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Loss of *Bacillus Badius* Phenylalanine Dehydrogenase Specificity towards Phenylalanine Substrate in Presence of CdTe Quantum Dots

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ABSTRACT

Phenylalanine dehydrogenase (PheDH) which is categorized in oxidoreductase enzymescatalyzes the NAD^+-dependent oxidative deamination of L-Phe to phenylpyruvate. This enzyme has widespread applications in industrial and medical fields such asdetermining the amount of phenylalanine for monitoring of phenylketonuria (PKU). Quantum dots (QDs) are known as semiconductors with many advantages including high photostability, unique optical characteristics, and symmetric emission spectrum. The protein-QD interactions have become an important interest due to its similarity with protein-ligand interactions. These interactions depend on many factors such as protein conformation and orientation and also can be lead to increase or decrease enzymatic activity due to NPs features.In this study, the interaction of *B. badius* Phenylalanine dehydrogenase (PheDH) and CdTe540 through examining of kinetic parameters of the enzyme for L-phenylalanine and L-tyrosine as substrateswereassessed to understand how this protein can interact with QD. After expression and purification of enzyme in prokaryotic *E. coli BL21* host, kinetic parameters of the enzyme such as K_m, V_{max}, K_{cat}, K_i and K_{cat}/K_m values for L-Phenylalanine and L-tyrosine substrates in the presence and absence of CdTe540 were calculated. The results showed that CdTe could have an inhibitory effect on PheDH enzyme. Fluorescence spectroscopy demonstrated that the binding of QDs with enzyme induced conformational changes in the enzyme in the presence of CdTeQD. It was concluded that a comprehensive characterization of stability of enzyme bound QDs could be a necessary step in their potential use in biomedical fields.

Keywords: Phenylalanine dehydrogenase, Quantum dots, CdTe, Specificity

INTRODUCTION

Phenylalanine dehydrogenases have a lot of applicable fields for different purposes such asdiagnostic tools for Phenylketonuria detection (PKU) [1,2]. Phenylalanine dehydrogenase (EC 1.4.1.) is considered to play a role in the degradation of substrates specially L-phenylalanine and other non-specific substrates such as L-tyrosine [3,4].

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Semiconductor quantum dots (QDs) are nanocrystals material with diameters in the range of 1-20 nm [5,6]. These nanoparticles consisted of elements such as Zn, Cd, Hg, Se, S and Te [7]. QDs provide unique physical and optical characteristics such as high quantum yield (QY), luminescence characterization depending on size, immense photostability [8], narrow and symmetric emission spectrum, and readily tunableemission for studying biorecognition molecules [9] such as proteins, nucleic acid or peptides [10] for different biological and medical purposes [11-13]. They have also been used to study

protein-protein interaction [14], peptide delivery [15,16] and cell imaging [12-19]. Therefore, bioconjugation of QDs with proteins [17], especially enzymes can have significant benefits [18,19]. Enzyme-QDs conjugates have been popular because of the catalytic ability of enzymes for bioanalytical and biotechnological targets [20].

Many strategies have been reported for Nanoparticle-Protein binding: 1. Electrostatic adsorption (Fig. 1a). 2. Conjugation via functionalized groups (Fig. 1b). 3. Linking by specific affinity of protein to cofactor (Fig. 1c). 4. Direct linkage with NP surface atoms (Fig. 1d) [21-23]. Among these methods, electrostatic adsorption is the simplest strategy because the chemical reaction is not needed and can be used to assemble proteinin polyelectrolyteenvironments [24]. Linking to the NP-ligand based on covalent bonds is another general method for protein-QDs bindings [23,25]. Depending on the different chemical groups, this method can result in coupling of proteins on the surface of QDs. For nanoparticles with TGA (thioglycolicacid) groups, binding can occur between polyhistidine tags in N-terminal of enzymes [26,27]. There are so manyenzymes that can use these strategies for bioconjugation such as binding of Lactate dehydrogenase and Pyruvate kinase (pyka) enzyme that been reported recently [28]. Because of changes in protein functionality due to binding on the QDs surface, it is essential to evaluate these behaviors. Measuring of kinetic parameters for enzyme activity as well as Fluorescence techniques can be useful methods for determining the changes of bioconjugation effects [29].

