PROTEIN-INORGANIC HYBRID SYSTEM FOR THE EFFICIENT HIS-TAGGED ENZYME AO:DCPIP-OR IMMOBILIZATION

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ABSTRACT

Enzyme immobilization is a widespread technique in enzymatic processes, permitting enhanced stability under aggressive reaction conditions. A new cross-linking immobilization technique has emerged, showing augmented stability and activity through protein self-assembly into flower-like structures. This work investigates the synthesis of protein-inorganic hybrids from recombinant thiamin-dependent enzyme Acetoin:dichlorophenolindophenol oxidoreductase (Ao:DCPIP-OR). Three different inorganic ions (Cu^{2+}, Zn^{2+}, and Co^{2+}) were added in PBS (100 mM, pH 7.4) containing 0.25 mg mL^{-1} of enzyme and incubated for 72 hours at 4 °C. The immobilized enzyme activity was tested using (*S*)-phenylacetylcarbinol synthesis. FT-IR spectroscopy and SEM analysis confirmed the protein-inorganic hybrids' (PIh) successful synthesis. Each inorganic ion showed different results, particularly the best results (relative activity of ~ 44% and immobilization yield of ~ 97%) obtained employing Co^{2+} ions, confirming cobalt selectivity toward recombinant protein His-tags. Based on the results, Co(II)Ao:DCPIP-OR hybrids were ulteriorly studied concerning the growth mechanism and relative activity after different incubation times.

Keywords: Enzyme immobilization. Thiamin-dependent enzyme. Protein-Inorganic hybrid. Recombinant enzyme. His-Tag enzyme.

1 INTRODUCTION

Enzymes have great potential and have been recognized and established in various industrial sectors for several years. Their applications are growing due to their versatility and advantages over chemical catalysts, such as milder operating conditions. Biocatalysts' high specificity minimizes industrial production costs by reducing by-product formation. Nevertheless, there are some limitations in large-scale enzyme employment, such as high costs and low stability under extreme reaction conditions. Enzyme immobilization is the preferred technique to resolve these problems, as it permits great stability and reusability. In most cases, enzyme activity results lower than the initial one due to increased mass transfer limitations of the substrate towards the enzyme active site.[1] Recently, a new immobilization technique using a protein-inorganic hybrid (PIh) system has gained great attention to overcome activity limitations.

The synthesis of PIhs was first reported by Ge et al.,[2] who obtained flower-shaped hybrid nanostructures with enhanced activity. Following the first work, many hybrid nanoflowers (hNFs) syntheses have been reported, showing great potential. They achieve not only greater stability but also enhanced activity related to free enzymes. hNFs' advantages are high surface area, simplicity of synthesis, higher stability, and excellent catalytic activity. Different metals have been employed in the simple hNFs' synthesis, such as Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, and Ca^{2+}. Employing an inorganic component over another depends on chemical and biochemical metal ion properties. For example, many copper-laccase nanoflowers have been synthesized due to Cu (II) action as a laccase cofactor, gaining great activity and stability from the metal biochemical role. Metal chemical properties have also been exploited to obtain metal binding selectivity when recombinant His-tagged enzymes are employed, as reported by Lòpez-Gallego & Yate.[3] This modern technique shows great potential, but the loading efficiency and immobilization time hinder hNFs industrial applications.

Based on previous works, in this study, the 72 h immobilization at 4 °C of the recombinant His-tagged thiamin-dependent enzyme Acetoin:dichlorophenolindophenol oxidoreductase (Ao:DCPIP-OR) using different inorganic ions (Cu^{2+}, Co^{2+}, and Zn^{2+}) have been performed. The resulting immobilized enzymes produced (*S*)-phenyllacetylcarbynol (PAC) by the carboligation reaction of 3-hydroxy-3-methyl-2-butanone (methylacetoin) and benzaldehyde.

2 MATERIAL & METHODS

The Ao:DCPIP-OR utilized in this work was obtained from *Bacillus licheniformis*, cloned and overexpressed in *Escherichia coli* Shuffle cells, as described by Bernacchia et al.[3] For raw purification, fractionate precipitation was performed based on a previous work of Giovannini et al.[4] After every protein purification, the recovered His-tag enzyme was lyophilized and stocked at -8 °C. For the immobilization, the His-tagged Ao:DCPIP-OR was employed as the organic component, while, on the other hand, three different inorganic components have been investigated: Cu (II), Co (II), and Zn (II). A classic immobilization was performed dissolving the amount of enzyme in PBS (100 mM, pH 7.4) necessary to obtain a protein concentration of 0.25 mg mL^{-1}. After forming primary crystals by adding the inorganic solution, the cofactors solution

(Thiamine diphosphate (ThDP) and $MgSO_4$ concentrations of 0.047 and 0.080 mM, respectively) was added. The immobilization mixture was then vortexed for 45 s, and after was incubated for 72 h at 4 °C, then recovered by centrifugation at 6,000 rpm for 3 min, permitting precipitate and supernatant separation.

