International Journal of Biological Macromolecules 91 (2016) 663–670

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

# Immobilization of horseradish peroxidase on amidoximated acrylic polymer activated by cyanuric chloride

Saleh A. Mohamed[a,b,*], Saeed S. Al-Ghamdi[a], Reda M. El-Shishtawy[c,d]

[a] Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
[b] Molecular Biology Department, National Research Center, Dokki, Cairo, Egypt
[c] Chemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
[d] Dyeing, Printing and Textile Auxiliaries Department, Textile Research Division, National Research Center, Dokki, Cairo, Egypt

ARTICLE INFO

*Article history:*
Received 4 January 2016
Received in revised form 31 May 2016
Accepted 1 June 2016
Available online 2 June 2016

*Keywords:*
Peroxidase
Acrylic polymer
Immobilization

ABSTRACT

Horseradish peroxidase (HRP) was immobilized on amidoximated acrylic fabric after being activated with cyanuric chloride. FT-IR spectroscopy and scanning electron microscopy were used to characterize fabrics. The maximum immobilization efficiency of HRP (70%) was detected at 4% cyanuric chloride and pH 7.0. The immobilized enzyme retained 45% of its initial activity after ten reuses. The immobilization of enzyme on the carrier is saturated after 6 h of incubation time. The pH was shifted from 7.0 for soluble HRP to 7.5–8.0 for the immobilized enzyme. The soluble HRP and immobilized HRP had the same optimum activity at 40 °C. The immobilized HRP is more thermal stable than soluble HRP. Substrate analogues were oxidized by immobilized HRP with higher efficiencies than those of soluble HRP. Km values of the soluble HRP and the immobilized HRP were 31 and 37 mM for guiacol and 5.0 and 7.8 mM for $H_2O_2$, respectively. The immobilized HRP had higher efficiency for removal of phenol than that of soluble HRP. The immobilized HRP had higher resistance toward heavy metal ions compared to the soluble enzyme. The immobilized HRP was more stable against high concentration of urea, Triton X-100 and isopropanol. The immobilized HRP exhibited high resistance to proteolysis by trypsin than soluble enzyme. In conclusion, the immobilized HRP could be used as a potential efficient catalyst for the removal of aromatic pollutants from wastewater.



## 1. Introduction

There are several reasons for using an enzyme in an immobilized form. Immobilized enzyme facilitated its separation from the product of the reaction, thereby minimizing or eliminating protein contamination of the product. Immobilization also facilitated the efficient recovery and reuse of the enzyme with the reducing of the production cost. Furthermore, it enhanced stability under storage and operational conditions such as its denaturation by heat or organic solvents [1]. Improved enzyme performance via enhanced stability and repeated re-use is reflected in higher catalyst productivities. Generally, three traditional methods of enzyme immobilization can be distinguished, binding to a support (carrier), entrapment (encapsulation) and cross-linking [2].

Acrylic resins such as Eupergit C are widely used as supports. Immobilization by covalent attachment to Eupergit C has been successfully applied to a variety of enzymes for industrial application [3,4]. Other polymers such as poly(2-naphthol) and chitosan beads were used as carriers for immobilization of peroxidase [5,6].

On the other hand, the use of hydroxylamine hydrochloride as a strong reducing agent useful in biochemical cross-linking applications has been reported. Amidoxime polyacrylonitrile (PAN) nanofibrous membranes could be prepared by treating PAN nanofibrous membrane in hydroxylamine hydrochloride aqueous solution [7]. The hydrophilic amidoxime groups could improve the chemical and biological properties of the PAN nanofibrous membranes. Surface modified PAN nanofibrous membranes have been widely used in biological and environmental applications such as metal ion adsorption, cell adhesion, and enzyme immobilization [8–11]. Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) has been used as a coupling reagent to cross link enzymes to supports [12–14]. The chlorine atoms in the molecule react with nucleophillic groups to form stable linkages.

* Corresponding author at: Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, 21589, Kingdom of Saudi Arabia, Box 80203, Saudi Arabia.
*E-mail address:* saleh38@hotmail.com (S.A. Mohamed).

http://dx.doi.org/10.1016/j.ijbiomac.2016.06.002
0141-8130/

Peroxidases are ubiquitous in fungi, plants, and vertebrates [15]. They oxidized a large number of organic compounds initiated by one electron oxidation step [16]. Peroxidases oxidized phenols and aromatic amines to products that are much less soluble in water. This property could be used in removing carcinogenic aromatic amines and phenols from industrial aqueous effluents. Peroxidases are used in bioremediation of aromatic compounds and other xenobiotics, including pesticides, polycyclic aromatic hydrocarbons, and dioxins [17–19]. Peroxidases are used as antioxidant [20], as indicators for food processing [21], in bioelectrodes [22] and in the synthesis of conducting materials [23]. Despite the importance of peroxidases, their commercial uses and application as biocatalysts in industrial processes are limited because they are low stability under operational conditions and inhibited in presence of high concentrations of hydrogen peroxide [24]. The improvement of the properties of peroxidases resulted in uses of these enzymes in commercial applications. In this study, acrylic fiber polymer was pretreated with hydroxylamine hydrochloride to get the corresponding amidoximated polymer. This polymer was also activated with different concentrations of cyanuric chloride. HRP has been immobilized onto activated acrylic polymer for the improvement its stability and retention of its activity under physical and chemical conditions.