In this study,the focus was on the kinetic properties of Phenylalanine dehydrogenase enzyme (PheDH, EC 1.4.1.20) of *Bacillusbadius*whichcatalyzesthe reversible oxidation-reduction for L-phenylalanine as specific substrate and L-tyrosine as non-specific in presence and absence of Cadmium telluride quantum dot. Also, thestructuralchanges of the enzyme with intrinsic fluorescence and UV-Vis techniqueswere evaluated.

MATERIAL AND METHODS

Materials

L-phenylalanine, L-tyrosine and α-nicotinamide adenine dinucleotide (NAD^+) were purchased from Sigma-Aldrich. Ni-NTA-Sepharose affinity column from Novagen, kanamycin, and lactose also from Sigma-Aldrich, were prepared. The water-soluble CdTe QD (2.8 nm) with excision wavelength of 540nm used in this study was prepared [30].

Expression and Purification of PheDH Enzyme

The *E. coli* BL21 strain was transformed by *Bacillus badius* PheDH coding gene plasmids (pET28a plasmid) as in our previously published study [31]. Histidine-tailed PheDH was expressed in 10 ml LB medium with 50 µg ml^{-1} kanamycin and incubated overnight at 37 °C at 220RPM. Then 1 ml of the seeding culture was added to 125 ml kanamycin included 2XYT medium and incubated again at 37 °C at 220RPM shaking.After almost 3.5-4 h, when OD_{600} ~ 0.6 reached, 2.5 microliters of 8mM lactose for the expression of enzyme was added to 2XYT medium and incubation at 20 min with 220RPM for 16 h was performed. Then after the cell pellets were harvested by centrifugation at 6000 g for 10 min at 4 °C. The suspension was sonicated by lysis buffer (50 mM Tris-Hcl (PH 7.8), and then centrifugation at 14000 g for 20 min, at 4 °C was done to collect the supernatant. Finally, purification with Ni-Sepharose column affinity chromatography was carried out by using 250 mM imidazole as elution buffer. Protein concentration was determined by Bradford assay using Coomassie blue and bovine serum albumin as standards.

Enzyme Assay

For measuring kinetic parameters of the enzyme, the activity of PheDH was determined at 25 °C in 100 mM glycine/KCL/KOH buffer (pH 10.5) by measuring of NAD^+ at 340 nm in 250 µl volume. K_m values of the enzymewere obtained by fixing the concentration of NAD^+ at 2.5 mM and L-phenylalanine and L-tyrosine were varied.

Assay of PheDH in Presence of QD

The enzyme activity was measured in presence of different concentrationof QDs prepared from the main concentration of cadmium tellurium (c = 9.2 µM) in which the equivalent molar of the enzyme was added in each solution. After incubation time (30 min) the activity of each

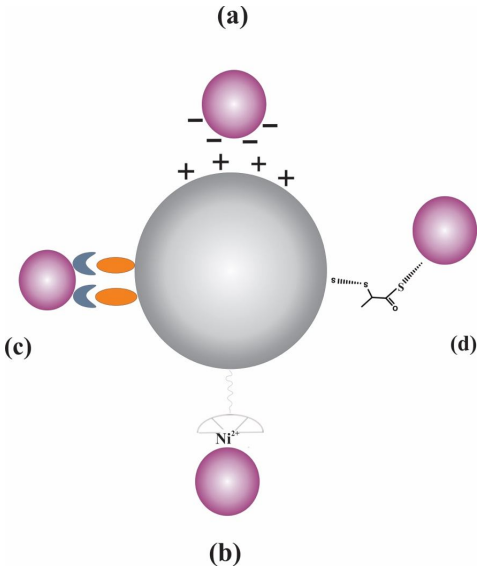

Fig. 1. Nanoparticle binding strategies with proteins: (a) Electrostatic adsorption (b) Conjugation *via* functionalized groups (c) Linking bythe specific affinity of protein to cofactor (d) Direct linkage with NP surface atoms.

assembly were evaluated by PerkinElmer Lambda 25 UV-Vis spectroscopy.

