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Expression and Purification of Firefly Luciferase and its Interaction with Cadmium Telluride Quantum Dot

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ABSTRACT

Firefly luciferase is a monomeric enzyme of 62 kDa that catalyzes emission of green to yellow region, typically 550-570 nm upon reaction with d-luciferin, ATP, and molecular oxygen. Semiconductor nanocrystal, also known as the quantum dots (QDs), are nanoscaled inorganic particles in size range of 1-10 nm. QDs have properties, such as sharp and symmetrical emission spectra, size-dependent emission, good chemical and photostability and high quantum yield. In this study, recombinant *P. pyralis* luciferase was expressd and then purified based on N-terminal His-tag. Then the effects of the Cadmium Tellurium QD (6.2 nm) on the tertiary structure, kinetic properties, Bioluminescence Resonance Energy Transfer, thermal stability and remaining activity of luciferase was assayed using fluorescence spectroscopy and bioluminescence assay. The results showed that the CdTe QD affects the tertiary structure of the luciferase enzyme. The kinetic parameters of the enzyme also changed, as well as the thermal stability. The remaining activity was decrease in the presence of quantum dot compared to the native enzyme and BRET also was not observed in the presence of QD.

Keywords: Bioluminescence, Luciferase enzyme, Quantum dot, Cadmium tellurium, Bioluminescence resonance energy transfer

INTRODUCTION

Bioluminescence is the emission of light by living organisms. Basically, the enzymes that catalyze the generation of light are called luciferases. Firefly luciferase is a peroxisomal enzyme that converts a cyclicsubstrate luciferin to an excited state oxyluciferin in the presence of ATP, Mg^{2+} and O_2 [1-4] . When excited state oxyluciferin molecules return to ground state, light is produced with high quantum yield [5]. Luciferase enzyme is applied in assesses the amount of ATP in the proportion of microbial contamination through luminescence, screening of drugs, making cancer detection kits, gene reporters [6], analyzing protein interactions [7], bioluminescence imaging in reporter systems [8], pyrosequencing method [9], *in vivo* imaging [10], measuring cell survival, biological sensors, and *etc*. [11]. Luciferase from *P. pyralis* produces light at pH ≈ 7.8 *in vitro*, in the range of green-yellow (λ_{max} = 560 nm) [12,13]. Luciferase from *P. pyralis* folds into two globular domains, a large N-terminal domain consisting of residues 44-435 and C-terminal domain consisting of residues 44-544 [14-16]. Quantum dot (QDs), are nanoscaled inorganic particles in size range of 1-10 nm [17,18]. QDs have properties, such as sharp and symmetrical emission spectra, size-dependent emission, good chemical and photostability and high quantum yield [19,20]. QDs are made up of three layers (Core, shell, cover). The core is selected from group 2 and 5 elements of the Mendeleev table [21]. The sell is selected from semiconductors like ZnS for Enhance optical activity and core protection. The last layer is organic coating which is the place to conjugate various biomolecules, including oligonucleotides, proteins, peptides and small molecules [22]. When electrons in QDs are stimulated by light, electric field, or heat, some of them rise to a higher energy level from a lower energy level (capacity layer). In this case, the capacity layer becomes empty and when the electrons return

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from the top layers to capacity layer the light emission released [23-26].

MATERIALS AND METHODS

Ni-NTA Sepharose column, lactose, ampicillin antibiotics, magnesium sulfate, and luciferin were purchased from Sigma Aldrich (UK). Cadmium tellurium Quantum dot coated with Thioglycolic acid (TGA) (6.2 nm) were prepared from Dr. Sajedi Tarbiat Modares university. All tests repeated in triplicate.

Expression and Purification of *E. coli* BL21 Luciferase Enzyme

Fire fly *P. pyralis* luciferase protein in *E. coli* strain BL21 containing the expression plasmid pET16b histidine-tailed were expressed in LB medium with 40 μg ml^{-1} ampicillin. For this purpose, at first, 10 ml of LB medium was inoculated by one colony containing *E. coli* BL21 Luciferase, and incubated overnight at 37 °C, with shaking at 180 rpm. Then, 1ml of the seeding was added to 250 ml of 2XYT medium with 250 μl ampicillin and incubated at 37 °C for 4 h. Subsequently, when OD_{600} reached ~ 0.6-0.8, 5 μl of 400 mM lactose was added to culture medium and incubated at 18 °C for 12 h. Then after the cell pellets were collected by centrifugation 6000 g for 20 min at 4 °C. The pellets were suspended in lysis buffer (NaCl 300 mM, Tris 50 mM, Imidazole 10 mM), pH 7.8. The suspension was sonicated on ice to disturb bacterial cells. Centrifugation 12000 g for 20 min, at 4 °C was done to collect supernatant, and then *E. coli* strain BL21 luciferase were purified by Ni-Sepharose affinity chromatography column. Protein concentration was determined by Bradford assay using coomassie blue and bovine serum albumin as standards [27].

Enzyme Assay

Quantitative measurement of luciferase activity was performed using a luminometer (Berthold Luminometer FB 14). Luciferase enzyme activity was measured by adding 5 μl substrate (Luciferin 2 mM, $MgSO_4$ 10 mM, ATP 4 mM, and Tris buffer 50 mM) to 5 μl enzyme in a Berthold Luminometer (FB 14) device.

