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Expression and purification of TetX2 monooxygenase enzyme and investigating the effect of HAuCl4 on its structure and activity

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ABSTRACT

TetX2 monooxygenase is an enzyme with a molecular weight of 44 kDa. This enzyme catalyzes the selective hydroxylation of tetracycline to 11a-hydroxytetracycline due to the presence of NADPH as a cofactor and O_2 as an electron acceptor. Gold is one of the metals with low toxicity, good solubility, and easy preparation. It is commonly used in the field of biological sciences, particularly in the production of diagnostic biosensors. In this study, recombinant Bacteroides thetaiotaomicron TetX2 monooxygenase was expressed and then purified based on N-terminal His-tags. The effect of $HAuCl_4$ on the tertiary structure of TetX2 monooxygenase, as well as its kinetic parameters, size, thermal stability, and remaining activity, was investigated using a spectrophotometer, fluorescence spectrophotometer, and dynamic light scattering. The results showed that km, Vmax of the enzyme decrease in the presence of $HAuCl_4$. Also, increasing the concentration of $HAuCl_4$ in the presence of TetX2 monooxygenase enzyme caused a decrease in the emission intensity of tryptophan amino acid residues in the protein structure at a wavelength of 295 nm. The remaining activity, thermal stability, and size of the enzyme in the presence of $HAuCl_4$ have also decreased compared to the native enzyme. Evidence showed that $HAuCl_4$ affects the structure of TetX2 monooxygenase enzyme.

Keywords: Bacteroides thetaiotaomicron, TetX2 monooxygenase, tetracycline, protein tertiary structure, gold

INTRODUCTION

The TetX enzyme is a FAD-dependent monooxygenase that extensively degrades tetracycline analogs. Monooxygenase TetX2 has been isolated from the transposon CTnDOT in Bacteroides thetaiotaomicron. The molecular weight of this enzyme is 44 kDa. It catalyzes the hydroxylation of tetracycline to 11a-hydroxytetracycline through the participation

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of NADPH as a cofactor and O_2 as an electron acceptor [1, 2]. The TetX gene encodes an enzyme that enhances tetracycline resistance. This enzyme contains 388 amino acids that require NADPH. The TetX gene was found in transposons Tn 4351 and Tn 4400 of the obligate anaerobic bacterium Bacteroides fragilis. Two orthologs of the main gene, TetX1 and TetX2, have also been identified in another bacteroid transposon, CTnDOT. TetX2 is %99 identical to the original TetX, while TetX1 is an N-terminal truncation (359 amino acids) with %66 similarity to other proteins lacking the FAD binding domain. The transfer of the TetX gene to *Escherichia coli* during aerobic growth leads to antibiotic resistance in bacteria. If tetracycline is present in the culture medium of this bacterium, it is degraded, causing a color change in the medium[3]. The potential of enzymes in relation to tetracycline antibiotics leads us to utilize them for identifying this analyte in the environment. Diagnostic methods based on enzyme reactions offer advantages such as sensitivity and rapid detection, but these techniques are more costly than microbiological methods. Additionally, environmental factors like temperature and pH can influence enzyme performance[4]. Therefore, it is crucial to offer solutions to enhance the efficiency of enzyme activity under various conditions. In recent years, researchers have paid special attention to gold nanoparticles to develop biological nano sensors. Gold metal is particularly significant because of its affordability and diverse applications in biological sciences[5-8]. In this research, after expressing and purifying the TetX2 monooxygenase enzyme, the impact of gold on the kinetic parameters and tertiary structure of the enzyme was investigated.

MATERIALS AND METHODS

TetX2 monooxygenase in *E. coli BL21*, containing the expression plasmid pET-28a with a histidine tag, was provided by Prof. Saman Hosseinkhani from Tarbiat Modares University. Ni-NTA Sepharose column and $HAuCl_4$ were purchased from Nora Gene Pishro Company in Tehran, Iran. Antibiotic kanamycin, lactose, and NADPH were purchased from Sigma Aldrich Company (USA). All experiments were repeated three times.

Expression and purification of TetX2 monooxygenase enzyme from E. coli BL21

The TetX2 monooxygenase protein was expressed in E. coli BL21, which contained the expression plasmid pet-28a with histidine sequences, in LB culture medium supplemented with the antibiotic kanamycin at a concentration of 50 mg/ml. For this purpose, 10 µl of kanamycin antibiotic were added to 10 ml of LB culture medium. Subsequently, a colony of E. coli bacteria was introduced, and the mixture was then incubated for 18 hours at 37 °c with a shaking rate of 180 RPM. In the next step, 1ml of culture medium containing cultured bacteria was added to 2XYT culture medium containing 250 µl of kanamycin antibiotic and incubated for 4 hours at 37°C with a shaking rate of 180 RPM. When the optical density (OD) of the medium reached between 0.6-0.8, we added 5 ml of 400 mM lactatos and incubated it at 16°C for 15 hours at 180 RPM. Bacterial cells were separated from the environment by centrifugation at 6000G for 2 minutes. Then, a bacterial suspension was prepared using lysis buffer (K2HPo4 50 mM, NaCl 500 mM, β-mercaptoethanol 5 mM, and imidazole 20 mM) at pH 8. To destroy the bacterial cell, it was exposed to Suspanone using a pulse sonicator for 20 minutes. It should be noted that this step was done on ice. In order to separate the supernatant, the sample was centrifuged for 20 minutes at 12,000g. Finally, affinity chromatography was employed to isolate the TetX2 monooxygenase protein. For every 900 µl of protein, 100 µl of glycerol (60%) were added to it. Protein expression was determined

using SDS-PAGE, and its concentration was quantified using the Bradford method by constructing a standard curve [2, 27].

