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Graft copolymerization of acrylamide on chitosan-*co*-chitin and its application for immobilization of *Aspergillus* sp. RL2Ct cutinase

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ABSTRACT

The synthesis of chitosan (Chs) and chitin (Chi) copolymer and grafting of acrylamide (AAm) onto the synthesized copolymer have been carried out by chemical methods. The grafted copolymer was characterized by FTIR, SEM and XRD. The extracellular cutinase of *Aspergillus* sp. RL2Ct (E.C. 3.1.1.3) was purified to 4.46 fold with 16.1% yield using acetone precipitation and DEAE sepharose ion exchange chromatography. It was immobilized by adsorption on the grafted copolymer. The immobilized enzyme was found to be more stable then the free enzyme and has a good binding efficiency (78.8%) with the grafted copolymer. The kinetic parameters K_M and V_{max} for free and immobilized cutinase were found to be 0.55 mM and 1410 µmol min^{-1} mg^{-1} protein, 2.99 mM and 996 µmol min^{-1} mg^{-1} protein, respectively. The immobilized cutinase was recycled 64 times without considerable loss of activity. The matrix (Chs-*co*-Chi-g-poly(AAm)) prepared and cutinase immobilized on the matrix have potential applications in enzyme immobilization and organic synthesis respectively.

1. Introduction

Immobilization of cells and enzymes is a well established technique in enzyme technology to harness maximum catalytic potential of a biocatalyst. A large number of methods and matrices are available for immobilization of enzymes but their selection depend on which one is simple to follow and provides more stability and reusability to enzymes upon immobilization. Cutinases are enzymes that catalyze the hydrolysis of ester linkages of cutin. Cutin is a major structural component of plant cuticle that protects the aerial parts of plants from dessication and microbial infestation [1]. However, some bacterial and fungal pathogens secrete cutinase to break cuticular barrier to infest the host plants [2,3]. Cutinases are finding important applications in chemistry to hydrolyse or synthesize ester bond. They are active in both aqueous medium as well as in water-lipid interface and can act on both soluble and emulsified triglycerides [4].

The use of free enzymes in reactions is their one time use that fails to harness maximum catalytic potential of the enzymes. Numerous methods have been developed to immobilize the enzymes which enhance stability, catalytic activity and selectivity of the enzymes [5,6]. The methods used for immobilization of cutinases comprise adsorption on polymethyl methacrylate (PMMA) latex particles [7], adsorption on NaY zeolite and polyamide-Accurel PA6 [8], cross-linked enzyme aggregates (CLEA) on Lewatit beads [9], encapsulation in reversed micelles of AOT (bis(2-ethylhexyl) sodium sulfosuccinate) for catalyzing various reactions [10]. Chitosan and chitin have been employed as potential biomaterial for immobilization of enzymes [11,12]. They are non-toxic, biodegradable, non-carcinogenic and have biocompatible properties [13,14]. Several studies have been carried out on chemical modification of chitosan [15–17]. Chellapandian and Krishnan [11] has covalently attached urease onto chitosan-poly (GMA) by the introduction of epoxy groups to the support. It has resulted in improved storage stability and pH of urease. Also, lipase from *Candida rugosa* was immobilized on chemically modified chitosan poly(acrylonitrile-*co*-maleic acid) membrane surface and properties of the immobilized enzyme was studied [18]. Most of the hitherto immobilized methods have significant loss of activity upon immobilization and limited recycling (up to eleven cycles) due to nature of the matrix and the method used for immobilization. In the present investigation an attempt has been made to overcome limitations and lacunae of previous research. The cutinase was stabilized in an active conformation by immobilization on grafted copolymer of chitosan and chitin, which increased its thermal stability over the course of the reaction and improved its efficiency (recycling up to 64 cycles). The use of this graft

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copolymer as matrix for immobilization of cutinase of a fungus *Aspergillus* sp. RL2Ct is being reported for the first time.

2. Materials and methods

2.1. Microorganism

Aspergillus sp RL2Ct earlier isolated [19] as cutinolytic organism in our laboratory was used as source cutinase in the present studies.

2.1.1. Production of Aspergillus sp. RL2Ct cutinase

In 50 ml of seed medium (potato dextrose broth), 2.11×10^7 spores (approx.) of *Aspergillus* sp. RL2Ct were seeded and incubated in gyratory incubator shaker at 30 °C for 24 h at 100 rpm. 1 ml of seed medium was added to 50 ml of production medium (minimal salt medium containing g/l of KH_2PO_4 0.5 g, K_2HPO_4 1.0 g, NaCl 0.2 g, $FeSO_4{\cdot}7H_2O$ 0.01 g and cutin 1 g, pH 7.0), incubated at 30 °C for 48 h. The enzyme was obtained by centrifugation (at 10,000*g* for 20 min at 4 °C) of culture broth and the supernatant was designated as crude enzyme and assayed for enzyme activity.