Measurement of the Luciferase Enzyme Absorption Spectra

Absorption spectrum of the expressed luciferase was measured with a Perkin Elmer spectrophotometer Lambda 25 apparatus. A volume of 25 μl of substrate mixture consisting of Luciferin 1 mM, $MgSO_4$ 10 mM, ATP 2 mM and Tris buffer 50 mM pH 7.8 was added to 25 μl of purified luciferase solution in quartz cell. Data were collected over the wavelength range between 280-400 nm.

Measurement of Bioluminescence Emission Spectra

Bioluminescence emission of the expressed luciferase was measured with a Perkin-Elmer spectrophotometer LS55 apparatus. A volume of 15 μl of substrate mixture consisting of luciferin 2 mM, $MgSO_4$ 10 mM, ATP 4 mM and Tris buffer 50 mM pH 7.8 was added to 15 μl of purified luciferase solution in quartz cell. Data were collected over the wavelength range between 450-650 nm without turning on the lamp of the device.

Measurement of the CdTe QD Absorption Spectra

Absorption spectra of the CdTe QD was measurd with a Perkin Elmer spectrophotometer Lambda 25 apparatus. A volume of 30 μl CdTe QD (0.16 μM) added to 30 μl 50 mM Tris buffer, pH = 7.8 at 25 °C in the quartz cell. Data were collected over the wavelength range 300-700 nm.

Measurement of CdTe QD Fluorescence Emission Spectra

Fluorescence emission of the CdTe QD was measured with a Perkin-Elmer spectrophotometer LS55 apparatus. A volume of 20 μl CdTe QD (0.16 μM) added to 140 μl of 50mM Tris buffer, pH = 7.8 at 25 °C in the quartz cell. Data were collected over the wavelength range 400-700 nm.

Determination of CdTe QD Size

The maximum absorption wavelength of CdTe QD was placed in the formula [28].

$$D = (9.8127 \times 10^{-7})\lambda^3 - (1.7147 \times 10^{-3})\lambda^2 + (1.00064)\lambda - (194.84)$$

Also, the size of CdTe QD at 25 °C and pH = 7.8 was measurd with Dynamic Light Scattering technique

(Malvern).

Intrinsic Fluorescence of Luciferase in the Presence of CdTe QD

Intrinsic fluorescence investigation for luciferase enzyme in the presence of CdTe QD was performed by PerkinElmer spectrophotometer (LS55) apparatus. At first, serial dilutions of the CdTe QD with a concentration of (0/16, 0.08, 0.04, 0.02, 0.01, 0/005, 0/0025, 0/00125, 0/000625, 0/00031 μM, 0/00015, 0/00078 and 0/000039 μM) was made by 50 mM Tris buffer, pH = 7.8. A volume of 30 μl from different concentrations of CdTe QD (0.16 μM) added to luciferase protein (0.6 μg ml^{-1}) in the quartz cell. Data were collected in 295 nm excitation wavelength and emission spectra were recorded between 250 and 450 nm [2,29-31].

Kinetic Properties of Luciferase Enzyme in the Presence and Absence of CdTe QD

Kinetic properties of native and CdTe QD included luciferase were measured at 25 °C. To determine luciferin Km, 5 μl of assay reagent (10 mM $MgSO_4$, 4 mM ATP in 50 mM Tris, pH 7.8) was mixed with 8 μl of various concentrations of luciferin (5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 and 0.00097 mM) in a tube. The reaction was initiated by adding 5 μl of enzyme to 5 μl of substrate and light emission was recorded by luminometer (FB 14 Berthold Detection System, Germany). The reaction in the presence of CdTe QD was initiated by adding 2.5 μl of enzyme to 2.5 μl of QD and 5μl substrate, and then light emission was recorded. Determination of ATP kinetic constant was performed in a similar way. 2 μl from various concentrations of ATP (40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312 mM, 0.156 and 0.078 mM) were mixed with 16μl assay reagent (10 mM $MgSO_4$, 2 mM luciferin in 50 mM Tris, pH 7.8). The reaction was initiated by adding 2 μl enzyme, and light emission was recorded. Also the reaction in the presence of CdTe QD was initiated by adding 1 μl of enzyme to 1 μl of QD and 18 μl substrate, then the and light emission was recorded. Apparent kinetic parameters were calculated by Michaelis-Menten and Lineweaver-Burk plots by using PRISM software.

Thermal Stability Studies of Luciferase Enzyme in the Presence of CdTe QD

Thermal stability of the luciferase enzyme in the presence and absence of CdTe QD was investigated. For the measurement of thermal stability, the purified enzyme (0.6 mg ml^{-1}) and enzyme (0.6 mg ml^{-1}) with (0.02 μM) quantum dot were incubated at 4, 20, 25, 30, 35, 40, 45 °C for 5 min. Before assaying the enzyme activity at room temperature, the enzymes were cooled in an ice-water (4 °C) bath for 2 min.

Remaining Activity Studies of Luciferase Enzyme in the Presence of CdTe QD

For the measurement of remaining activity, the purified enzyme (0.6 mg ml^{-1}) and enzyme(0.6 mg ml^{-1}) with (0.02 μM) QD were incubated at 4, 25, 32 °C for 60 min and after about 5 min, some of it was taken. Before assaying the enzyme activity at room temperature, the enzymes were cooled in an ice-water (4 °C) bath for 2 min.