For enzyme activity measurement, based on Giovannini et al.,[4] the synthesis of (*S*)-PAC from methylacetoin (1 mmol) and benzaldehyde (0.2 mmol) was utilized. Typically, free, or immobilized enzyme was dissolved in PBS solution (100 mM, pH 7.4), then methylacetoin and DMSO (10% v/v) were added. Finally, benzaldehyde was added to the reaction mixture, and that moment was taken as the starting point.

To investigate immobilization success, two types of characterization techniques were employed: (i) Fourier transform infrared spectroscopy (FT-IR) was used to obtain molecular information on the immobilized PIhs from the stretching and bending vibrations of specific protein, and inorganic component, bonds; (ii) scanning electron microscopy (SEM) was performed to observe the sponge-like structure of the hybrid biocatalysts.

3 RESULTS & DISCUSSION

The synthesis of PIhs using different inorganic ions was then carried out (72 h of incubation at 4 °C), and the enzyme activity and relative enzyme activity were evaluated (Table 1). From the presented results, Co-PIhs showed the best activity (26.5 U g^{-1}) and relative activity (43.6%) concerning Cu-PIhs, and Zn-PIhs; in particular, no activity was observed in Cu-PIhs after 72 h incubation. Co-PIhs' better results concerning Cu-PIhs and Zn-PIhs, are related to Co^{2+} ions' selectivity for Ao:DCPIP-OR His-tags. As reported by Lòpez-Gallego & Yate,[3] Co^{2+} has shown excellent selectivity, leaving the active site free for catalysis in the His-tags enzyme and permitting it to obtain higher relative activity.

Table 1 Evaluation of enzymatic activity and relative enzymatic activity for synthesizing PIhs using different inorganic ions.

PIhs	Activity (U g^{-1} ± SD[a])	Relative activity (% ± SD[a])[b]
Cu	0	0
Co	26.52 ± 8.26	43.66 ± 10.02
Zn	17.24 ± 7.29	28.35 ± 11.99

[a] Standard deviation. [b] Relative activity was calculated as the ratio between PIh and lyophilized enzyme.

Based on these results, Co (II) was selected as an inorganic component for the following kinetic immobilizations. The formation of the Co-PIhs was evaluated after 24, 48, and 72 h of incubation. Results obtained for activity, immobilization yield (IY), and relative activity are shown in Table 2.

Table 2 Co-PIhs synthesis kinetic study showing enzymatic activity, IY, and relative enzymatic activity.

Time (h)	Activity (U g^{-1} ± SD[a])	Relative activity (% ± SD[a])	IY (% ± SD[a])
24	17.93 ± 8.34	32.63 ± 13.13	78.72 ± 16.86
48	35.60 ± 9.42	64.79 ± 17.85	84.12 ± 9.76
72	26.52 ± 8.26	43.60 ± 10.02	97.11 ± 4.09

[a] Standard deviation.

According to the presented results, Co-PIhs synthesized with 48 h incubation time exhibited higher activity than 24 and 72 h. In particular, the 48 h incubation time PIhs better results compared to the 72 h incubation time can be ascribed to mass-transfer limitations to the active site caused by the excessive cobalt phosphate reticulation after the additional 24 h incubation. On the other hand, 72 h incubation time resulted in the best immobilization yield of 97.11%, indicating the complete immobilization of Ao:DCPIP-OR through sponge-like structures anisotropic growth. Moreover, PIhs obtained with a 24 h incubation time showed the worst activity and immobilization yield, indicating that this time is not enough for the correct growth of PIhs. Combining IY results (Table 2) and SEM images (Figure 1) Co-PIhs growth mechanism can be divided, as reported by Ge et al.,[2] into three steps, which can be monitored at 24, 48, and 72 h. In the first stage, primary cobalt phosphate crystals are formed, and protein molecules start forming complexes with Co (II). Afterward, in the second stage, the formation of large agglomerates of protein molecules and primary crystals (Figure 1a,b) can be observed, showing an IY increase in the growth process. Last, the third stage, PIhs complete immobilization occurred after 72 h (Table 2; Figure 1c).

