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Polyacrylamide beads: Polymer entrapment increases the catalytic efficiency and thermal stability of protease

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ABSTRACT

Immobilization is a unique method for the improvement of product yield. During solid phase catalysis, the chemistry of the synthetic matrix plays an essential role in the performance of the biocatalyst. In the current study, immobilized protease within polyacrylamide macrosphere beads exhibited 76.0% entrapment yield with a remarkable stability of enzyme (33.0%) after 30 days of storage period at 4 °C. The entrapment of free enzyme within polyacrylamide also increased the optimal reaction temperature by 55 °C and provided a broad range of pH optima. Moreover, a significant enhancement in thermal stability was also detected. Polyacrylamide entrapped protease revealed up to 30.93% activity after incubation period of 30.0 min at 70 °C whereas, the free enzyme was completely inactivated at this temperature. Additionally, entrapped protease displayed an efficient recycling capacity and retained approximately 24.0% of its initial activity after eight successive reaction cycles. After entrapment of protease, the anchoring of substrate to the active site of the free protease exhibited change in K_m and V_{max} values. Therefore, owing to economic feasibility, the polyacrylamide entrapped protease might be a promising candidate for various applications in different industrial sectors.

1. Introduction

Casein is a complex milk protein and is an aggregate of micelle structures consisting of α-, β-, and κ-casein along with colloidal phosphate calcium [1] Casein has been used for non-food applications predominantly, as binding materials for plastics, fibers and for preparation of novel cotton fibers [2,3]. Apart from these applications, casein hydrolysate is also used to generates value added products that has significance commercial applications such as calcium binding peptides [4] and stabilizers to improve emulsion stability of food products [5]. This complex structure of casein can be hydrolyzed by a special mechanism in which proteolytic enzyme cleave the peptide bonds within the polyamine chain and generate different peptides of varying length. Therefore, protease has attractive characteristics that contributes to numerous industrial applications such as tenderization of meat in meat industry, formulation in pharmaceutical industries, dehairing in leather processing industry [6] and bio-hydrolysis of nylon fibers in textile industry [7]. Additionally, specific proteases have been used in food biotechnology industries [8].

Besides vast commercial significance of protease, there are some major applicability difficulties with reference to free protease. The limitation of using free protease includes extra cost of substrate, media components and continuous maintenance of culture for next inoculation batch [9]. Along with these issues, the catalytic efficiency of soluble enzyme is also reduced which drastically impact its commercial applications. For these reasons, novel processing techniques like immobilization of enzymes, was developed. Immobilization is a remarkable approach that not only confines or localize an enzyme molecule within the matrix but it also preserves the catalytic properties of the enzyme [10,11]. In addition, immobilization approach increases the operational stability and makes the enzyme feasible to be reused for continuous industrial process. As a result, a reduction in process cost is noticed. There are various methods of immobilization including adsorption, crosslinking and entrapment of enzyme within polymeric matrix. Nevertheless, entrapment is mostly favored due to its simplicity and cost effective approach. It also creates negligible impact on the conformation of the enzyme in contrast to other immobilization methods that causes structural and functional changes in enzyme molecules [12,13]. Among various synthetic matrices that are currently being used, the most suitable one is polyacrylamide. Polyacrylamide is a water-insoluble polymer with pronounced application in agricultural and industrial sectors [14,15]. The attractive properties of

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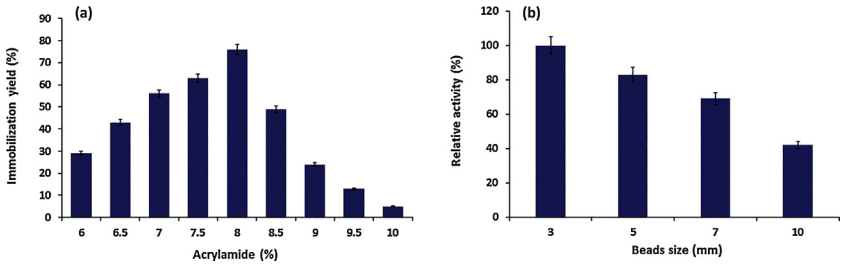

Fig. 1. Optimization of entrapment conditions for maximum yield of immobilized protease. (a) Effect of acrylamide concentration on immobilization yield of protease; (b) Effect of polyacrylamide bead size on entrapment of protease. The values are shown as mean of three independent experiments (n = 3; Standard deviation ± SD: 2%; p-value < 0.005).

polyacrylamide have gained much attention in the field of biomedical applications as well [16]. The unique feature of polyacrylamide comprises of the negative charges that are created during the gelling process of the polymer. The carboxylic group within the polyacrylamide provides a suitable internal macroenvironment for the enzyme [17]. Silva et al. [18] reported that the thermal stability of polyacrylamide might be due to the presence of strong interaction of the hydrogen bonds present within this matrix. This also reduces the mobility of the polymer chain and therefore, increase the thermal strength. It can be suggested that polyacrylamide network improves the stability of an enzyme molecule under hostile industrial conditions.