Bioluminescence Resonance Energy Transfer Studies

BRET emission spectrum investigation between luciferase enzyme and CdTe QD was performed by PerkinElmer spectrophotometer (LS55) apparatus and Checked in three modes. A solution of the (0.6 mg ml^{-1}) luciferase protein was incubated with (0.02 μM) CdTe QD for 3hours at room temperature (25 °C) and then the mixture wax added to substrate mixture consisting of luciferin 2 mM, $MgSO_4$ 10 mM, ATP 4 mM and Tris buffer 50 mM (pH 7.8). After that the device was placed in bioluminescence mode and data were collected over the wavelength range 450-650 nm. Once again, the (0.6 mg ml^{-1}) enzyme and (0.02 μM) QD were combined and the process was evaluated without incubation time. In another case, the (0.6 mg ml^{-1}) enzyme and (0.02 μM) QD were mixed then nickel (100 μM) and substrate was added and emission recorded.

RESULTS

Luciferase Enzyme Expression and Purification

The expressed proteins were purified based on the

6His-tagged fusion by affinity (Ni-NTA-Sepharose) chromatography. SDS-PAGE analysis of eluted fractions showed that the enzyme with molecular weight of 62 kDa was efficiently purified (Fig. 1).

Luciferase Enzyme Absorption Spectra

The *in vitro* absorption spectra of luciferase enzyme with the substrate were measured at 25 °C and pH 7.8. The luciferase showed spectrum with a single peak at 340 nm (Fig. 2).

Bioluminescence Emission Spectra of Luciferase Enzyme

The *in vitro* bioluminescence spectra of luciferase enzyme with the substrate were measured at 25 °C and pH 7.8. The luciferase exhibit spectrum with a single peak at 560 nm (Fig. 3).

CdTe QD Absorption Spectra

Absorption spectra of TGA coated CdTe QD were measured at 25 °C (pH 7.8). The CdTe QDs exhibited spectrum with a single peak at 400 nm (Fig. 4).

Fluorescence Emission Spectra of CdTe QD

Fluorescence emission spectra of TGA coverd CdTe QD were measured at 25 °C and (pH 7.8). The CdTe QD exhibited spectrum with a single peak at 610 nm (Fig. 5).

Intrinsic Fluorescence Measurements

The tertiary structural changes were monitored by the intrinsic fluorescence analysis for native luciferase enzyme and luciferase enzyme in the presence of CdTe QDs. Luciferase contains several tryptophan residues, which can be excited at 295 nm. Purified native luciferase alone and in the presence of CdTe QDs were compared by fluorescence spectroscopy. Alteration in the emission spectra from tryptophan can be seen in response to the local environment changes surrounding the aromatic ring of tryptophan. Any changes in the enzyme conformation and oxidation states usually affects tryptophan fluorescence. As shown in Figs. 6 and 7, luciferase in the presence of CdTe QDs demonstrated the lower fluorescence emission as the QD concentration increases at 295 nm in comparison with native protein.

Determination of Kinetic Properties

Enzyme substrate affinity (Km) value was calculated for both native luciferase enzyme and luciferase in the presence of CdTe QD with different concentrations of ATP substrate (Figs. 8a and b) and luciferin substrate (Figs. 8c and d), after drawing Line weaver-Burk plot. Km and V_{max} for ATP and luciferin was calculated in the presence of CdTe QD which showed significant changes compared to the native enzyme. Km values of luciferase enzyme for ATP and luciferin in the presence of CdTe QD were decreased. V_{max} values of luciferase enzyme in the presence of CdTe QD for ATP substrate were decreased and for luciferin substrate were increased. Kinetic properties (Km and V_{max} for luciferin and ATP) of native and enzyme in the presence of QDs is summarized in Tables 1 and 2.

Thermal Stability of the Native and Luciferase in the Presence of CdTe QD

The thermal stability analysis of native and luciferase enzyme-QD showed a decrease in thermostability for luciferase enzyme-QD compared to the native, as indicated in Fig. 9.

Remaining Activity of the Native and Luciferases in the Presence of CdTe QD

The remaining activity analysis of native and luciferase enzyme-QD during 60 min at 4, 25 and 32 °C showed a decrease in activity for luciferase enzyme-QD compared to the native (Fig. 10).

BRET Process

As the bioluminescence spectrum of the luciferase was not matched to the CdTe QD excision wavelength, BRET was not observed in any of the modes and only the bioluminescence spectra of luciferase enzyme were observed at 560 nm (Fig. 11).

DISCUSSIONS

Bioluminescence has found extensive application in biotechnology and molecular biology due to easy detection of the light emission [28]. Luciferase has wide applications such as including infection disease monitoring,

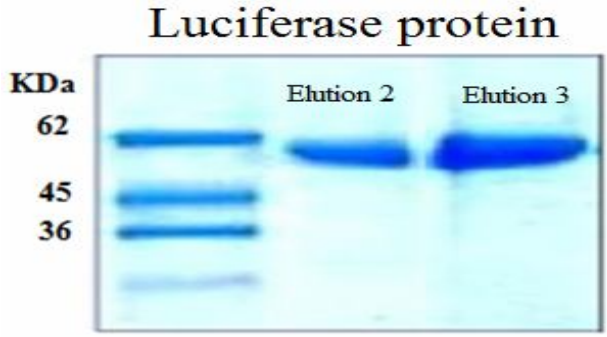

Fig. 1. The SDS-PAGE analysis of luciferase protein purification.