Fluorescence Spectroscopy

Intrinsic Fluorescence emission spectroscopy of enzyme in the presence of different concentrations of CdTe was measured by PerkinElmer LS 55. The excitation wavelength of *Bacillus badius* PheDH was 295 nm and the intrinsic emission spectra were recorded between 250 and 395 nm. All the Fluorescence measurements carried out at room temperature in final volume of 60 microliters. The excitation-emission slit widths were set at 5 and 10 nm respectively, and protein concentration was 0.6 mg ml^{-1} for each assay.

RESULTS

Bacillus Badius PheDH Expression

The expressed protein was purified based on the 6His-tagged fusion by affinity (Ni-NTA-Sepharose) chromatography. The SDS-PAGE analysis of the eluted enzyme showed that the enzyme was admittedly purified to homogeneity (Fig. 2).

Determination of Kinetic Properties

Kinetic properties of enzyme in the presence and absence of QD were studied after drawing line Weaver-Burk plot. Comparison of kinetic values for both L-phenylalanine and L-tyrosine in the presence and absence of CdTe540 showed interesting results (Tables 1 and 2). PheDH enzyme in presence of CdTe showed a decrease in the specific activity (K_{cat}/K_m) toward these substrates in comparison to the absence of CdTe. Also, V_{max} and K_{cat} were decreased and it can be concluded that the CdTe may have an inhibition effect on Phenylalanine dehydrogenase.

Intrinsic Fluorescence Changes of PheDH in Presense of Different Concentration of Cadmium Telluride

Intrinsic fluorescence spectra of the enzyme in different concentrations of quantum dots was measured (Fig. 3). Enzyme fluorescence intensity was reduced by increasing concentration of CdTe. It is noteworthy that PheDH activity was also decreased. The rate of fluorescence reduction of the enzyme in the presence of different concentrations of CdTe suggesting that change in the tertiary structure of the enzyme resulting in the exposure of inner aromatic residues to the solvent.

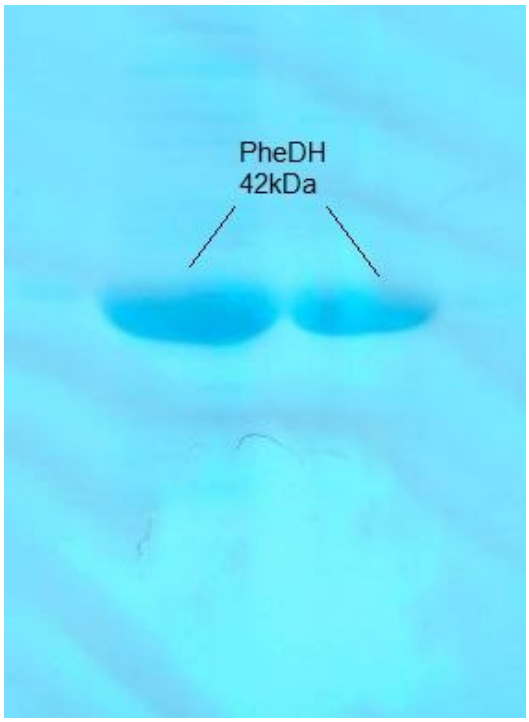

Fig. 2. SDS.PAGE analysis of *B. badius* PheDH enzyme.

Table 1. Kinetic Parameters of *B. badius* PheDH Enzymes for L-phenylalanine as Substrate in Presence and Absence of CdTe QD

	K_m (μM)	K_{cat} (units/s^{-1})	K_{cat}/K_m	V_{max} (unit/mg)	K_i
PheDH	90	87.2	0.968	83	
PheDH + CdTe540	390	83	0.212	79	0.879

Table 2. Kinetic Parameters of *B. badius* PheDH Enzymes for L-tyrosine as Substrate in Presenceand Absence of CdTe QD

	K_m (μM)	K_{cat} (units/s^{-1})	K_{cat}/K_m	V_{max} (unit/mg)	K_i
PheDH	7500	28	0.003	27	
PheDH + CdTe540	13500	35	0.002	20	0.0468

