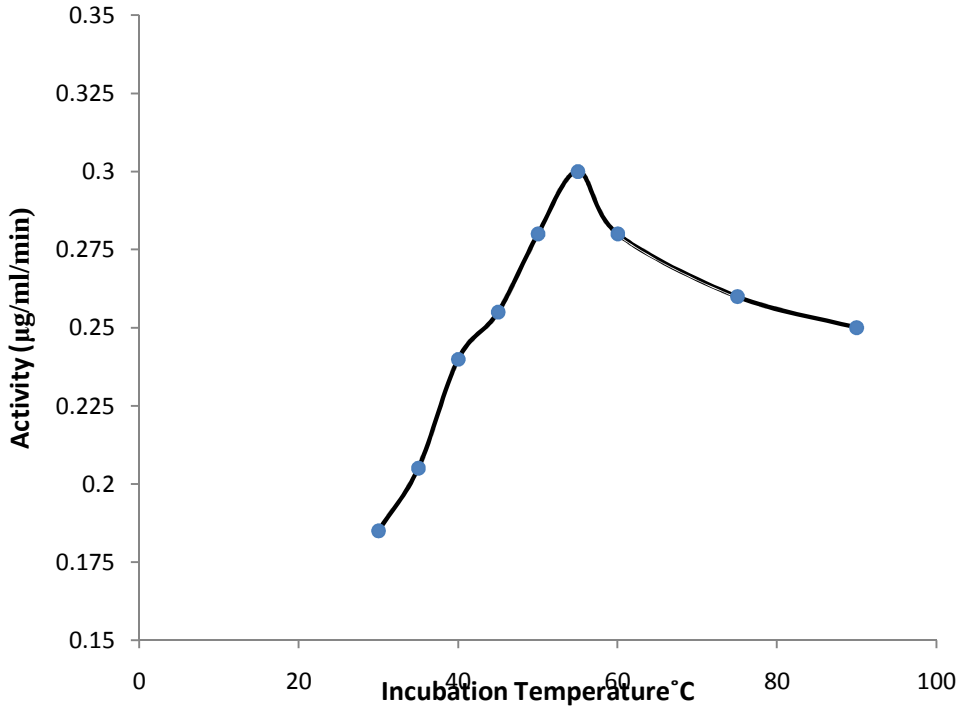

Fig. 2-Effect of temperature on activity of immobilized protease

The optimum pH of the enzyme after immobilization can be changed or unchanged. The optimum pH the protease immobilized on plastic brushwas shifted by 1.2 unit towards the alkaline when compared to the free enzyme (Table 1). The pH optima of the free and immobilized alkaline protease were 6.3and 7.5 respectively (Fig. 1). The activity of the immobilized protease was decreased in the acidic region when compared to the free enzyme. In earlier studies, an increase in optimum pH was observed withtrypsin immobilized on sand [14], neutral protease onvermiculite [6] andalkaline protease on zirconiumcoated porous glass [5]. This change inoptimum pH isusually explained by an alteration in the microenvironment of the enzyme due to immobilization or support. This effect is caused by partitioning of protons in the vicinity of the active site of the enzyme when coupled to a carrier possessing electrostatic interactions.The stability of the immobilized protease as a function of pH was investigated and compared with that of free alkaline protease. Normally an increase in temperature increasesenzyme activity up to a maximum level and thereafter decline in activity is observed due to denaturation of the protein. The activity immobilized protease increased up to 55°C andthendecreased with further increase in temperature (Fig. 2). The optimum temperature was found to be 50°C and 55°C for free and immobilized alkaline protease respectively. Similarresults were obtained with alkaline protease immobilized on bentonite [15], controlled-pore glass[5]and nylon [7]. The optimum time of incubation was found to be 90 min and 60 min for free and immobilized alkaline protease respectively(Fig.3). Thermal stability of the immobilized enzyme was one of the most important criteria with respect to applications. The thermal stability of enzymes after immobilization can be enhanced, decreased or remain unchanged. In this study, the proteaseimmobilized on plastic brush showed a considerable increase in thermal stability when compared to the free enzyme.The immobilized protease retained 81 and 52% of its original activity after 60 days when stored at 4°C and 25°C in sodium phosphate buffer (pH 7.0) (Fig.4). Thedecrease in activity on storage at 25°C is due to denaturation of immobilized protease. It has been reported that the activity of the immobilized protease stored at 25°C was less than that stored at 4°C [7].