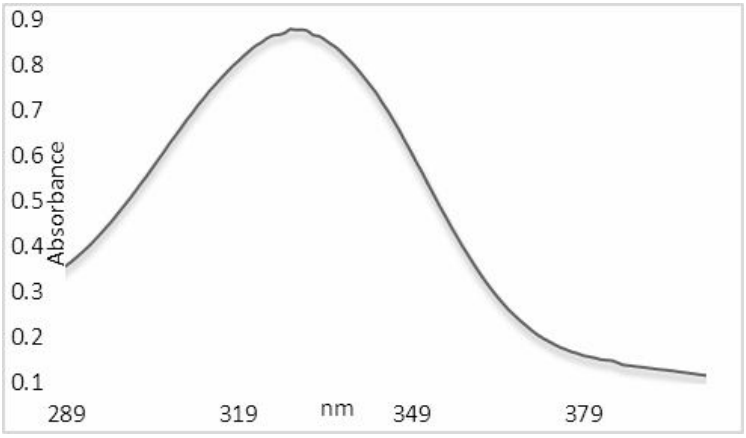

Fig. 2. Absorption spectra produced by the native *P. pyralis* luciferase.

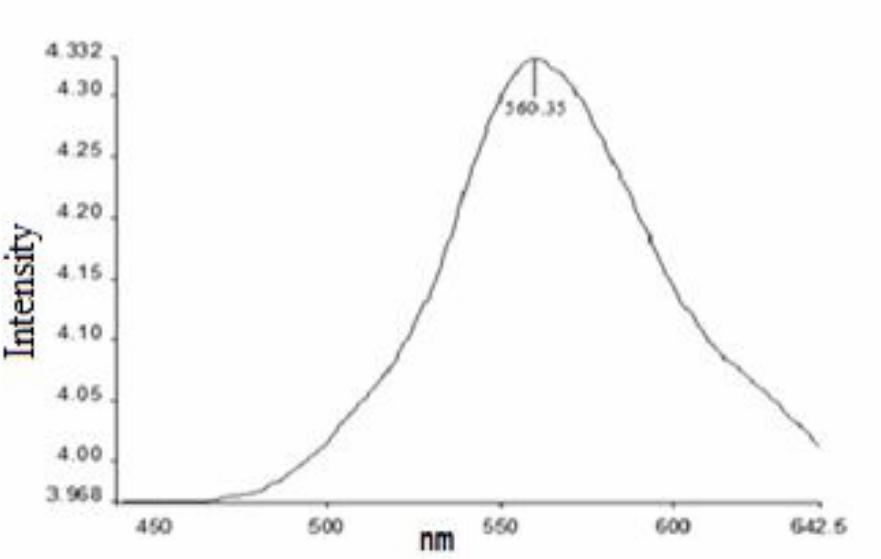

Fig. 3. Bioluminescence emission spectra produced by the native *P. pyralis* luciferase.

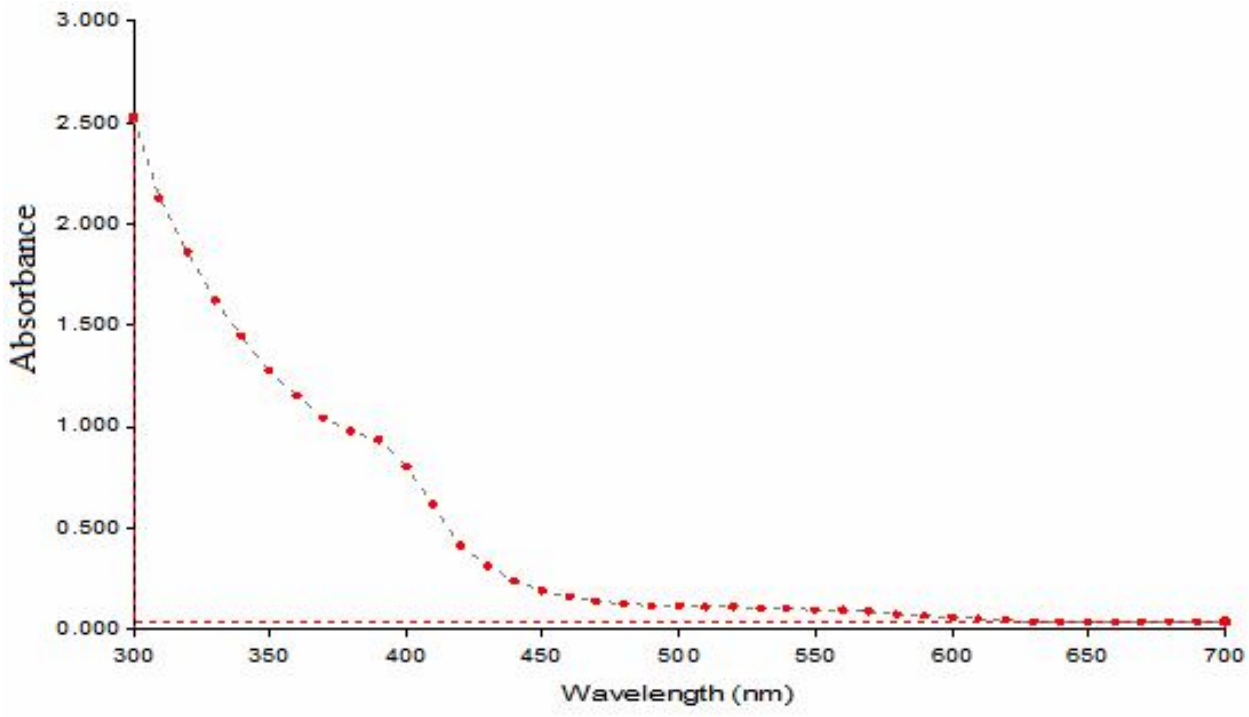

Fig. 4. Absorption spectra produced by the native CdTe QDs.

