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# Covalent Immobilization of *Lepidium draba* peroxidase recombinant on zinc metal-organic framework using glutaraldehyde

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## Introduction

Peroxidases play an essential role in clinical assays such as creatinine, glucose, and uric acid (Yujun *et al.*, 2010; Dhruvaraj, 2017). *Lepidium draba* peroxidase (LDP) shows a high similarity with the horseradish peroxidase (HRP) enzyme sequence (Fattahian *et al.*, 2017).

However, enzymes are limited for use in industrial and medical environments because of the impossible to reuse enzymes in the aqueous environment and the difficulty of their purification steps. One of the methods that help to overcome these limitations is the immobilization of the enzyme on solid support (Radzicka & Wolfenden, 1995).

Metal-organic frameworks (MOFs) are a suitable option for immobilizing enzymes due to their pore size, large surface area, and high thermal and chemical stability (Hassanzadeh *et al.*, 2018). Recently, the immobilization of this enzyme on Zn-MOF was done using the physical adsorption method. The results showed an increase in activity and in the substrate affinity for the stabilized enzyme compared to the free ones. While the thermal stability at high temperatures showed a decrease compared to the free enzyme (Farhadi *et al.*, 2021). In this research, in order to create a covalent bond between LDP and Zn-MOF, the stabilization conditions were optimized using glutaraldehyde. Thereafter the immobilization effect was evaluated on its physicochemical, kinetic, and stability properties.

**Methods and Materials**

The expression and purification of the recombinant LDP were carried out according to the Fattahian *et al.* method (Fattahian *et al*. 2017). Overexpression was done in the presence of 1 mM IPTG, and the protein was purified using Ni-NTA affinity chromatography column. The quality and quantity of the purified protein ~~was~~ were estimated by the use of SDS-PAGE gel electrophoresis and the Bradford method (Bradford, 1976).

Enzyme activity assay was measured according to the method of Krainer *et al.* (Krainer *et al.*, 2014). The reaction mixture included $H_2O_2$ (1 mM), TMB (0.6 mM) as the enzyme substrates and enzyme (0.5 mg) in the final volume of 1000 µL of phosphate buffer (50 mM and pH: 6.5). The extinction coefficient of oxidized TMB at the wavelength of 653 nm ($3.9\times1104$ x $mol^{-1}$ $cm^{-1}$) was used to calculate the enzyme activity.

For immobilizing enzymes on Zn-MOF, some parameters including MOF, glutaraldehyde concentrations, and the time of incubation were optimized. Optimization of the amount of MOF was carried out as follows, 0.75 mg/ml of enzyme, 1 dM glutaraldehyde in 50 mM phosphate buffer, pH 6.5 was mixed with different amounts of Zn-MOF (0.015 to 0.05 g) after being incubated at 4°C. Then, to separate the stabilized enzyme, washing was done twice using phosphate buffer. Finally, its specific activity and the amount of loading on the substrate were measured (Xia *et al.*, 2017).

To optimize the immobilization time, LDP enzyme (0.75 mg/ml), MOF-Zn (the optimized amount), and 1 dM glutaraldehyde at different time intervals (0.5-5 hours) were incubated at 4°C. Thereafter washing was done by the use of phosphate buffer (50 mM and pH 6.5) before its specific activity was measured.

To optimize the concentration of glutaraldehyde, different concentrations of it (0.6 to 2 dM) were added in phosphate buffer (50 mM and pH 6.5) containing Zn-MOF (the optimized amount) and enzyme (0.75 mg/ml) before incubated at 4 °C. Thereafter washing was done twice with phosphate buffer. At least its specific activity was calculated.

The percentage of immobilization yield was obtained as described Zhang *et al.*, (Zhang *et al.*, 2011). At least, the effect of immobilization were determined on the physicochemical properties (optimum temperature and optimum pH), kinetic parameters ($K_m$ and $V_m$) as well as the enzyme stability against heat and pH.

