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Designing and surface modification of zinc oxide nanoparticles for biomedical applications

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ABSTRACT

The present study aimed to work out a simple and high yield procedure for the immobilization of β galactosidase on bioaffinity support, concanavalin A (Con A) layered zinc oxide nanoparticles (ZnO-NP). Thermogravimetric analysis of bioaffinity support revealed 4% loss in weight at 600 °C whereas its thermal decomposition was observed at 530 °C by differential thermal analysis. No significant change was noticed in the band intensity of pUC19 plasmid after its treatment with Con A layered ZnO-NP. Comet assay further exhibited negligible change in tail length of comet after treating the lymphocytes by bioaffinity matrix. The bioaffinity matrix binds 89% of the enzyme activity. Atomic force microscopy analysis showed that the prepared matrix has an advantageous microenvironment and large surface area for binding significant amount of the enzyme. The functional groups present in native and parent compound were monitored by Fourier transform-infrared spectroscopy. Michaelis constant, K_m was 2.38 and 5.88 mM for free and immobilized β galactosidase, respectively. V_{max} for the soluble and immobilized enzyme was 0.520 mM/min and 0.460 mM/min, respectively. Concanavalin A layered ZnO-NP bound β galactosidase exhibited a shift in the temperature-optima and retained nearly 86% activity even after its 6th repeated use.

1. Introduction

Biotechnology is currently considered as a useful alternative to conventional process technology in industrial and analytical fields. Over the last few decades, intense research in the area of enzyme technology has provided many approaches that facilitated their practical applications (Ravindra et al., 2007). Among them, the newer technological developments in the field of immobilized biocatalyst can offer the possibility of a wider and more economical exploitation of biocatalyst in industry, waste treatment, medicine and in the development of bioprocess monitoring devices like biosensors (Logoglu et al., 2006; Zuo et al., 2009). The selected procedure of immobilizing enzyme should be able to stabilize the macromolecules to allow easier diffusion of substrates and products (Mahmoud and Helmy, 2009; Husain, 2010). However, only few immobilization methods can control the spatial distribution of catalyst. Stabilization of enzymes against several physical and chemical denaturants has been accomplished using a multitude of immobilization strategies including covalent coupling, adsorption, microencapsulation, polymer entrapment and chemical aggregation (Haider and Husain, 2007; Iyer and Ananthanarayan, 2008; Mateo et al., 2007; Di Marco et al., 2010).

Recently, zinc oxide nanoparticles have attracted the attention of the biotechnologist as they can be surface functionalized with a wide range of metal and semiconductor core materials thereby imparting useful properties with potentially wide-ranging therapeutic applications (George et al., 2010; Yan et al., 2011).

Several techniques that have been employed previously for the immobilization of β galactosidase include entrapment (Betancor et al., 2008), crosslinking (Zhang et al., 2010), adsorption (Gurdas et al., 2010) and magnetic Fe_3O_4-chitosan nanoparticles (Pan et al., 2009). However, bioaffinity based procedures of enzyme immobilization are emerging as powerful tools due to reversibility, lack of chemical modification and the usually accompanying stability enhancement (Ansari and Husain, 2010). Moreover, this procedure gives oriented immobilization to enzymes that facilitated good expression of activity and reusability (Akhtar et al., 2005; Sardar and Gupta, 2005). This method of enzyme immobilization

Abbreviations: AFM, atomic force microscopy; Con A, concanavalin A; FT-IR, fourier transform-infrared spectroscopy; IβG, Con A layered ZnO-NP adsorbed β galactosidase; ONPG, *o*-nitrophenyl β-D-galactopyranoside; SβG, soluble β galactosidase.

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retained very high enzyme activity as no modification/distortion occurs at the active site of the enzyme. Since the active site is less hindered by nano matrix, the steric accessibility allows more free access for incoming substrate and outgoing products. In addition, affinity binding procedure offers very mild, controlled adsorption of biocatalysts onto the supports and is likely to be of continuing value for the immobilization of sensitive biocatalyst (Ellis et al., 2008; Haider and Husain, 2009; Husain, 2010).