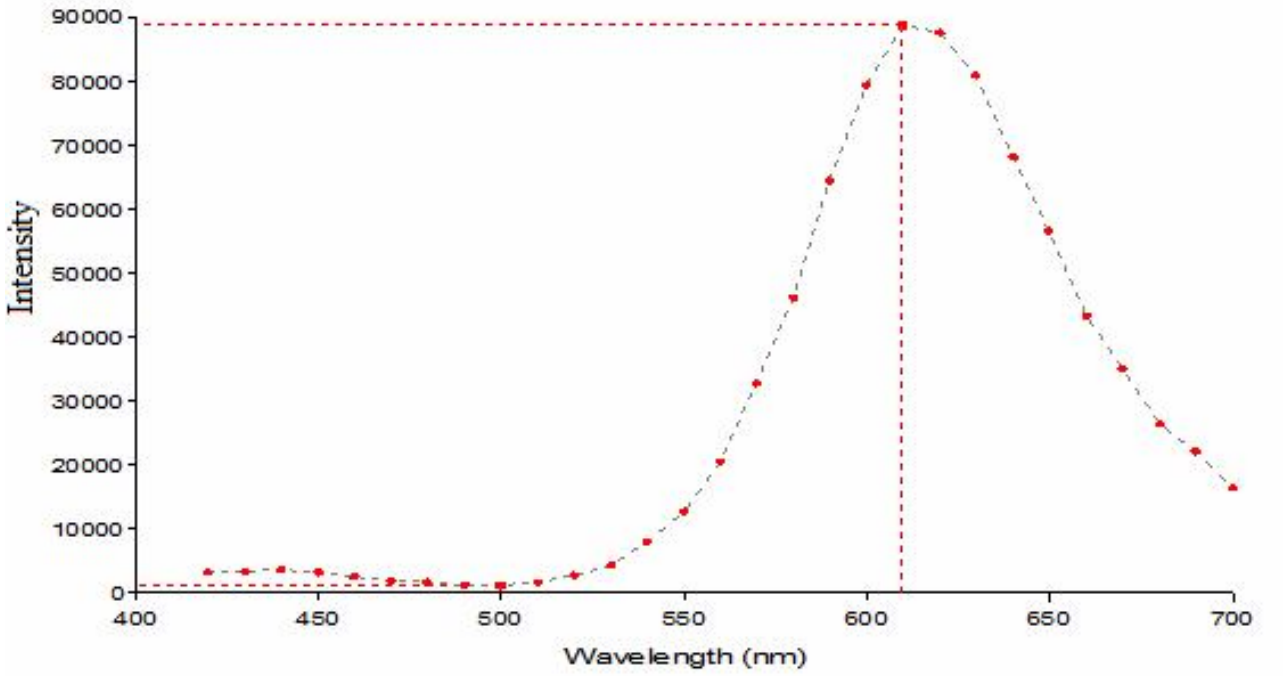

Fig. 5. Fluorescence emission spectra produced by the native CdTe QD.