## 2. Materials and methods

### 2.1. Horseradish peroxidase

Horseradish peroxidase (HRP) was previously purified from horseradish cv. Balady. The detailed process was reported in our previous paper [25].

### 2.2. Acrylic fabrics

The acrylic fabrics used in this study was a 1/1 woven acrylic (40.65 × 40.65 threads/inch for both weft and warp) with 0.36 g/cm$^3$ density, supplied by Misr El-Mehalla Co., Egypt. The fabric was washed with ethanol three times and dried at room temperature.

### 2.3. Peroxidase assay

Peroxidase activity was carried out according to Yuan and Jiang [26]. The reaction mixture containing in one ml: 8 mM $H_2O_2$, 40 mM guaiacol, 50 mM sodium acetate buffer, pH 5.5 and least amount of enzyme preparation. The change of absorbance at 470 nm due to guaiacol oxidation is 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.

### 2.4. Preparation of treated acrylic polymer

A known mass of acrylic fiber was pretreated with 1% hydroxylamine hydrochloride using aqueous solutions of 2% ammonium acetate at a liquor-to-goods ratio of 50:1 at 85 °C for 60 min. The pretreated samples were thoroughly rinsed with water and air dried. The amidoxime acrylic polymer was activated with different weight per cents of cyanuric chloride (2–8% w/w of substrate) in acetone/water mixture (50% v/v) of pH 4 at 0 °C for two hours, after which the substrate was thoroughly rinsed with cold water, acetone and dried in ventilated refrigerator ready for enzyme immobilization.

### 2.5. Immobilization procedure

The enzyme immobilization was carried out by end over end onto treated polymer with HRP dissolved in 50 mM sodium acetate buffer pH 4.0 or Tris–HCl buffer pH 7.0 or 8.5 at room temperature during overnight. Aliquots of the supernatant were drawn up and the polymer is dried at room temperature to verify the advancement of the immobilization. The immobilization efficiency% was calculated from the following formula

$$\text{Immobilization efficiency \%} = \text{Activity of immobilized enzyme/Initial activity of enzyme} \times 100.$$

### 2.6. Structure characterization

The FTIR spectra of immobilized enzyme were performed on a PerkinElmer spectrum 100 FT-IR spectrometer. The morphology of immobilized enzymes was examined with a scanning electron microscope Quanta FEG 450, FEI, Amsterdam, Netherland.

### 2.7. The reuse of immobilized enzyme

The reusability of immobilized enzyme was studied by repeated usage for about 10 times. The activity determined for the first time was considered as control (100%) for the calculation of remaining percentage activity after each use.

### 2.8. Physicochemical characterization of the enzyme

Kinetic studies were determined by using different concentrations of guaiacol and $H_2O_2$ as substrates. The Km was calculated according to the Lineweaver–Burk double reciprocal plots. The optimal temperature and pH for soluble HRP and immobilized HRP were made by incubation the enzyme in pH ranged from 4.0 to 9.0 and temperature ranged from 30 °C to 80 °C. The thermal stability was determined by incubation the enzyme in different temperature for 15 min. The enzyme was taken out and left for 5 min in ice bath. Then $H_2O_2$ and guaiacol were added for determination the enzyme activity. The highest activity is taken as 100%.

### 2.9. Determination of removal of phenol

Phenol solution (2.0 mM) was transferred to a test tube contained soluble and/or immobilized HRP and the reaction was started by addition of $H_2O_2$ solution (4 mM). These experiments were carried in 20 mM Tris–HCl buffer, pH 7.0. The reaction mixture was taken at 2 min intervals for determination of% removal of phenol. The phenolic compound assay contained the reaction mixture, 2.0 mM *p*-aminoantipyrine and 6 mM potassium ferricyanide in 20 mM Tris–HCl buffer, pH 7.0. The absorbance of the assay mixture was measured at 510 nm using a spectrophotometer after 15 min of incubation at room [27].

### 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 before different temperature for 15 min before adding the $H_2O_2$ and guaiacol. The activity in absence of metal ions is taken as 100%.