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D-Amino acid oxidase bio-functionalized platforms: Toward an enhanced enzymatic bio-activity

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ABSTRACT

The purpose of this work is to study the adsorption process and surface bio-activity of His-tagged D-amino acid oxidase (DAAO) from *Rhodotorula gracilis* (His_6-*Rg*DAAO) as the first step for the development of an electrochemical bio-functionalized platform. With such a purpose this work comprises: (a) the His_6-*Rg*DAAO bio-activity in solution determined by amperometry, (b) the adsorption mechanism of His_6-*Rg*DAAO on bare gold and carboxylated modified substrates in the absence (substrate/COO^-) and presence of Ni(II) (substrate/COO^- + Ni(II)) determined by reflectometry, and (c) the bio-activity of the His_6-*Rg*DAAO bio-functionalized platforms determined by amperometry. Comparing the adsorption behavior and bio-activity of His_6-*Rg*DAAO on these different solid substrates allows understanding the contribution of the diverse interactions responsible for the platform performance. His_6-*Rg*DAAO enzymatic performance in solution is highly improved when compared to the previously used pig kidney (*pk*) DAAO. His_6-*Rg*DAAO exhibits an amperometrically detectable bio-activity at concentrations as low as those expected on a bio-functional platform; hence, it is a viable bio-recognition element of D-amino acids to be coupled to electrochemical platforms. Moreover, His_6-*Rg*DAAO bio-functionalized platforms exhibit a higher surface activity than *pk*DAAO physically adsorbed on gold. The platform built on Ni(II) modified substrates present enhanced bio-activity because the surface complexes histidine–Ni(II) provide with site-oriented, native-like enzymes. The adsorption mechanism responsible of the excellent performance of the bio-functionalized platform takes place in two steps involving electrostatic and bio-affinity interactions whose prevalence depends on the degree of surface coverage.

1. Introduction

Enzyme bio-functionalized platforms are of major importance in biosensing, drug delivery, and decontamination systems as well as in various industrial processes from pharmaceutical and food processing to waste treatment [1–3]. The challenge behind these applications is to integrate the highly specific molecular recognition of native enzymes to the platform. Enzymes adsorb to the majority of solid substrates, mainly through electrostatic and hydrophobic interactions [4–8]. Usually, hydrophobic interactions confer some degree of denaturalization to the adsorbed enzymes; thus attempts have been proposed to induce favorable electrostatic interactions. Since most of the used solids are negatively charged at pH conditions in which enzymes are active, as well as many of the residues of the proteins (pH > IEp), different strategies have been recently proposed to confer positive charge to the solid substrates: pre-adsorption of metal ions [9], cationic polymers [10] or cationized proteins [11,12]. On the other hand, covalent bonds between the enzyme and the substrate also represent a way to minimize the protein denaturalization produced by physical (mostly hydrophobic and electrostatic) interactions [12]. However, covalent linkage requires the modification of one or both parties which may also affect the biological activity. In many cases, these methods give rise to high enzyme loadings maintaining the native structure and biological activity, even increasing the response of the adsorbed enzymes compared to the activity in solution [13–15]. However, none of these methods can control the orientation of the enzyme on the solid substrates, the other key factor that determines the enzyme performance in terms of active site accessibility. In this regard, the interaction between His-tag (usually His_6) proteins and surface metal

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sites (Ni^{2+}, Cu^{2+}, Co^{2+}, or Zn^{2+}) generates high-affinity surface chelate complex of oriented enzymes [16,17]. His-tags can be genetically introduced into recombinant enzymes at the N- or C-terminal as well as in exposed loops of the protein without affecting the biological activity [4,18]. Therefore, this bio-affinity reaction between the histidine residues of the protein and the cation on the surface offers a gentle site oriented bio-functionalization procedure, providing important advantages over other strategies [16,17,19,20].

Bio-affinity interactions between a His-tag antigen and Ni(II)-modified solid substrates (silica and gold) were proven to provide a very good performance of the bio-functional platform [21,22]. Bio-affinity interactions result in site-oriented antigens on the surface with a strong coordinate bond between the His-tag at the N-terminal of the protein and the Ni(II) surface sites which can only be removed with high concentration of specific competitors (i.e. histidine or imidazole solutions). The assembly is the result of two-stage competitive mechanism ruled by electrostatic interactions followed by the surface complex formation between the His-tag and Ni(II) sites. This two-stages process is controlled by the characteristic filling (τ_f) and optimization (τ_{op}) times, related to the first electrostatic approach and the bio-affinity interactions, respectively. The two time constants appear because of the small size of the tag compared to the whole antigen that limits the complex formation.

D-Amino acid oxidase (DAAO, EC 1.4.3.3) catalyses the oxidation of D-amino acids to the corresponding α-keto acids in the presence of O_2 to produce H_2O_2 and ammonia [23–28]. This redox reaction coupled to a DAAO bio-functionalized platform has been employed to detect D-amino acids with electrochemical biosensors [27,28], to treat tumors with the H_2O_2 produced *in vivo* from exogenous molecules [25] and to manufacture the mother nucleus of cephalosporin antibiotics [23]. Therefore, several solid substrates together with DAAO from different sources have been proposed to improve the enzymatic response of the bio-functionalized platforms. Recently, we studied the surface bio-activity of DAAO from pig kidney (*pk*DAAO) on negatively charged hydrophilic (silica) and hydrophobic (gold) solid substrates [29]. Although *pk*DAAO adsorbs on both solid substrates even under unfavorable electrostatic conditions, the surface bio-activity is highly dependent on the ratio between τ_f and τ_{op}. The optimization step is electrostatic in nature on silica and hydrophobically driven on gold. Accordingly, the bio-activity of the native *pk*DAAO is preserved at any degree of surface coverage on silica whereas on gold it is only retained at high degree of surfaces coverage. However, these *pk*DAAO bio-functionalized gold platforms cannot be coupled to the commonly used amperometry technique because the analyte (D-alanine) interference increases the detection limit of the method. Consequently, different enzyme sources together with another adsorption strategy are required to improve the catalytic performance of DAAO bio-functionalized platforms.