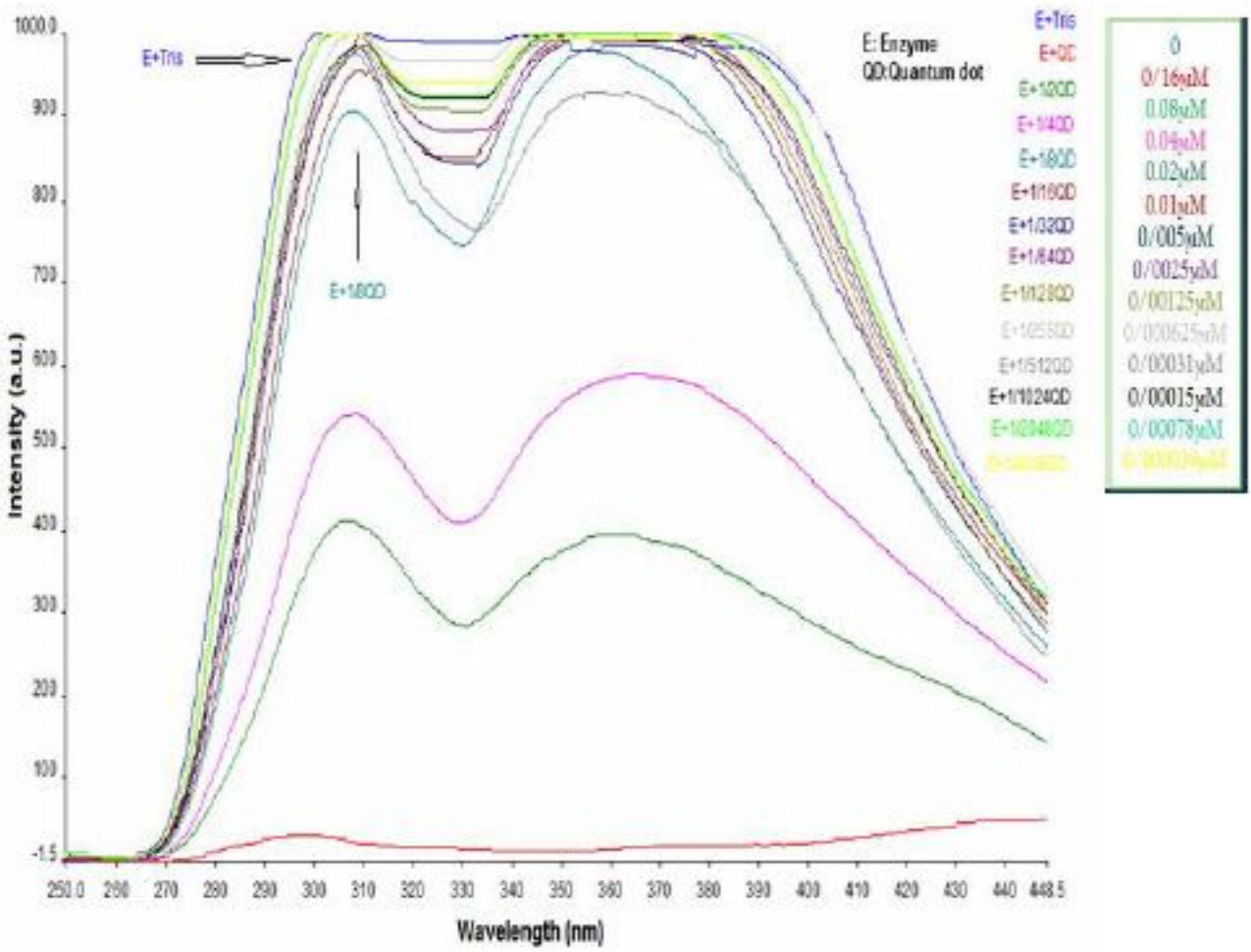

Fig. 6. Intrinsic fluorescence spectra of native luciferase enzyme and luciferase in the presence of CdTe QD. Spectra were recorded at 25 °C and pH 7.8. The concentration of proteins was 0.06 mg ml^{-1}. The excitation wavelength was 295 nm.

environmental pollutions diagnosis, gene therapy and *in vivo* tumor studies to split-luciferase-based biosensors [32-36]. Instability of native luciferase at room temperature as a limiting factor causes fast decrease in enzyme activity which in turn results in decrease in accuracy and sensitivity of measurements based on luciferase [12]. Quantum dots have many benefits such as stem cell identification, cell identification in biomonitoring, imaging and targeting of cancer tumors, intracellular study, pathogenesis, protein labeling, cellular proteins, energy transfer analysis, resonance fluorescence, diagnosis of pathogens of vetoxins, cell imaging and *etc.* [7,37,38]. Quantum dots have the ability to diffuse light and also connect to proteins at their surface and enter to the specific cells [39].

Study of the Kinetic Parameters

Kinetic properties of luciferase enzyme showd that Km values of luciferase enzyme for ATP and luciferin in the presence of CdTe QD (6.2 nm) were decreased. V_{max} values of luciferase enzyme in the presence of CdTe QD for ATP substrate were decreased and for luciferin substrate were increased. Previously the effects of silver nanoparticles on the activity of luciferase enzyme activity was investigated by A. K¨akinen and colleagues showed that the silver nanoparticle decreases the activity of the enzyme. Overall Ag^{+} showed a higher affinity for luciferase than for ATP or

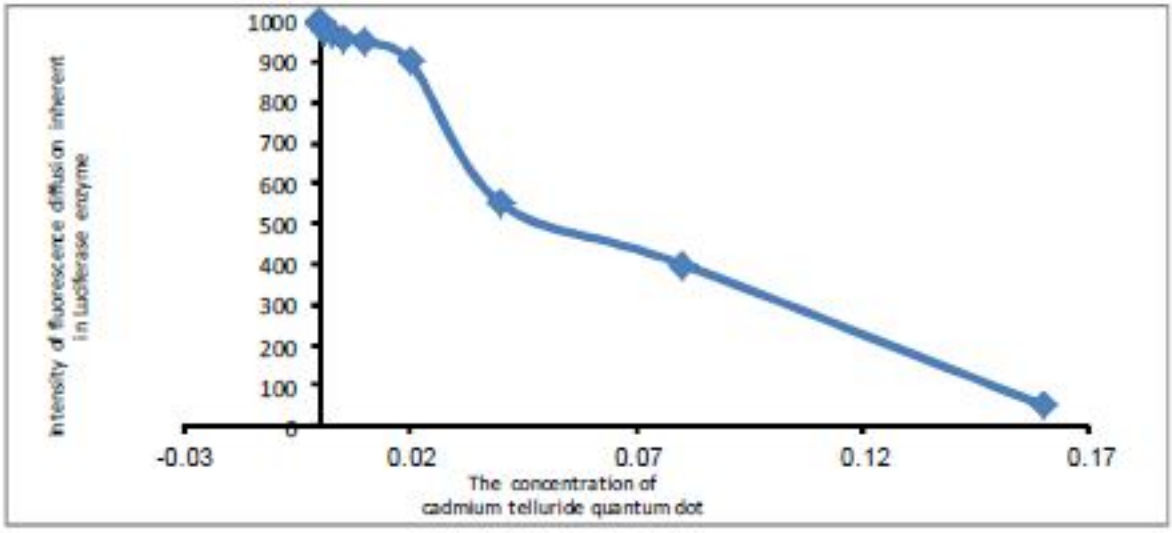

Fig. 7. Intrinsic fluorescence spectra of native luciferase enzyme and luciferase in the presence of CdTe QD.

