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Multifunctional nanoenzymes from carbonic anhydrase skeleton

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ABSTRACT

Carbonic anhydrase (carbonic dehydratase) (CA) is a metalloenzyme that contains zinc (Zn^{2+}) ion in its active site. CA catalyzes the reversible conversion of carbon dioxide and water to bicarbonate and protons. Zn^{2+} ions, which are present in the active site of the enzyme, interact with the substrate molecules directly and cause catalytic effect.

In this study, a nano-enzyme system was designed in aqueous solutions at room temperature and under nitrogen atmosphere to use the CA enzyme without any pre-treatment and deformation in its structure. The novel concept ANADOLUCA (AmiNo Acid (monomer) Decorated and Light Underpinning Conjugation Approach) was used for this process, nano CA enzyme of size 93 nm was synthesized. The activity of the synthesized nano CA was measured following the change in absorbance during the conversion of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion at 348 nm for a period of 10 min at 25 °C compared with free CA enzyme. Km and Vmax values for nano CA enzyme were found to be 0.442 mM and 1.6×10^{-3} mM min^{-1}, respectively, whereas Km and Vmax values for free CA were found to be 0.471 mM and 1.5×10^{-3} mM min^{-1}, respectively. In addition to these, the Zn^{2+} ion present in the active site of the nano CA enzyme was replaced by rodium metal. This nanorodium-substituted CA has been investigated as a new reductase enzyme for the stereoselective hydrogenation of olefins. Then, the Zn^{2+} ion in the active site of the nano CA enzyme was replaced with manganese metal to enhance the enzyme structure, thereby gaining characteristics of peroxidase. This newly synthesized nano manganese-substituted CA enzyme was investigated for its role as a peroxidase, which could be an alternative for hydrogen peroxidases.

1. Introduction

Carbonic anhydrase (CA; carbonic dehydratase, EC 4.2.1.1) is a zinc-containing metalloenzyme. The CA enzyme catalyzes the reversible hydration of carbon dioxide to form bicarbonate and protons [1,2]. CA increases rate the of the reaction in the forward direction and produces a reaction rate of 10^4-10^6 per min [3,4].

In the biological system, CA is found in association with all tissue and organ systems involved in the transport and excretion of CO_2, ranging from the site of CO_2production, i.e., metabolically active tissues such as the muscle, to circulating red blood cells in the vasculature, to the various organs of gas exchange, i.e., the lungs and gills. It is found in the kidneys, eye lens, gastric mucosa, salivary glands, muscles, brain, nerve myelin sheath, pancreas, prostate and endometrial tissues and some of them are purified and analyzed for their biochemical properties [5–7].

CA is grouped into three different gene families, namely, α, β, γ CA isoenzymes in prokaryotes and eukaryotes. The gene families do not have similarities with regard to amino acid sequences. Vertebrates, bacteria, algae and green plants with cytoplasm have α-CA; bacteria, algae, and some plants with chloroplasts have β-CA; and some bacteria have γ-CA [8]. The common property of all CA isoenzymes is that they have Zn^{2+} ions bounded to histidine amino acids. Zn^{2+} ions directly interact with the substrate molecules and increase the catalytic activity of the enzyme, thus producing a powerful hydrolysis of water to $H^+$and a highly reactive Zn − OH molecule [9–11].

Modification of enzymes with cofactors or complexes that have metal ions is one of the steps of the process that leads to the production of new catalytic activities. According to this aspect, different catalytic forms of the same enzyme can be obtained by replacing the metal ions already present in the active site of an enzyme with another metal ion. The activities of enzymes with a broken structure decrease during these procedures. It is important that these enzymes are designed for one-time usage. Earlier studies on metal ion binding in the CA enzyme structure showed that the Zn^{2+} ion in the CA enzyme could be removed from the enzyme structure using dialysis versus some cheleting agents. Inactive CA enzyme structure, caused by removing the Zn^{2+} ion, could be activated again by adding the Zn^{2+} ion in the enzyme structure [12].