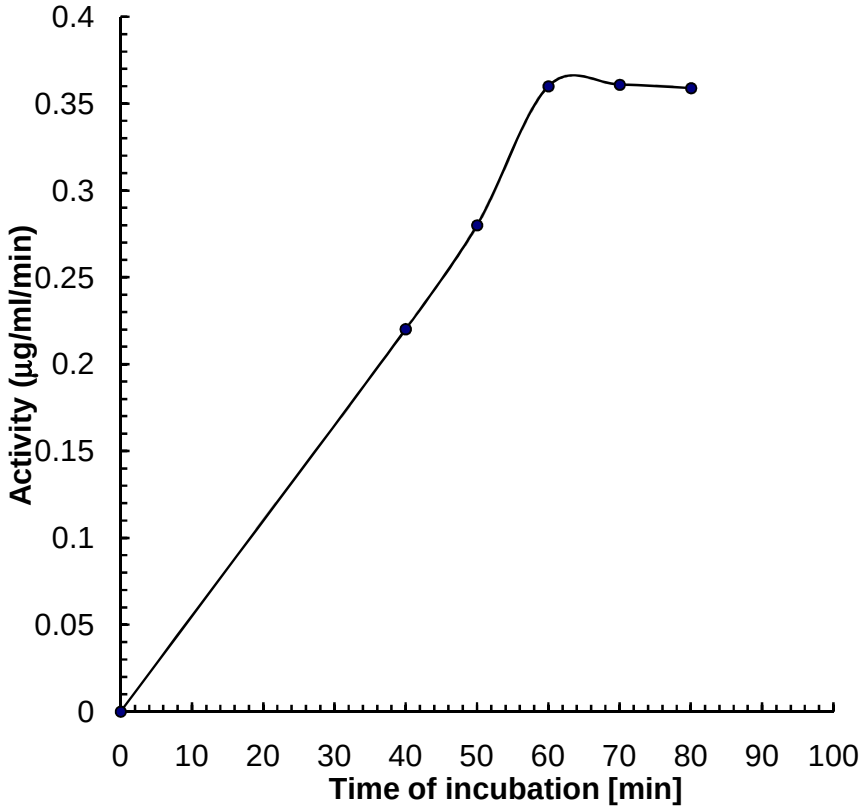

Fig. 3-Effect of time of incubation on activity of immobilized protease

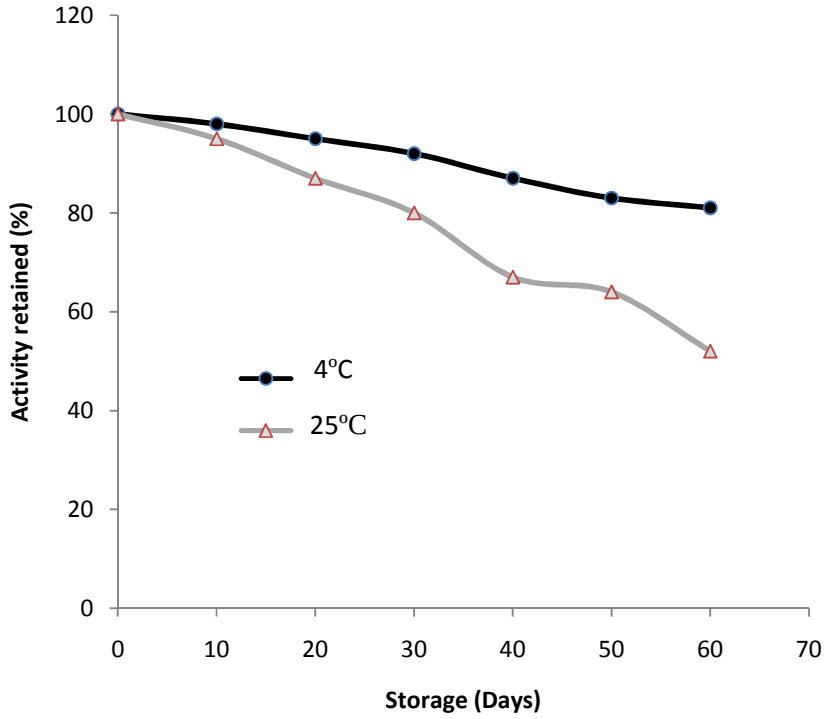

Fig. 4-Storage stability of soyabean protease immobilized on plastic brush

CONCLUSION

Soyabean protease was coupled to plastic brush by covalent binding using glutaraldehyde. The optimum pH, temperature of the immobilized enzyme and saturation concentration of substrate was shifted to a higher value while the optimum time of incubation of immobilized protease was decreased. The thermal and storage stability of the protease was considerably improved by immobilization.

REFERENCES

[1] TWGusekand JEKinsella. *Food Technol*., **1988,***42*, 102-107.
[2] AWiseman,*Chemistry in Britain*,**1994,** *30*, 571-573.
[3] BKrajewska and W Zaborska, *Biocybernetics and Biomed Eng.*, ***1989,****9*, 91-102.
[4] H Mustapha and LGYves, *J of Marine Biotechnol.*, **1993,***1*, 51-54**.**
[5] S. A. Wilso, K. Peek andR. M. Daniel. *Biotechnol. Bioeng*., **1994**, *43*, 225-231**.**
[6] MChellapandianandCASastry,*Bioprocess Eng.*,**1992**,*8*, 21-31**.**
[7] MChellapandian and CASastry,*Bioprocess Eng.*, **1994**,*11*, 17-21.
[8] MHasegawa, H Kitano, RNishida and TKobashi,*Biotechnol. Bioeng.*, **1990**,*36*,219-223**.**
[9] SGanapathi;DAButterfield and D. Bhattacharya,*J.Chem.Technol. Biotechnol.*,**1995,***84*, 157-164.
[10] M Pugniere; CS Juan and MA, Coletti-Previero, *BiosReport.*,**1988**,*8*, 263-269.
[11] OHLowry;WJRosenberg;A LFarr;RJ,Randell, *JBiol Chem.*,**1951,***193*, 265-275**.**
[12] VHooda;AGahlaut;HKumar and CSPundir,*SensActuators B Chem.*,**2009**,136, 235-241**.**
[13] O RZaborsky,*In Immobilized Enzymes* CRC Press, Cleveland OH, **1974,**49-50.
[14] RPuvanakrishnan and S M Bose,*BiotechnolBioeng.*, **1980**,*22*, 919-928.
[15] SSreenivasulu;SCDhar and RPuvanakrishnan, *Leather Science.*,**1985**,32, 75-80.