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Usage of immobilized papain for enzymatic hydrolysis of proteins

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ABSTRACT

In this presented work, magnetic poly(HEMA-GMA) nanoparticles were synthesized by using emulsion polymerization technique. These magnetic nanoparticles were then functionalized with the affinity dye ligand, Cibacron Blue F3GA. The dye attached magnetic nanoparticles were characterized by using FTIR, ESR, SEM, AFM and EDX analysis. Synthesized magnetic nanoparticles demonstrated magnetic behavior and their size was about 200 nm. Dye loading on the nanoparticle was calculated as 173.96 μmol/g polymer by using sulfur stoichiometry. Effects of medium pH, initial papain concentration and ionic strength on the papain adsorption onto dye attached magnetic nanoparticles were also investigated and maximum papain adsorption onto the nanoparticles was found to be 764.0 mg/g polymer in pH 7.0 HEPES buffer. Kinetic constants of the free and immobilized papain were compared and their pH, thermal, operational and storage stabilities were investigated. It was demonstrated with these studies that, immobilized form of papain was much more stable than that of free form. At the end of the study, catalytic efficiency of immobilized papain was investigated toward the different type of proteins such as casein, BSA, IgG and cytochrome *c* and it was demonstrated that, highest catalytic efficiency was achieved with IgG.

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1. Introduction

Papain is an enzyme belonging to the group of hydrolyses possessing a polypeptide chain including 212 amino acid residues, with a molecular weight of 23,406; isoelectric point at 8.75; and optimal catalytic activity at pH 5–8. The molecule comprises a single polypeptide chain including three disulfide bonds and one sulfohydride group. The active center includes cysteine and histidine residues, which are activated by mercaptans (and other reducing agents) and inhibited by oxidizers and heavy metal ions. The enzyme catalyze the hydrolysis of proteins, peptides, amides, esters, and thioesters [1]. Papain presents anti-inflammatory, antibacterial, and antioxidant properties and can be used in the treatment of large skin lesions [2]. In food industry, papain is used to tenderize meat and related derivatives, to produce protein hydrolysate, to clarify juice and bear in brewing industry, for cheese production in dairy industry, in baking industry, and for the extraction of flavor and color compounds from plants [3]. Papain has been used in cell isolation studies and in the separation of biomolecules in large-scale industry processes. Besides, papain is widely used in the leather, cosmetic, textiles, detergents, food, and pharmaceutical industries [4].

Magnetic nanoparticles are well-established nanomaterials that offer controlled size, ability to be manipulated by an external magnetic field, and enhancement of contrast in magnetic resonance imaging. As a result, these nanoparticles could have many applications including bacterial detection, protein purification, enzyme immobilization, contamination decorporation, drug delivery, hyperthermia, etc. [5]. Recently, the use of magnetic nanoparticles in enzyme immobilization has become very popular [6–9]. Using magnetic nanoparticles as the supports for immobilized enzymes has some advantages: (1) higher specific surface area obtained for the binding of a larger amount of enzymes, (2) lower mass transfer resistance and less fouling and (3) the selective separation of immobilized enzymes from a reaction mixture by the application of a magnetic field [5,10].

Over the past decades, dye-ligands have gained wide interest for offering advantage over biological ligands (antibodies, enzymes, etc.) in terms of economy, ease of immobilization, stability and high adsorbent capacity [11–16]. Among all of the triazine dyes, Cibacron Blue F3GA is one of the most widely preferred ligands in the dye-affinity separation of proteins [17]. Cibacron Blue F3GA is a monochlorotriazine dye which contains three acidic sulfonate groups and four basic primary and secondary amino groups [18]. By the nucleophilic reaction between chloride of the triazine ring and reactive group of the supports including hydroxyl (–OH) or amino

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(NH_2), Cibacron Blue F3GA can be immobilized onto the supports for protein affinity adsorption [11]. The interaction between the dye ligand and proteins can be complex combination of electrostatic, hydrophobic and hydrogen bonding [19–23].

This article reports on the adsorption of papain by using dye ligand affinity technique with magnetic nanoparticles. The poly(HEMA-GMA) magnetic nanoparticles were prepared by surfactant-free emulsion polymerization of 2-hydroxylethyl methacrylate (HEMA), glycidyl methacrylate (GMA) and Fe_3O_4. Cibacron Blue F3GA was then covalently attached to these magnetic nanoparticles. Synthesized magnetic nanoparticles were then characterized by using Fourier transform infrared spectroscopy (FTIR), electron spin resonance (ESR), scanning electron microscope (SEM), atomic force microscope (AFM) and energy dispersive X-ray (EDX) analysis. Papain adsorption on the dye attached magnetic nanoparticles was performed with different papain concentrations, different buffers and pHs, and ionic strengths. Desorption of papain and reusability of the dye attached magnetic nanoparticles was also investigated. Kinetic constants of soluble and immobilized papain were determined. Additionally the catalytic efficiency of immobilized papain over some different proteins was examined.

2. Experimental

2.1. Materials

Papain (from *Carica papaya*, EC 3.4.22.2), casein, trichloroacetic acid, 2-hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA), magnetic nanopowder (Fe_3O_4; average diameter, 20–50 nm), *N,N,N′,N′*-tetramethylene diamine (TEMED), ethylene glycol dimethacrylate (EGDMA), ammonium persulfate (APS) and Cibacron Blue F3GA were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and used without further purification. All solutions were prepared with deionized ultrapure Milipore Simplicity® (18.2 MΩ cm) water.