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Protein modifications using cofactors or complexes that contain metals had been widely studied in derivating new catalytic enzymes. A research study combined the metal hydrogen catalysts with proteins to reduce the organic compounds with enzymes [13]. Different approaches have been used in other studies when synthesizing a new reductase enzyme from other enzymes. One of these approaches is the indirect binding method. This method involves the binding of a metal catalyst to a ligand followed by binding of this ligand–catalyst complex to protein [14]. Another study described the direct binding of hydrogenation catalyst to protein surface. In this method, rhodium or similar hydrogenation catalysts were bound to the protein surface at different places. Hence, the synthesized catalyst was non-selective [15].

These studies on synthesizing a new generation hydrogenase or peroxidase from enzymes have led to a new perspective which involves replacing the metal ions in metalloenzyme structure with rhodium or similar reducing metal [16,17]. In this aspect, the CA enzyme is suitable for generating a new peroxidase or reductase enzyme because the Zn^{2+} ion in the active site could be replaced with rodium ion because of the similarity of atomic radius. Removal of the Zn^{2+} ion from the CA enzyme structure would result in the apo-CA enzyme structure. Binding studies have show that during the binding process of rhodium in the apo-CA enzyme, the shape of the active site would not be destroyed.

Synthesizing a new oxidase enzyme without cofactors or coenzymes from other enzymes has led to accelarate studies in this area. For example, hydrogen peroxide is a strong oxidating agent, but it requires a coenzyme to perform the reaction [18]. To develop the characteristic of an oxidant characters in the reaction media, Zn^{2+} ion in the CA enzyme was replaced with the Mn^{2+} ion. Mn^{2+} substituted CA enzyme was used as a catalyst in epoxilation reactions of alkenes [19].

The increased stability, easy recovery and continuous usage of enzymes depends on the method adopted for synthesis [20–22]. Enzymes, proteins, antibodies, and other bio molecules could be synthesized at nano scale without any deformation in their structure by using the ANADOLUCA (AmiNo Acid (monomer) Decorated and Light Underpinning Conjugation Approach) concept [23]. Particle size is an important factor for reactions of differents materials, as smaller particles have larger specific surface area. This property provides more enzyme activities on their surface and less restriction for diffusion of substrate and product. Hence, according to the ANADOLUCA concept, microemulsion polymerization media has been prepared. Then, biomolecules were allowed to interact with ruthenium-based aminoacid monomers to acquire features such as extra stability and fluorescence in their structure. Nano bioparticles were obtained in microemulsion polymerization media by mixing for 48 h at room temperature, in daylight and under nitrogen atmosphere. An excellent property of these nano bioparticles was that they could be used many times without any deformation in their structure. Nano bioparticles could be removed from the reaction media by centrifugation, and after the washing process, they could be used repeatedly. In addition to this, the nano-bio molecules that were synthesized according to the ANADOLUCA method could be stable in different reaction meadia such as alkaline media, lower or higher temperature values or more acidic reaction media [24–29].

In this study, first, a multifunctional bionano CA platform from CA skeleton was synthesized using a ANADOLUCA hapten cross-linker-$(MAT)_2Ru(bipyr)_2$ by photoinduced electron transfer polymerization. Then, different catalytic features such as oxidase and reductase forms of this synthesized nano enzyme were investigated by replacing the cofactor of the nano CA enzyme, Zn^{2+}, with Mn^{2+} and Rh^{2+} ions.

2. Methods

2.1. Materials

Ruthenium based aminoacid monomer, bis (2-2′-bipyridyl)-Methacryloylamido tyrosine ruthenium(II), (MATyr-Ru$(bipyr)_2$-MATyr), was synthesized according to a previously published procedure [23]. CA, from bovine erythrocytes ≥ 95% and Bradford reagent were purchased from Sigma. 4-Nitrophenylacetate (NPA) and Poly-vinyl alcohol (PVA; (MW = 27,000 Da) were purchased from Fluka BioChemica. Ammonium persulfate (APS) was supplied from a local source. 2-(N-morpholino) ethane sulfonic acid (MES), 2,6-pyridine dicarboxylic acid (2,6 PDCA), bis (1,5-cyclooctadien) rhodium (I) tetrafluoroborate and $Mn(NO_3)_2.4H_2O$ were purchased from Sigma-Aldrich.