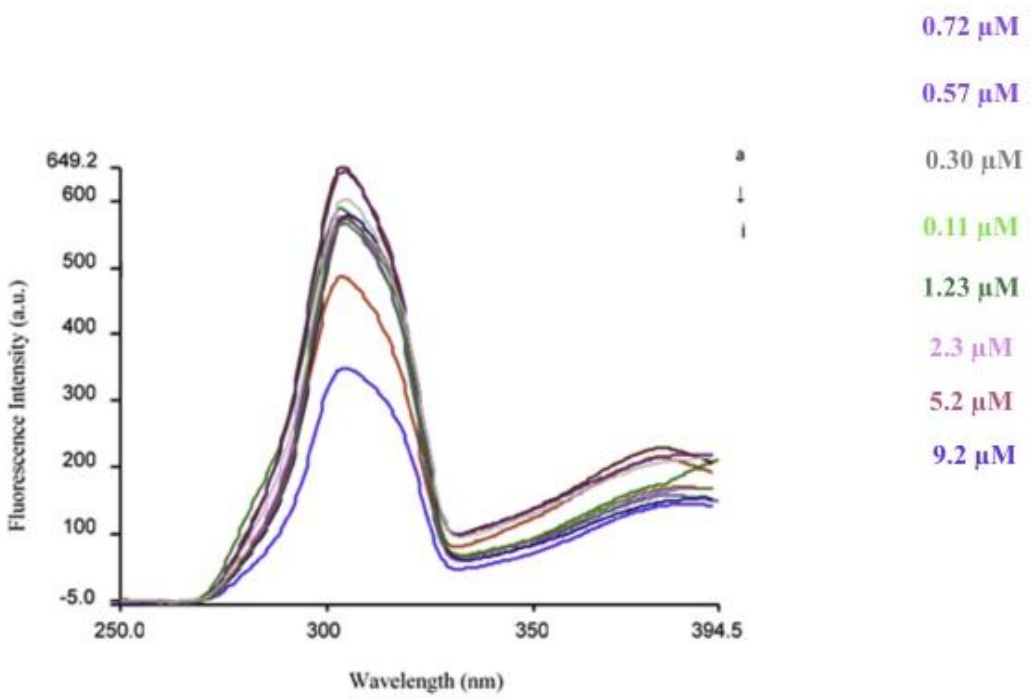

Fig. 3. Intrinsic Fluorescence changes upon the interaction of PheDH with different concentration of CdTe540. (a)-(i) the concentration of CdTeare 0.72, 0.57, 0.30, 0.11, 1.23, 2.3, 5.2 and 9.2 µM, respectively.

Table 3. The Ratio Specific Constants for the L-phenylalanin as Specific Substrate and L-tyrosine as non-specific substrate in presence and absence of CdTe540

	S Specific Activity (F)/Specific Activity (Y)
PheDH	322.6
PheDH + CdTe540	106

As was presented in Table 3, specificity of the enzyme for phenylalanine in the presenceof CdTe540 in was decreased compared to free enzyme.

DISCUSSION

There are many potential applications for nanoparticles-enzyme bioconjugations, including probes for monitoring enzyme activity, signal transduction in assays, bioremediation materials, biocatalysts, and biosynthesis. The phenomenon of enzyme activity in the presence of nanoparticle interface is something that hasmany different results, and thus provide these materials with even more potentials in the field of biomedical sciences. Some of these phenomena can result in increase or decrease of enzyme activity in reaction conditions [32-34]. Yeast alcohol dehydrogenase immobilization on Fe_3O_4 magnetic nanoparticles showeda 10-fold increase in

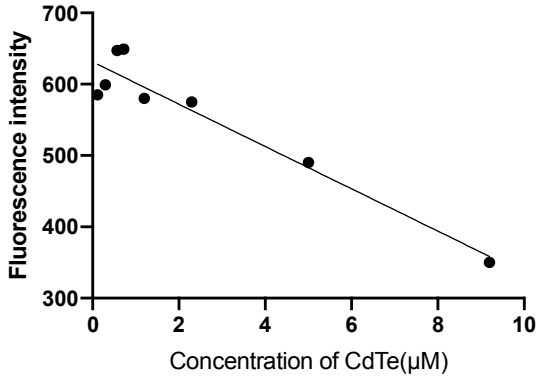

Fig. 4. Intrinsic fluorescence intensity of the PheDH at various concentrations of CdTe.