**Results & Discussion**

In this research, LDP enzyme was immobilized on Zn-MOF nanostructures using glutaraldehyde as a linker. One of the main advantages of covalent immobilization is the firm connection between enzyme and support (Sakai-Kato *et al.*, 2004). In this study, in order to increase the immobilization efficiency, some parameters such as Zn-MOF concentration, glutaraldehyde concentration, and incubation time were optimized. According to the results, the amount of 0.02 g of Zn-MOF, with a concentration of 1.2 dM glutaraldehyde and incubation time of 3 hours at 25 °C was considered the optimal condition for immobilization, which was used for further studies. In these conditions, the amount of enzyme loading was calculated as 57 mg/g and the stabilization efficiency was 67.3%. Notably, the stabilization of this enzyme was done through physical binding with a loading of 109.9 mg/g and a stabilization efficiency of 93.3% (Farhadi *et al.*, 2021). Hydrolase enzyme immobilization with this linker has been done with a loading of 87 mg/g (Cao *et al.*, 2016a) and the efficiency of HRP enzyme immobilization with this linker has been reported as 83% (Altikatoglu Yapaoz & Attar, 2020).

Based on the results of physicochemical studies, it was found that the stabilization of LDP enzyme does not affect the optimal temperature and pH of the enzyme. The results showed that the affinity of the enzyme to the substrates TMB and $H_2O_2$ changed and increased and decreased, respectively, compared to the free enzyme after immobilization. Changes in the kinetic parameters of an enzyme after immobilization have also been reported by other researchers. Chang *et al.* reported that after immobilizing HRP on magnetic core-shells made of iron oxide nanoparticles, using glutaraldehyde, the $K_m$ of the enzyme increased (Chae *et al.*, 1998). Also, Cao *et al.* immobilized soybean epoxide hydrolase enzyme on UiO-66-NH2 MOF with 1.3 dM glutaraldehyde and

reported that the affinity of the enzyme to the substrate increased after immobilization, which is the reason change in the tertiary structure of the enzyme (Cao *et al.,* 2016a). A decrease in the affinity of the enzyme to $H_2O_2$ can be due to the change in the structure of the enzyme after immobilization (Cao *et al.,* 2016, Xia *et al.,* 2017, Farhadi *et al.,* 2021).

Since enzyme immobilization on the support can be done from any side, the specific activity of the immobilized enzyme can change compared to the free enzyme (Rodrigues *et al.,* 2013). As shown in the results, the specific activity of the immobilized enzyme has increased about 2 times compared to the free enzyme; which is consistent with the results obtained from the physical immobilization of this enzyme on Zn-MOF (Farhadi *et al.*, 2021). Also, Nadar & Rathod reported an increase in lipase enzyme activity after enzyme stabilization on the organic-metallic structures of zeolite (Nadar & Rathod, 2020). The immobilization of enzymes on support can be accompanied by changes in the structure of the enzyme (Rodrigues *et al.,* 2013).

Additionally, the stability of the stabilized enzyme against temperature and pH was also investigated. The results indicate that the residual activity of the immobilized enzyme at temperatures above 25°C is lower than that of the free enzyme.Additionally, the stability of the enzyme in acidic pHs is lower than that of the free enzyme, while in alkaline pHs, it is higher . Whilst, the physical immobilization of this enzyme on Zn-MOF is accompanied by increasing its stability against alkaline pHs and its structural stability against heat compared to the free enzyme (Farhadi *et al.,* 2021).

**Conclusion**

In general, although immobilization with glutaraldehyde is stronger than the physical adsorption method, in this study, the stability of the immobilized enzyme was not improved against pH and temperature changes. Therefore, to increase the stability of the stabilized enzyme with respect to pH and temperature changes, it is suggested to immobilize this enzyme on other support or to use other linkers.

***Keywords:*** *Kinetics, Optimization, Specific Activity, Stability.*

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