2.2. Synthesis of bio-nano CA platform from CA skeleton

The bio-nano CA platform was synthesized by the microemulsion polymerization process. The microemulsion medium was prepared by dispersing 0.5 *g* of PVA in 45 mL of deionized water. On the other hand, 3 mL of 2000 ppm CA in pH 8.0 buffer was allowed react with 25 μL of MATyr-Ru$(bipyr)_2$-MATyr (25 mg MATyr-Ru$(bipyr)_2$-MATyr in 10 mL deionized water). Then, this complex was added into 25 mL of microemulsion media. Finally, 20 mL of initiator solution prepared by dissolving 0.02 *g* of APS in 45 mL of deionized water was added into the polymerization reaction mixture. Polymerization was carried out for 48 h under nitrogen atmosphere at room temperature, in daylight. Nano CA particles were separated from the reaction solution by centrifugation at 6000 rpm for 20 min and washed with deionized water to remove unreacted substances.

The protein content of the synthesized nano CA was determined spectrophotometrically at 595 nm by the Bradford method, using bovine serum albumin as the standard [30]. The Bradford method was applied for the determination of protein concentration at the end of the synthesis of nano CA enzyme. For this purpose, the residual liquid reaction media were centrifuged and all the nano enzyme particles were precipitated. Initial concentration of CA was 2000 ppm in the reaction media. After the precipitation of nano enzymes, protein content of supernatant was determined Bradford method. Hence, unbonded protein concentration CA enzyme was calculated.

2.3. Synthesis of rhodium-substitued bio-nano CA (nano CA-Rh)

Before the synthesis of Rh-substitued nano CA (Nano CA-Rh), nano apo-CA was synthesized. For this process, the nano CA enzyme was dispersed in 5 mL of 0.1 M MES buffer and dialyzed for 24 h against 2,6-PDCA solution. Dialysis solution was prepared by dissolving 3.34 mmol 2,6-PDCA in 500 mL of 0.1 M sodium acetate of pH 5.5. Then, the nano enzyme was washed with 500 mL of 50 mM MES buffer (pH 6.0) for removing 2,6-PDCA. The Zn^{2+} ions removed from the nanoCA enzyme was determined by atomic absorption spectrometry (AAS).

Two different methodology were applied for the synthesis of rhodium-substitued bio-nano CA. In the first method, the obtained nano apo-CA enzyme was allowed to interact with 0.5 mmol of bis-(1,5-cyclooctadien) rhodium-(I) tetrafluoroborate in 500 mL of MES buffer (pH 6.0) for 10 h for substitution of Rh^{2+} in the nano apo-CA. After this process, nano-enzyme particles were allowed to interact with 50 mM MES buffer (pH 6.0) for removing the excess Rh^{2+} ions from the nano CA-Rh structure. The amount of substituted Rh^{2+} ions was determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Perkin Elmer).

In the second method, firstly free CA enzyme was dialyzed and apo CA was obtained. Then, Rh^{2+} ion bound to apo CA. Obtained CA-Rh complex was converted to the nano form by ANADOLUCA method.

2.4. Synthesis of manganese substitued nano CA (nano CA-Mn)

The synthesized nano apo-CA enzyme was allowed interact with Mn $(NO_3)_2.4H_2O$ in 0.1 M MES buffer (pH 7.2) for 10 h for substitution of Mn in the nano apo-CA enzyme. Then, the resulting nano CA-Mn was dialyzed against MES buffer (ph 5.5) to remove excess Mn^{2+}. The amount of substituted Mn^{2+} ions was determined by ICP-OES.