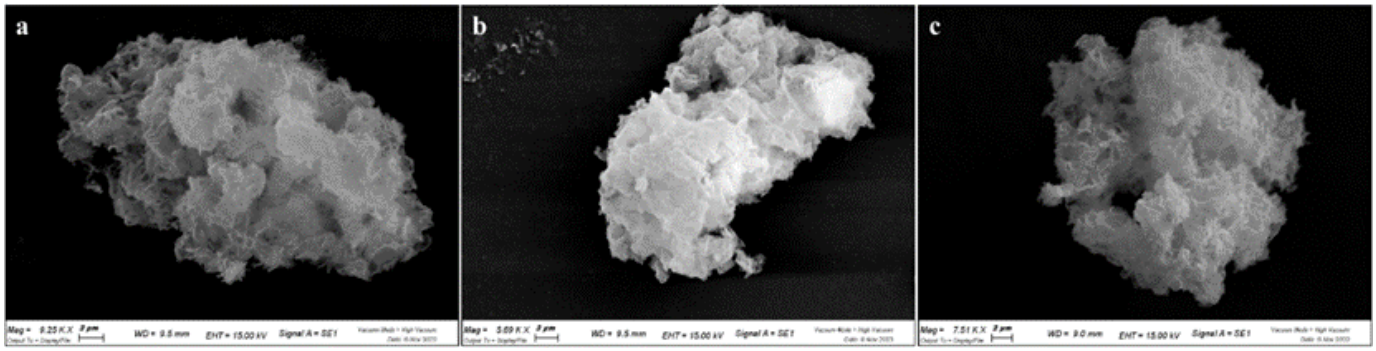

Figure 1 SEM images of 24 (a), 48 (b), and 72 h (c) incubation time Co-PIhs.

FT-IR spectroscopy, which can be identified by the incorporation of the enzyme in the inorganic structure, also confirmed the immobilization success. This was shown by the absence of the nucleophilic groups' typical peaks at 3191 and 3016 cm^{-1} (N-H and O-H) and the presence in all samples of the enzyme peak at 1072 cm^{-1}. IR spectra of the enzyme and Co-PIhs are reported in Figure 2.

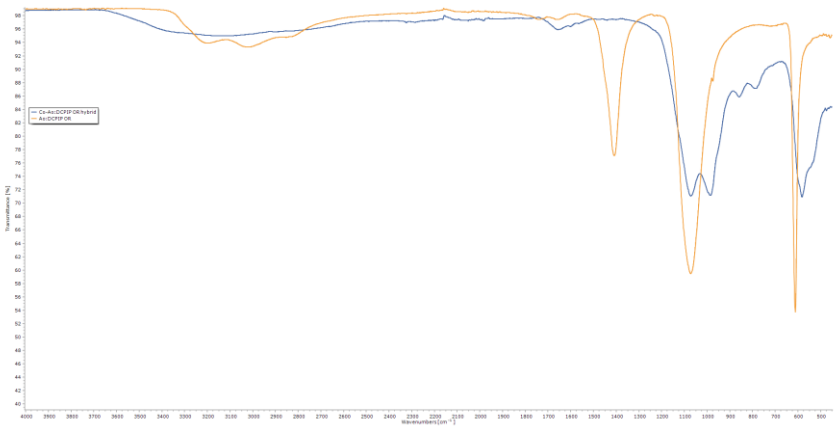

Figure 2 FT-IR compared spectra of Ao:DCPIP-OR (yellow) and Co-PIhs (blue).

Ultimately, Co-PIhs stability has also been proved by reutilizing the same immobilized enzyme after 45 days (stocked at 4°C), showing the same activity as the first employment.

4 CONCLUSION

It was possible to synthesize and fully characterize PIhs from the His-tag enzyme Ao:DCPIP-OR. In particular, Cu-, Co-, and Zn-PIhs have been investigated, with the best results, based on relative activity and IY, obtained with Co(II)Ao:DCPIP-OR hybrids, confirming Co(II) selectivity towards enzyme's His-tags. Co-PIhs showed great stocking stability at 4°C compared to free Ao:DCPIP-OR, reporting total retained activity after 45 days. In conclusion, the simple and efficient synthesis of PIhs from the His-tagged enzyme Ao:DCPIP-OR has been reported, showing promising properties for further studies.

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