In the present work, an attempt has been made to prepare a novel and efficient bioaffinity support, concanavalin A layered zinc oxide nanoparticles (Con A layered ZnO-NP) to bring forth its exploitation in several biomedical applications. ZnO-NPs were characterized by transmission electron microscope (TEM). Thermal behavior of bioaffinity support was studied by thermogravimetric analysis (TGA) and differential thermal analysis (DTA). Genotoxicity of the bioaffinity support was checked by pUC19 plasmid nicking assay and Comet assay. β galactosidase from *Aspergillus oryzae* was successfully immobilized on this support. Immobilized β galactosidase was characterized by atomic force microscopy (AFM) and fourier transform-infrared spectroscopy (FT-IR). The effect of various denaturing agents on the activity of soluble and immobilized β galactosidase was monitored. K_m and V_{max} for soluble and immobilized enzyme were examined. Reusability of immobilized β galactosidase has also been evaluated.

2. Materials and methods

2.1. Materials

A. oryzae β galactosidase (Activity: 1200 U/mg), RPMI-1640 medium and Histopaque were obtained from Sigma Chem. Co. (St. Louis, MO, USA). 2-Nitrophenyl β-D galactopyranoside (ONPG) and Concanavalin A from jack bean (*Canavalia ensiformis*) were purchased from SRL Chemicals (Mumbai, India). pUC19 plasmid was procured from Genei, Bangalore India. All reagents were prepared in double distilled water with chemicals of analytical grade.

2.2. Synthesis and characterization of ZnO nanoparticles

Zinc oxide nanoparticles (ZnO-NP) were synthesized by sol–gel method. The sol was prepared by using zinc acetate dihydrate (99.5%), ethylene glycol, 2-propyl alcohol and glycerol. Zn $(CH_3COO)_2$. $2H_2O$ (100 g) was mixed with 25 mL ethylene glycol at 150 °C for 15 min to obtain a uniform transparent solution. The solution was solidified to a transparent brittle solid on cooling down to room temperature. This solid was then dissolved in 200 mL of 2-propyl alcohol. The resulting solution was highly hydrophobic and converted into gel upon addition of few drops of water. Triethylamine was added to facilitate the hydrolysis of zinc acetate. The solution gets transformed into light brown powder upon incubation at 200 °C for 6 h. The resulting powder was initially heated in a programmable furnace at 450 °C for 8 h and then at 700 °C for 5 h to remove all organic impurities yielding white pure ZnO powder. The characterization of the obtained ZnO-NP was performed by X-ray diffraction according to the procedure described by Azam et al. (2010). The morphological characterization of ZnO-NPs was carried out by a transmission electron microscope (TEM) study in the Department of Anatomy at the All India Institute of Medical Sciences (AIIMS, New Delhi).

2.3. Preparation of ZnO-NP bioaffinity support

ZnO-NP (100 mg) was added to 1.0 mL of 0.1 M sodium phosphate buffer, pH 6.2 containing Con A (60 mg/mL). The reaction was allowed to proceed overnight at 30 °C with gentle stirring. Con A layered ZnO-NP was collected by centrifugation at 3000 *g* for 20 min and was washed 2–3 times with 0.1 M sodium phosphate buffer, pH 6.2.

2.4. Thermogravimetric analysis and differential thermal analysis of Con A layered ZnO-NP

Thermogravimetric analysis was performed with a Mettler-3000 thermal analyzer using 2 mg sample with heating rate of 10 °C/min in N_2 atmosphere. Differential thermal analysis was also carried out in similar heating range by using TA Instruments Q200 Differential Scanning Calorimeter (DSC).

2.5. Genotoxicity test of the bioaffinity support by plasmid nicking assay

Plasmid nicking assay was performed by pUC19 DNA with slight modifications according to the procedure described by Kitts et al. (2000). Reaction mixture (30 μL) containing 10 mM Tris–HCl buffer (pH 7.5), pUC19 plasmid DNA (0.5 μg) and Con A layered ZnO-NPs (10 μL) was incubated for 2 h at 37 °C. Ten micro liter of the solution containing 40 mM EDTA, 0.05% bromophenol blue (tracking dye) and 50% (v/v) glycerol was added after incubation and the solution was then subjected to electrophoresis in submarine 1% agarose gel. Ethidium bromide stained gel was then viewed and photographed on a UV-transilluminator.