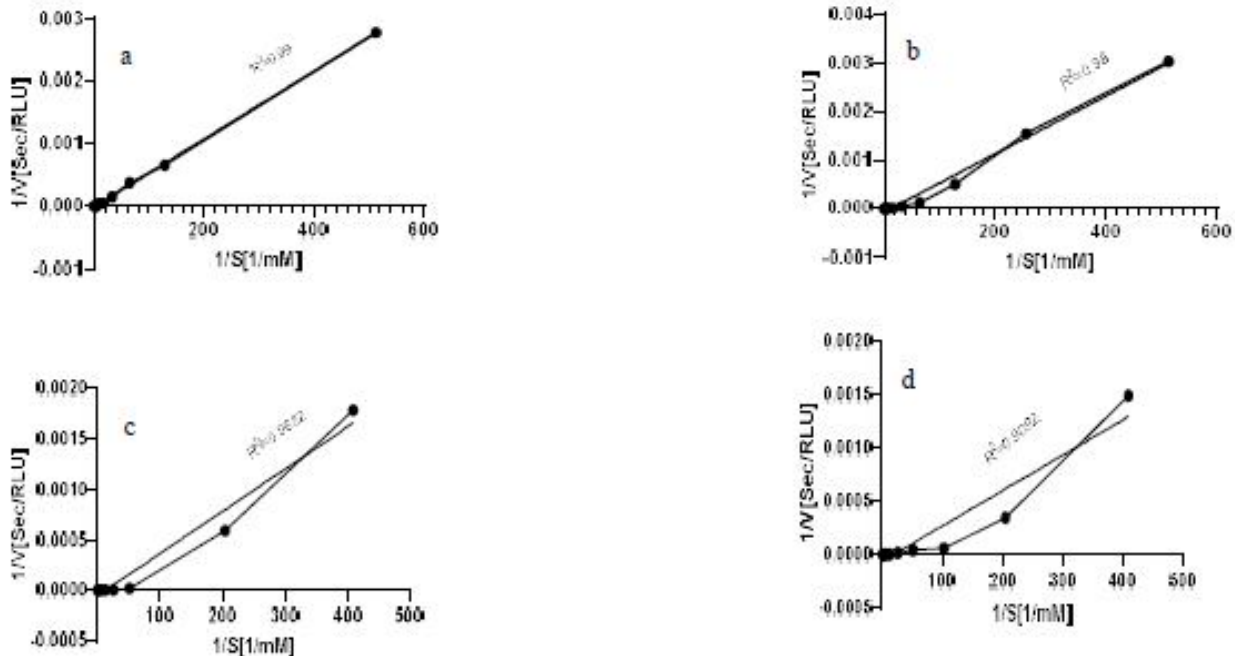

Fig. 8. a) Line weaver-Burk plot of native luciferase enzyme at different concentrations of ATP; b)Line weaver-Burk plot of luciferase in the presence of CdTe QD at different concentrations of ATP; c) Line weaver-Burk plot of luciferase in the presence of CdTe QD at different concentrations of ATP; d) Line weaver-Burk plot of native luciferase enzyme at different concentrations of luciferin.

Table 1. Kinetic Properties of Native and Luciferase in the Presence of CdTe QD with Different Concentration of ATP Substrate

Different concentrations of ATP	K_m (μM)	V_{max} (RLU mg^{-1} s^{-1})
Native enzyme	163	3.27×10^6
Enzyme in the presence of quantum dot	81.29	1.35×10^6

Table 2. Kinetic Properties of Native and Luciferase in the Presence of CdTe QD with Different Concentration of Luciferin Substrate

Different concentrations of Luciferin	K_m (μM)	V_{max} (RLU mg^{-1} s^{-1})
Native enzyme	53.39	1.29×10^9
Enzyme in the presence of quantum dot	52.34	1.58×10^9

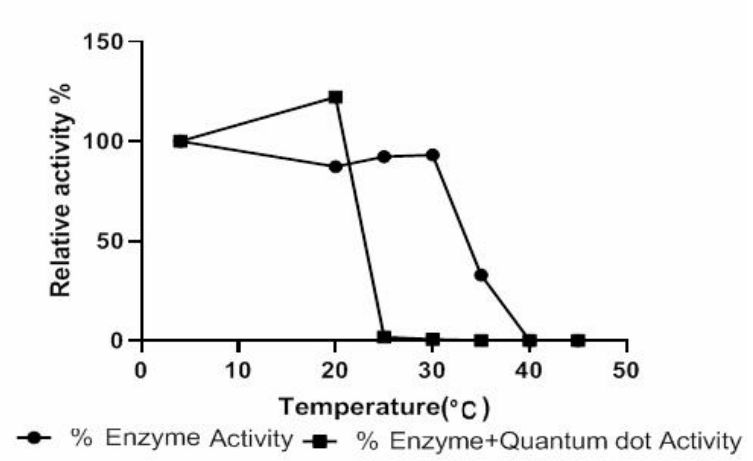

Fig. 9. Comparison of the thermal stability between native luciferase and luciferase in the presence of CdTe QD.

luciferin, judged by the corresponding spectral changes for these ligands [40] and study by A. Noori on the magnetic nanoparticles supported ionic liquids improve firefly luciferase properties showd that kinetic constants for luciferin in the presence of magnetic nanoparticles supported ionic liquids were almost the same as that of the luciferase in the absence of these compounds. The Km values of luciferase for ATP in the presence of magnetic nanoparticles decreased [41].