The current investigation aims to develop a suitable process that provides mechanical strength to enzyme and maintains the catalytic efficiency as well as the thermal stability under extreme industrial conditions. Protease from *Aspergillus niger* KIBGE-IB36 was subjected to entrapment using polyacrylamide as a synthetic matrix.

2. Material and methods

2.1. Production and purification of protease

For production of protease, *Aspergillus niger* KIBGE-IB36 [Genbank: KF905651] was used which was previously isolated from an indigenous source [19] The selected strain was subjected to submerged fermentation at 30 °C for 120 h in medium containing ($g\,L^{-1}$): casein, 2.5; peptone, 20; yeast extract, 0.50; glucose, 2.5; dipotassium hydrogen phosphate, 1.0; magnesium chloride, 0.10 and calcium chloride, 0.10 and keeping the pH at 6.0. After fermentation, the cells were harvested by using filter paper and further mycelium spores were separated by centrifugation at 40,000*g* for 15 min at 4 °C. The clear supernatant containing extracellular enzyme was precipitated by using 40% ammonium sulphate saturation [20]. The precipitates were solubilize in Tris-HCL buffer (pH: 5.0, 50 mM). The solubilized precipitates were desalted and then protease activity and total protein of the samples were calculated and expressed in term of specific activity ($U\,mg^{-1}$). The enzymatic activity of partially purified protease from *Aspergillus niger* KIBGE-IB36 used in the current study was 860.0 $U\,mg^{-1}$ [20].

2.2. Immobilization of protease within polyacrylamide gel

The immobilization of protease within polyacrylamide was performed by polymerization of acrylamide and *N,N′*-methylenebisacrylamide. For this purpose, acrylamide (8.0%), bisacrylamide (0.4%), of partially purified protease (5000 μl) by the addition of ammonium persulphate (20%) and tetramethylethylenediamine (TEMED) (0.015 μl). The prepared solution was mix thoroughly for even distribution of enzyme and was poured immediately into a petri plates (60 × 15 mm) for solidification. The prepared plates were kept at 4 °C for up to 6 h. The solidified gel with entrapped enzyme was cut into equal sizes with the help of metallic borer (macrosphere diameter: 5.0 mm; macrosphere width: 1.0 mm) and washed with Tris-HCl buffer (pH: 5.0, 50 mM) to remove unbound enzyme from the surface of the gel. The gel was also prepared for control studies in which same buffer was used instead of enzyme.

2.3. Enzyme assay

The activity of free and entrapped protease was estimated by Anson method [21] with slight modifications in which the casein (0.2%) was used as a substrate. Tyrosine was used as a standard. For the enzymatic assay of free protease, 0.5 ml of enzyme was mixed with 1.0 ml of casein while, for entrapped protease, 0.5 g of macrosphere beads were mixed with same amount of casein solution prepared in Tris-HCl buffer (pH:5.0, 50 mM) and incubated at 50 °C for 15 min. After 15.0 min, macrosphere were removed carefully and the enzyme-substrate reaction was stopped by adding 5.0 ml trichloroacetic acid solution (10% TCA). The tubes were incubated at 37 °C for 30 min. The undigested casein precipitates were removed by Whatman® filter paper No. 1. After filtration, 2.0 ml of filtrate was withdrawn from both test and control tubes. The reaction mixture was incubated with 5.0 ml of sodium carbonate (500 mM) and 1.0 ml of Folin & Ciocalteu's phenol reagent (1:4) and was further incubated at 37 °C for 15 min in dark. The digested casein released tyrosine which reacts with Folin & Ciocalteu's phenol reagent and generates a measurable color that is visualized under spectrophotometer (660 nm) [22]. One unit of protease can be defined as *the amount of enzyme required to release 1.0 μM of tyrosine per minute under standard assay conditions.*

2.4. Immobilization yield of protease

The immobilization yield of entrapped protease can be calculated according to a following equation:

$$\text{Immobilizationyield}\,(\%) = \frac{\text{Activity of Immobilized Protease}}{\text{Activity of Free Protease}} \times 100$$

2.5. Optimization of immobilization conditions for maximum yield of entrapped protease

The optimum concentration of acrylamide is essential to maintain the structure and porosity of the gel. Therefore, various concentration of acrylamide (6.0%–10%) were investigated to form a suitable matrix for protease entrapment. The beads size of entrapped enzyme greatly effects on the yield of entrapped protease. Therefore, various sizes of beads (3 mm–10 mm) were also prepared.