2.2. Preparation of magnetic nanoparticles

Surfactant free emulsion polymerization technique was used for the synthesis of magnetic poly(HEMA-GMA) nanoparticles. Firstly, HEMA and GMA ratios were studied in order to reach maximum polymerization yield. The optimized conditions were presented as follows. Initially, 0.5 g of PVA was dissolved in 45 mL of deionized water and transferred to a polymerization reactor. Then, monomers (0.6 mL of HEMA and 50.0 μL of GMA) were added to the PVA solution and slowly shaken for 30 s. After that, 0.2 g of Fe_3O_4 and 0.0198 g of potassium peroxodisulfate were added to the polymerization mixture and this solution was incubated for 4 h at 70 °C. At the end of this polymerization process, reaction mixture was cooled down to room temperature and washed with ethanol and water several times in order to remove the unreacted monomers.

2.3. Cibacron Blue F3GA attachment to magnetic nanoparticles

Magnetic nanoparticles (0.1 g/100 mL) were treated with 100 mL of Cibacron Blue F3GA solution (5.0 mg/mL) containing NaOH (4 g) at 80 °C for 4 h. After incubation, the Cibacron Blue F3GA attached magnetic nanoparticles were washed with distilled water and methanol several times until all the physically attached Cibacron Blue F3GA molecules were removed. The modified magnetic nanoparticles were than stored at 4 °C with 0.02% sodium azide to prevent microbial contamination.

2.4. Characterization of dye attached magnetic nanoparticles

The FTIR spectrum of magnetic poly(HEMA-GMA) and Cibacron Blue F3GA attached magnetic poly(HEMA-GMA) nanoparticles were analyzed by using a FTIR spectrophotometer (Varian FTS 7000, USA). For this, dried magnetic nanoparticles (0.1 g) were mixed with 0.1 g of KBr which is inert, forms excellent pellet and does not show any absorption spectrum in infrared region. Then, the mixture was pressed into a pellet form and FTIR spectra of samples were recorded. The presence of magnetite particles in the synthesized nanoparticles was showed with an ESR spectrophotometer (EL9, Varian, Palo Alto, CA). The size and the morphology of the magnetic nanoparticles were investigated by SEM analysis. For this, nanoparticles were covered with a thin layer of gold and then mounted in a SEM device (Philips, XL-30S FEG, the Netherlands). Surface morphology of the dye attached magnetic poly(HEMA-GMA) nanoparticle was investigated by AFM (Digital-Instruments, MMSPM, Nanoscope IV, USA). Cibacron Blue F3GA loading to the magnetic nanoparticles was determined by EDX analysis (LEO, EVO 40, Carl Zeiss NTS, USA) and the amount of attached Cibacron Blue F3GA was calculated from these data considering the sulfur stoichiometry.

2.5. Papain adsorption–desorption studies from aqueous solutions

Adsorption of papain on the dye attached magnetic nanoparticles was performed in a batch experimental set-up. Adsorption experiments were conducted for 120 min at 25 °C with continuous stirring. The effects of pH and buffer type, initial papain concentration and ionic strength on adsorption of papain onto dye attached nanoparticles were investigated. The papain concentration was measured at 280 nm by using a double beam UV/vis spectrophotometer (Model 1601; Shimadzu, Tokyo, Japan). The amount of adsorbed papain on the dye attached magnetic nanoparticles was determined by measuring the initial and final concentration of protein. Additionally papain desorption was investigated with 1.0 M NaCl in pH 4.0 acetate buffer. In order to show the reusability of the dye attached magnetic nanoparticles, the adsorption–desorption cycle of papain repeated for ten times by using the same magnetic nanoparticles.

2.6. Activity of papain

Papain activity was measured at room temperature in 50 mM phosphate buffer (pH 7.5) containing 38 mM EDTA and 34 mM cysteine chloride, using casein as substrate, by the modified method of Kemphavi et al. [24]. For this, 400 μL of 1% (w/v) aqueous solution of casein was added to 50 μL of enzyme solution diluted in 350 μL of buffer, and the reaction mixture was incubated at room temperature for 10 min. After the incubation period, casein proteolysis was stopped by addition of 800 μL of 10% trichloroacetic acid solution. The mixture was incubated at room temperature for 30 min, centrifuged at 12,000 × *g* for 10 min (Universal 32R, Hettich, Tuttlingen, Germany) and the absorbance of the supernatant measured at 280 nm. One unit of enzyme was taken as the amount of enzyme that hydrolyze casein to produce equivalent absorbance to 1 μmol of tyrosine/min with tyrosine as a standard. Total protein concentrations were measured by the Bradford method.

These activities were performed in a pH range of 4.0–8.0 and in a temperature range of 4–85 °C and in a casein concentration range of 0.1–10 mg/mL. The thermal stability of free and adsorbed papain were investigated by measuring the residual activity at 75 °C in HEPES buffer (0.1 M, pH 7.0). In order to test the operational stability of immobilized papain, papain adsorption studies were carried out for 10 adsorption–desorption cycles and enzyme activities were

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