Intrinsic Fluorescence of Luciferase

Fluorescence is a useful technique for the evaluation of three-dimensional changes in protein structure because the intrinsic fluorescence of Trp residues is particularly sensitive to the CdTe QD. According to Fig. 6, intrinsic fluorescence spectra of native enzyme and luciferase in the presence of CdTe QD demonstrated that increasing the QD concentration cause a lower fluorescence emission at 295 nm in comparison with native protein. But in some

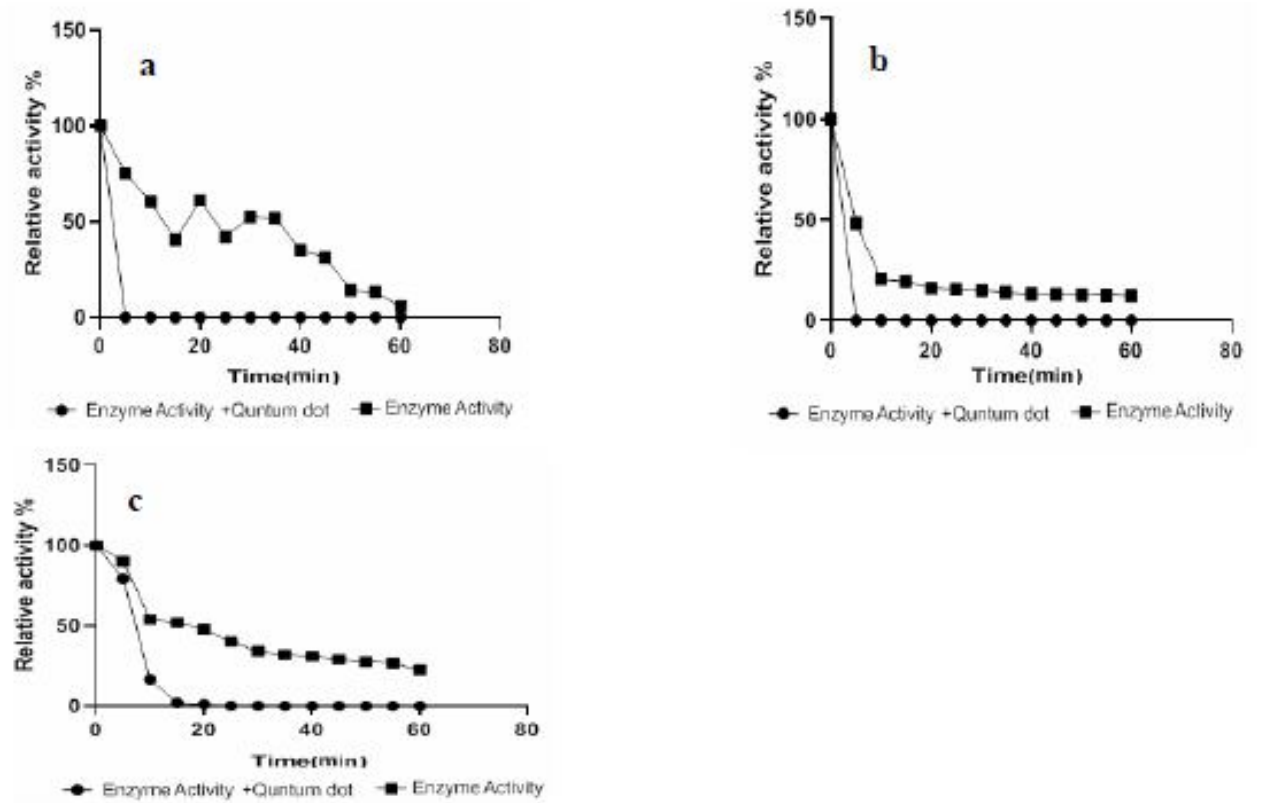

Fig. 10. a) Comparison of the remaining activity between native luciferase and luciferase in the presence of QDs during 60 min at 32 °C (pH 7.8). b) Comparison of the remaining activity between native luciferase and luciferase in the presence of QDs during 60 min at 25 °C (pH 7.8). c) Comparison of the remaining activity between native luciferase and luciferase in the presence of QDs during 60 min at 4 °C (pH 7.8).

previously published studies the increase in the fluorescence intensity of luciferase in the presence of magnetic nanoparticles has been reported [41].

Thermal Stability and Remaining Activity of Luciferases

The thermal stability analysis of native and luciferase enzyme-QD showed a decrease in thermostability for luciferase enzyme-QD compared to the native, indicated in Fig. 9. The remaining activity analysis of naked and luciferase-QD during 60 min at 4, 25 and 32 °C showed also a decrease in activity for luciferase enzyme-QD compared to the native, indicated in Fig. 10. But the results of the thermal stability of luciferase in the presence of magnetic nanoparticles showed that their original activity remained approximately 20 and 18% after incubation at 35 °C for 5 min in the presence of magnetic nanoparticles were preserved, respectively, whereas the native enzyme lost nearly all of its activity [41].

Bioluminescence Resonance Energy Transfer

The bioluminescence spectrum of the luciferase did not match the quantum dot excision wavelength and BRET was not observed in any of the modes, indicated in Fig. 11. But in another study commercially available QD_S with carboxylic acid presented at the surface (QD-COOH) was used. In the presence of Ni^{2+} cations, as the carboxylic acid on the QD_S bound the metal ions and formed complexes with the 6 × His tag on the luciferase fusion protein, BRET was seen and light emission from the QDs was showed [42].

CONCLUSIONS

The CdTe QD (6.2 nm) probably affected the tertiary

structure of the luciferase enzyme. The kinetic parameters of the enzyme also changed, as well as the thermal stability and the remaining activity. Decrease in activity of the enzyme in the presence of quantum dot compared to the native enzyme was also observed. These data can be used as a platform for design and build remarkable QD-protein bioconjugates for developing useful bioluminescence-based biosensors in the future.

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