enzymaticstability and a 2.7-fold increase in activity of enzyme compare to the free enzyme in solution [35,36]. Immobilization ofnative and adamantine-modified L-phenylalanine dehydrogenase on β-cylodextrin coated gold nanosphere *via* supramolecular association retained high catalytic activity and enzyme affinity for the substrate increased with similar fluorescence spectra to free protein [37]. Evaluating of enzyme activity for other enzyme like Keratinase also has been reported. Konwarth and co-workers immobilized Keratinase on PEG-coated Superparamagnetic nanoparticles and observed a 4-fold increase in enzyme activity compared to free enzyme in solution [38]. Wang and co-worker created a $CHPO_4$-α-amylase nanoparticle conjugated and also observed an increase in catalytic activity compared to that of the free enzyme [39].

PheDH is a protein with 42kDa and the $(His)_6$ tails at N-terminal of enzyme and is responsible for oxidation/ reduction of substrate such as L-phenylalanine and L-tyrosine. One of the most important roles of phenylalanine dehydrogenase is determining L-phenylalanine levels in phenylketonuria disease (PKU) [40,41], level of L-phenylalanine in PKU because of enzymatic failure of Hydroxylase increases and if this aromatic amino acid not be diagnosed in newborn infants, a dangerous complication such as mental retardation can happen. Recently many studies like calorimetric methods [42] and protein engineering for increasing affinity of the enzyme toward L-phenylalanine[31] has been carried out.

QDs are semiconductor NPs and have numerous advantages over traditional fluorescent dyes for biological applications. They have unique characteristicssuch as high quantum yield, luminescence characterization, photostability, narrow and symmetric emission spectrum, and readily tunableemission which is of great importance in the high-sensitivity detection both *in vivo* and in vitro systems. So it is worth studying the bioconjugation of QDs with proteins especially enzymes [43].

There is not enough research about the interaction of PheDH and quantum dots to estimating of what effect quantum dots can have on the enzyme, for this purpose we studied the interaction of *B. badius* phenylalanine dehydrogenase with CdTeQDs through activity measurement and fluorescence spectroscopy.

According to the kinetic studies (Table 3), a specific constant ratio of L-phenylalanine to L-tyrosine for the enzyme in the presence of CdTe540 has decreased. Also, specific activityof L-phenylalanine and L-tyrosine as the substrates in the presence of CdTeQDs were decreased (Tables 1 and 2). Also other kinetic parameters such as K_m and V_{max} value of enzyme against L-phenylalanine and L-tyrosine as substrates calculated. K_m value of enzyme for these two substrates has decreased in presence of CdTe and with decreasing V_{max} for both of these substrate. From these data, it can be concluded that CdTe may hasinhibitory effects on the enzyme.Inhibitory affectsare shown asanon-competitive pattern.Besides , K_i as an inhibitory kinetic parameter was obtained (Table1,2).Besides calculating kinetic parameters, changes tertiary structure of Phenylalanine dehydrogenase in different concentration of

CdTeQDs for tryptophan residue (at 295 nm wavelength) wereshown that by increasing concentrations of CdTe, the emission spectrum of the enzyme, continuously has decreased (Figs. 3 and 4). That CdTe caused changes inthe structure of the enzyme. Generally, the results suggest that CdTe could have an inhibitory effect on *B. badius* phenylalanine dehydrogenase enzyme activity.

In conclusion, based on the results in this manuscript it may be concluded that in spite of some reported enzymes, incubation of CdTeQDs with Phenylalanine dehydrogenase brought about with loss of specificity towards phenylalanine which has a negative impact on its application, even exhibited a linear fluorescence decrease which show quantitative measurement of enzyme.

Compliance with Ethical Standards

Conflict of interest. The authors declare that they have no conflict of interest.

Funding. No funding was received for this work

Ethics approval. Not applicable

Availability of data and material. Not applicable

Code availability. Not applicable

Informed consent. No human participants were involved in this study

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