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Horseradish peroxidase and chitosan: Activation, immobilization and comparative results

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ABSTRACT

Recently, horseradish peroxidase (HRP) was immobilized on activated wool and we envisioned that the use of chitosan would be interesting instead of wool owing to its simple chemical structure, abundant nature and biodegradability. In this work, HRP was immobilized on chitosan crosslinked with cyanuric chloride. FT-IR spectroscopy and scanning electron microscopy were used to characterize immobilized HRP. The number of ten reuses of immobilized HRP has been detected. The pH was shifted from 5.5 for soluble HRP to 5.0 for immobilized enzyme. The soluble HRP had an optimum temperature of 30 °C, which was shifted to 35 °C for immobilized enzyme. The soluble HRP and immobilized HRP were thermal stable up to 35 and 45 °C, respectively. The apparent kinetic constant values (K_m) of soluble HRP and chitosan–HRP were 35 mM and 40 mM for guaiacol and 2.73 mM and 5.7 mM for H_2O_2, respectively. Immobilization of HRP partially protected them from metal ions compared to soluble enzyme. The chitosan–HRP was remarkably more stable against urea, Triton X-100 and organic solvents. Chitosan–HRP exhibited large number of reuses and more resistance to harmful compounds compared with wool–HRP. On the basis of results obtained in the present study, chitosan–HRP could be employed in bioremediation application.

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1. Introduction

Biocatalysts are increasingly being employed because of their high selectivity and potential as a greener alternative to chemical catalysts. Therefore, interest for enzymatic processes is over growing. The soluble enzymes makes their uses for large-scale relatively costly and their recoveries are difficult [1]. Numerous efforts have been devoted to the development of insoluble immobilized enzymes for various applications. There are several benefits of using immobilized enzymes as the reusability of enzyme with the reducing of the production cost, stable and reusable analytical devices for analytical and medical applications, purification of proteins and enzymes and as effective microdevices for controlled release of protein drugs [2–5].

Natural polymers, alginate, agarose, chitin, and chitosan are used as carrier materials in immobilization of enzymes. Chitosan and chitin possesses distinct chemical and biological properties. In its linear polyglucosamine chains of high molecular weight, chitosan has reactive amino and hydroxyl groups, which are potentially capable of being crosslinked with different substances [6,7]. Along with unique biological properties include biocompatibility, biodegradability to harmless products, nontoxicity, remarkable affinity to proteins. Crucially, bio- and biodegradable polymers chitosan materials are eco-friendly, safe for humans and the natural environment [8,9]. Chitosan has been used as a support for immobilization of various enzymes [10–12].

Horseradish peroxidase (HRP) is one of the most extensively studied enzymes because of its growing number of applications. HRP has been used for removal of phenols from wastewater [13], organic syntheses [14], and applications for analytical purposes [15]. Applications of HRP have been developed because of its high activity, simple detection of products, relative stability, ease of immobilization and the stability of the immobilized preparations [15]. HRP has been immobilized with chitosan using distinct methods: crosslinking with gels [16], β-cyclodextrin [17], metallic nanoparticles [18] and polyethyl acrylate [19].

Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) has been used as a coupling reagent to cross link enzymes to supports [20,21].

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The chlorine atoms in the molecule react with nucleophillic groups (thiol, amino, imino and hydroxyl functions) to form stable linkages. The first chlorine reacts readily at low temperatures and pH, the second at 25–40 °C in alkaline pH and the third will react mainly with thiol groups at high temperature and pH [22]. The variations in the reactivity of the 1st and 2nd chlorine atoms and near inactivity of the 3rd atom make cyanuric chloride an efficient heterobifunctional coupling reagent for linking hydroxyl, amino and thiol groups.

Recently, we immobilized HRP on wool activated with cyanuric chloride [23]. In continuation of our interest for enzyme immobilization it was necessary to select chitosan instead of wool. It is known that this typology of solids is not easy to recover. However, chitosan has reactive amino and hydroxyl groups, which are potentially capable of being crosslinked with different substances. In this study, an effort has been made to immobilize HRP on chitosan crosslinked with cyanuric chloride. The number of enzyme reuse and the effect of some compounds on chitosan–HRP were compared with wool–HRP [23].