This work is aimed at studying the adsorption process and surface bio-activity of His-tagged D-amino acid oxidase (DAAO) from *Rhodotorula gracilis* (His_6-*Rg*DAAO) as the first step for the development of an electrochemical bio-functionalized platform. With such a purpose the bio-recognition element was expressed following the reported results by Pollegoni et al. [30,31] in order to determine the bio-activity of the native and adsorbed enzyme by amperometry and the adsorption mechanism on bare gold and carboxylated modified substrates in the absence (substrate/COO^-) and presence of Ni(II) (substrate/COO^- + Ni(II)) by reflectometry. Comparing the adsorption behavior and bio-activity of His_6-*Rg*DAAO on these different solid substrates allows understanding the contribution of the diverse interactions responsible for the platform performance.

2. Experimental

2.1. Materials

All reagents were of analytical grade and were used without further purification: D-alanine (Fluka), H_2O_2, $KMnO_4$ (Cicarelli), KH_2PO_4, K_2HPO_4, $K_4P_2O_7$, $HClO_4$, NaOH, $KClO_4$, and KOH (Baker), $Na_2C_2O_4$ (Riedel-de-Haën), $KClO_4$ (Erba), plasmid pET-15b (Novagen), isopropil-β-Dtiogalactósido (IPTG) (Biodynamics), and Ni(II)-nitrilotriacetic acid (Ni-NTA) agarose (Invitrogen). Aqueous solutions were prepared by using 18 MΩ cm^{-1} resistance water (Milli-Q, Millipore; Billerica, MA). H_2O_2 concentration was determined by titration with 0.1 M $KMnO_4$ which was standardized against $Na_2C_2O_4$. 5 mM buffer solutions (PB) were prepared by dissolving the desired amount of KH_2PO_4, K_2HPO_4, $K_4P_2O_7$ in water and adjusting the pH with either 2 M KOH or 2 M $HClO_4$ to reach pH 5.0, 7.0, or 8.5. The pH measurements were performed with a combined glass electrode and a digital pH meter (Orion 420A+, Thermo; Waltham, MA, USA). Unless noted, all experiments were performed at room temperature (26 ± 2 °C).

2.2. Cloning, expression, and purification of recombinant His_6-Rg*DAAO*

The expression and purification of the recombinant *Rg*DAAO engineered with a His_6-tag at the N-terminal of the enzyme were performed following the reported results by Pollegoni et al. [30–32]. The coding sequence of *Rg*DAAO (EC 1.4.3.3, DAAO) was ordered from Genscript, with its codon usage optimized for *Escherichia coli* expression. The fragment was introduced by cloning into pET15b (NOVAGEN) which drives the expression of fusion proteins with a polihistidine tag at the N-terminus, to generate pJV398. This plasmid was transformed into *E. coli* BL21 strain for subsequent expression experiments. *Escherichia coli* cells were grown in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl) supplemented with 0.1% ampicillin and 0.5% glucose at 37 °C with rotary shaking. When the cell optical density (OD = 600) reached 0.6, His_6-*Rg*DAAO expression was induced with the addition of 1 mM IPTG for 3 h at 37 °C. The bacterial pellet was resuspended in lysis buffer (15% glycerol, 0.5 M NaCl, 20 mM Tris–HCl pH 7.5) and the cells were lysed in an Emulsiflex High pressure homogenizer (AVESTIN). The homogenate was centrifuged at 10,000 rpm for 30 min. The supernatant containing recombinant His_6-*Rg*DAAO was subject to purification by metal chelation chromatography using Ni(II)-NTA Agarose matrix (QUIAGEN). The protein bound to the resin was rinsed several times with imidazole gradient (20–200 mM). The imidazole was eliminated by gel filtration and the protein was eluted with water and then lyophilized. Purity was checked by SDS-PAGE followed by Coomasie staining.

2.3. Bio-functional platform

2.3.1. Solid substrates

As previously described [22], the surface properties of modified silica and gold solid substrates were proven to be indistinguishable [22]. The adsorption experiments were performed with silicon wafers (100 mm, Silicon Valley Microelectronics Inc.; Santa Clara, CA, USA) oxidized at 1000 °C for 1 h (thickness was verified by ellipsometry) in order to obtain a silica layer of about 100 nm thick (essential for obtaining a high sensitivity in reflectometry experiments [33]) and cut in strips (1 cm × 4 cm) following the crystallographic plane of silicon (1 0 0). Prior to each adsorption experiment, these strips were cleaned with boiling piranha solution (2:1 H_2SO_4:H_2O_2) and rinsed thoroughly with deionized water. (*Caution! Piranha solution is a powerful oxidizing agent that reacts violently with organic compounds; it should be handled with extreme*