2.1.2. Enzyme assay and protein estimation

The cutinase activity was determined as described by Winkler and Stuckmann [20] with minor modification. The stock solution of the substrate (20 mM of *p*-nitrophenyl butyrate) was prepared in ethanol. Reaction mixture contained 5 mM of substrate, 20 µl of enzyme preparation and 2.8 ml of 80 mM potassium phosphate buffer (pH 7.0). The reaction was carried out at 30 °C for 10 min. The accumulation of *p*-nitrophenol was measured spectrophotometrically at 405 nm and concentrations were calculated by comparison to *p*-nitrophenol standard. Protein concentration was determined using the Bradford method [21]. One unit of cutinase activity was defined as the amount of enzyme that converts 1 µmol of substrate into product in one minute. The immobilized enzyme activity was estimated with 25 mg matrix containing immobilized cutinase (0.007 mg protein) in 2.5 ml of 80 mM potassium phosphate buffer (pH 7.0) containing 5 mM of *p*-nitrophenyl butyrate. The immobilized enzyme was separated by centrifugation at (5000*g* for 5 min at 4 °C) and *p*-nitrophenol formed was estimated in the supernatant by recording absorbance at 405 nm.

2.1.3. Purification of Aspergillus sp. RL2Ct cutinase

The proteins were precipitated by adding chilled acetone to 20% (v/v) in the crude enzyme preparation and stirred for 1 h on ice. The precipitates were separated by centrifugation (at 15,000*g* for 25 min at 4 °C) and discarded. To the supernatant chilled acetone was added at 60% (v/v) and stirred on ice for 1 h. It was centrifuged (at 15,000*g* for 25 min at 4 °C) and the sedimented proteins were suspended in 80 mM potassium phosphate buffer, pH 7.0. Those were applied to DEAE-anion exchange column. The column was washed with 80 mM potassium phosphate buffer, pH 7.0 until there was no further elution of protein. The enzyme was eluted with a linear gradient of NaCl (from 0 to 0.5 M) in the same buffer over a period of 100 min and fractions of 2.0 ml were collected and these were analysed for protein concentration and cutinase activity. The enzyme preparations of various stages of purification were subjected to native- and sodium dodecyl sulfate polyacrylamide gel electrophoresis (Native and SDS-PAGE) in 12% polyacrylamide gels using the procedure of Laemmli [22]. Gels were silver stained following method described by Chevallet et al. [23]. The relative molecular mass of the enzyme was determined by comparison with the relative mobilities of the standard proteins (ranging from 14.3 to 97.4 kDa) from Banglore Genei Pvt. Ltd., Banglore (India) and Sigma Aldrich Co Ltd (an affiliate of Merck KGaA, Darmstadt, Germany).

2.2. Synthesis of Chs-co-Chi polymer and graft copolymerization with acrylamide

Chs-*co*-Chi copolymer (pristine copolymer) was prepared following previously described procedure [24]. For grafting of pristine copolymer with acrylamide (AAm), pristine copolymer (1.0 g), APS (1.09×10^{-1} mol/l) and acrylamide [AAm, (3.52×10^{-1} mol/l)] were added to water (4 ml). The reaction mixture was placed in a water bath at 80 °C for 5 h and the product formed was washed thoroughly with water to remove any homopolymer, poly(AAm) formed during the reaction. The grafted copolymer Chs-*co*-Chi-g-poly(AAm) (grafted copolymer) thus obtained was dried and weighed to a constant weight. Graft copolymerization with AAm has been carried out under various reaction conditions acrylamide concentrations, ammonium persulphate (APS) concentrations, varied reaction temperature) that seem to affect graft copolymerization and the reaction conditions were optimized have been established on the basis of the swelling behaviour of the product in water as a function of time at room temperature.

2.2.1. Swelling studies of pristine and grafted copolymer in water

One gram each of pristine and grafted copolymers were separately immersed in beakers containing 30 ml of distilled water and kept at room temperature. The copolymers as removed from water after every half an hour and weighed. Increase in weight due to water sorption was measured till it became constant and percentage of swelling was calculated as follows:

$$\text{Percentage of swelling} = \frac{W_s - W_d}{W_d} \times 100$$

where W_s and W_d are the weights of swollen and original dried samples respectively.

2.2.2. Characterization

The pristine and grafted copolymer were characterized by Fourier transform infrared (FTIR), scanning electron microscopy (SEM) and X-ray diffraction (XRD).

2.3. Immobilization of purified cutinase on grafted copolymer

Fifty milligram of grafted copolymer *i.e.*, matrix was incubated in 0.5 ml of 80 mM potassium phosphate buffer pH 7.0 for 12 h in a glass vial and to it purified cutinase (protein content 0.014 mg/ml and specific activity 1856 U/mg protein) was added followed by incubation for 1 h at 30 °C. The matrix was then given two washings with potassium phosphate buffer (80 mM, pH 7.0) to remove unbound enzyme. The protein immobilized on matrix was assessed by subtracting unbound protein in the supernatant from the total protein taken for immobilization. The cutinase immobilized on grafted copolymer hereafter was termed as immobilized cutinase.

2.3.1. Binding efficiency

The binding efficiency was calculated as the ratio of activity exhibited by the bound enzyme to the total activity used for immobilization. The immobilization yield was calculated by the following equation:

$$\text{Immobilization yield}\,(\%) = \frac{\text{Immobilized enzyme activity}}{\text{Free enzyme activity}} \times 100$$

2.4. Enzymological characterization of purified and immobilized cutinase

Various parameters of purified and immobilized cutinase were studied to determine ideal reaction conditions for the assay of