2. Materials and methods

2.1. Materials

Chitosan (with specification: 85% deacetylated, medium molecular weight and medium viscosity), cyanuric chloride and all other reagent grade chemicals were purchased from Sigma Aldrich and were used as received.

2.2. Horseradish peroxidase

Horseradish peroxidase (HRP) was previously purified from horseradish cv. Balady with specific activity 26,805 units/mg protein [24].

2.3. Peroxidase assay

Peroxidase activity was assayed according to [25]. The reaction mixture contains in 1 ml: 8 mM H_2O_2, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5 and soluble HRP or immobilized HRP. The change of absorbance at 470 nm due to guaiacol oxidation was followed at 30 s intervals. One unit of peroxidase activity is defined as the amount of enzyme which increases the O.D. 1.0 per min under standard assay conditions. The relative activity% was

$$\text{Relative activity}\,(\%) = \frac{\text{activity}}{\text{maximum activity}} \times 100$$

2.4. Activation of chitosan with cyanuric chloride

An ice-cooled solution of cyanuric chloride (2–8%, w/v) in 100 ml of acetone–water mixture (1:1) was prepared. Chitosan (2 g) was added into this solution and left with shaking for 30 min at 0 °C. Sodium bicarbonate solution (10%, w/v; 100 ml) was drop wisely added to the above reaction mixture while shaking within 30 min at 0 °C. The reaction mixture was further kept under shaking and at 0 °C overnight. The chitosan sample was removed from the shaker bath and washed several times with acetone, water and acetone, and dried in ventilated hood and kept in stored in a plastic bag under refrigeration until enzyme immobilization [22].

2.5. Immobilization procedure

Enzyme immobilization was performed by end over end at 90 rpm onto the activated chitosan using a solution of HRP (2000 units which represented 26,805 units/mg protein) made in 50 mM sodium acetate buffer, pH 5.5 or Tris–HCl buffer, pH 7.0 at room temperature during overnight. The solid modified chitosan beads immobilized with HRP were filtered and washed. The immobilization efficiency% was calculated from the following formula

$$\text{Immobilization efficiency\%} = \frac{\text{activity of immobilized enzyme}}{\text{initial activity of soluble enzyme}} \times 100$$

The activity of enzyme has been also measured in the washing buffer.

2.6. ATR-FTIR analysis

The attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra for chitosan samples were performed on a PerkinElmer spectrum 100 FT-IR spectrometer.

2.7. SEM analysis

Scanning electron microscopy (SEM) images of chitosan samples were examined with a scanning electron microscope Quanta FEG 450, FEI, Amsterdam, Netherlands. The microscope was operated at an accelerating voltage of 10, 20 kV. The samples were placed on the double side carbon tape on Al-Stub and sputtered with a 20 nm thick gold layer (JEOL JFC-1600 Auto Fine Coater).

2.8. Reusability of immobilized HRP

After each assay the immobilized HRP preparation was taken out, washed with 50 mM sodium acetate buffer, pH 5.5 and stored overnight at 4 °C. The immobilized enzyme recovered by this procedure was used repeatedly. The activity determined for the first time was considered as control (100%) for the calculation of remaining percentage activity after each use.

2.9. Enzyme characterization

Estimates of optimal temperature and pH for soluble HRP and immobilized HRP were made by using a temperature ranged from 10 °C to 70 °C and a pH ranged from 4.0 to 8.5. The thermal stability was investigated by measuring the residual activity of soluble HRP and immobilized HRP after 15 min of incubation at different temperatures. The K_m values were determined from Lineweaver–Burk plots by using different concentrations of guaiacol and H_2O_2 as substrates.

2.10. Effect of metal ions

The effects of various metal ions on enzyme activity of soluble HRP and immobilized HRP were determined by pre-incubating the enzyme with 2 mM metal ions for 15 min and then assaying the enzyme activity. The activity in absence of metal ions is taken as 100%.

2.11. Effect of urea, organic solvents and Triton X-100

The soluble HRP and immobilized HRP were incubated with urea or Triton X-100 or isopropanol or butanol or dioxan for 1 h at 37 °C. The enzyme activity of soluble HRP and immobilized HRP was determined by assaying the enzyme in the presence of these compounds. Activity of enzyme without exposures to these compounds was taken as control (100%).

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