2.6. Comet assay (single cell gel electrophoresis) of the bioaffinity support

Comet assay was performed according to the procedure described by Klaude et al. (1996). Fully frosted microscopic slides pre-coated with 1.0% normal melting agarose (PBS buffer which doesn't contain Ca^{2+} and Mg^{2+} ions) were used at 50 °C. Around 10,000 cells were mixed with 80 μL of 1.0% low melting point agarose to form a cell suspension and pipetted over the first layer and covered immediately by a cover slip. The slides were placed on a flat tray and kept on ice for 10 min to solidify the agarose. The cover slips were removed and a third layer of 0.5% low melting point agarose (80 μL) was kept. Cover slips were placed over it which was then allowed to solidify on ice for 5 min. The cover slips were removed and the slides were immersed in cold lysis buffer containing 2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10. Triton X-100 (1.0%) was added before use for a minimum of 1 h at 4 °C. After lysis, DNA was allowed to unwind for 30 min in alkaline electrophoresis solution consisting of 300 mM NaOH and 1.0 mM EDTA, pH > 13. Electrophoresis was performed at 4 °C in field strength of 0.7 V/cm and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with 75 ml ethidium bromide (20 mg/mL) and covered with a cover slip. The slides were then placed in a humidified chamber to prevent drying of the gel and analyzed the same day. Slides were scored using an image analysis system (Comet 5.5; Kinetic Imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope (Olympus Optical Co., Tokyo, Japan) and a COHU 4910-integrated CC camera (equipped with 510–560 nm excitation and 590 nm barrier filters) (COHU, San Diego, CA, USA). Comets were scored at 100× magnification. Images from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess lymphocytes DNA damage was tail length (migration of DNA from the nucleus) and was automatically generated by the Comet 5.5 image analysis system.

2.7. Biospecific adsorption of β galactosidase on Con A layered ZnO-NP

β Galactosidase (35,550 U) was mixed with Con A layered ZnO-NP and the mixture was stirred overnight in sodium acetate buffer, pH 4.5 at 30 °C. The bioaffinity adsorbed β galactosidase was collected by centrifugation at 3000 *g* for 20 min. Con A layered ZnO-NP bound β galactosidase was washed thrice with 0.1 M sodium acetate buffer, pH 4.5 and finally suspended in assay buffer and stored at 4 °C for further use (Ansari and Husain, 2010).

2.8. Atomic force microscopy

Tapping mode AFM experiments of Con A, ZnO-NP, Con A-ZnO-NP and Con A-ZnO-NP adsorbed β galactosidase were performed using commercial etched silicon tips as AFM probes with typical resonance frequency of ca. 300 Hz (RTESP, Veeco, Japan).

2.9. FT-IR spectra

FT-IR spectra for Con A, ZnO-NP, Con A-ZnO-NP and Con A-ZnO-NP adsorbed β galactosidase were monitored with INTERSPEC 2020 model FT-IR instrument, USA. The calibration was done by polystyrene film. The samples were injected by Hamiet 100 μL syringe in ATR box. The syringe was first washed with acetone followed by distilled water. FT-IR analysis was done to monitor functional groups of the compounds.

2.10. Effect of pH

The activity of soluble and immobilized β galactosidase (2.0 U) was assayed in the buffers of different pH (3.0–9.0). The buffers used were glycine–HCl (pH 3.0), sodium acetate (pH 4.0, 5.0), sodium phosphate (6.0, 7.0) and Tris–HCl (pH 8.0, 9.0). The molarity of each buffer was 0.1 M. The activity at pH 4.5 was taken as control (100%) for the calculation of remaining percent activity.

2.11. Effect of temperature

The activity of soluble and immobilized β galactosidase (2.0 U) was measured in sodium acetate buffer (0.1 M, pH 4.5) at various temperatures (30–70 °C). The enzyme activity at 50 °C and 60 °C were taken as control (100%) for the calculation of remaining percent activity for soluble and immobilized enzyme, respectively.