Enzyme Assay

The TetX2 monooxygenase enzyme activity was assessed by measuring the consumption of NADPH over a three-minute period. For this purpose, the activity of the enzyme was assessed by measuring the absorbance at 340 nm using a WPA spectrophotometer after adding the substrate (NADPH 0.5 mM, tetracycline 25 µM, and Na2HPo4 0.1 M) to 13 µL of the enzyme(0.7mg/ml).

Determination of TetX2 monooxygenase enzyme size in the presence of HAuCl4

The size of TetX2 monooxygenase protein (0.7 mg/mL, pH 8.5) in the presence and absence of HAuCl4 (0.2 mM, pH 8.5), as well as the size of HAuCl4 (0.2mM, pH 8.5) alone at 25°C, was measured using the dynamic light scattering technique.

Intrinsic Fluorescence of TetX2 monooxygenase in the Presence of HAuCl4

The intrinsic fluorescence of the TetX2 monooxygenase protein was investigated at room temperature and in the presence of various concentrations of HAuCl4 using a PerkinElmer spectrophotometer (LS55). For this purpose, various concentrations of HAuCl4 (5, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5, and 0.2 mM) at pH 8.5 were prepared using double-distilled water. In the next step, various concentrations of HAuCl4, each with a volume of 30 µl, were added to 30 µl of TetX2 monooxygenase protein (0.7 mg/mL, pH 8.5) and then introduced into the quartz cuvette. Finally, the data were collected at wavelengths ranging from 300 to 600 nm, with an excitation wavelength of 295 nm The fluorescence emission intensity graph of the TetX2 monooxygenase enzyme was recorded in the presence and absence of various concentrations of HAuCl4 at the maximum emission wavelength around 310 nm[5, 9-13].

Kinetic Properties of TetX2 monooxygenase Enzyme in the Presence and Absence of HAuCl4

The kinetics of the native enzyme and the enzyme in the presence of HAuCl4 were measured at 25°C and pH 8.5. To determine the Km of tetracycline, various concentrations of tetracycline (25, 20, 15, 10, 5, 2.5, 1.5, and 0.5 µM) were added to the enzyme assay reagent (NADPH 0.5 mM, Na2HPO4 0.1 M). Then, 87 µl of assay reagent were added to 13 µl of TetX2 monooxygenase (0.7 mg/ml), and data were collected at a wavelength of 340 nm over 5 minutes using a WPA spectrophotometer. In the next step, 15 µL of TetX2 monooxygenase (0.7 mg/ml) was added along with 15 µL of HAuCl4 (0.2 mM). Subsequently, 70 µL of the prepared assay reagent was added, and data was collected at a wavelength of 340 nm over a period of 5 minutes.

Thermal Stability Studies of TetX2 monooxygenase TetX2 monooxygenase Enzyme in the Presence of HAuCl4

The thermal stability of TetX2 monooxygenase was measured in the presence and absence of HAuCl4 (pH 8.5). For this purpose, TetX2monooxygenase (0.7mg/ml) and TetX2 monooxygenase (0.7mg/ml) were incubated with HAuCl4 (0.2 mM) at different temperatures (4, 25, 37, 47, 57 °C) for 5 minutes. To adjust the temperature, the sample was immersed in ice water (4°C) for two minutes. Subsequently, the enzyme activity was measured for five minutes at a wavelength of 340 nm using a spectrophotometer.

Remaining Activity Studies of TetX2 monooxygenase Enzyme in the Presence of HAuCl4

The remaining activity of the enzyme was measured in the presence and absence of HAuCl4 (pH 8.5) at a wavelength of 340 nm over a 5-minute period using a spectrophotometer. For this purpose, TetX2 monooxygenase (0.7 mg/ml) and TetX2 monooxygenase (0.7 mg/ml) were incubated with HAuCl4 (0.2 mM) at different temperatures of 4, 25, 37, and 47 °C for 60 minutes. Every 15 minutes, some of the samples were removed and placed on ice water (4°C) for two minutes, and then their activity was measured.

RESULTS

TetX2 monooxygenase Enzyme Expression and Purification

After expression, TetX2 monooxygenase proteins were separated by histidine tags through binding to an affinity chromatography column (Ni-NTA Sepharose). The analysis of the SDS-PAGE gel results of the elutions collected from the chromatography column showed that TetX2 monooxygenase proteins with a molecular weight of 44 kDa were expressed with high specificity (Figure. 1).

Intrinsic Fluorescence Measurements

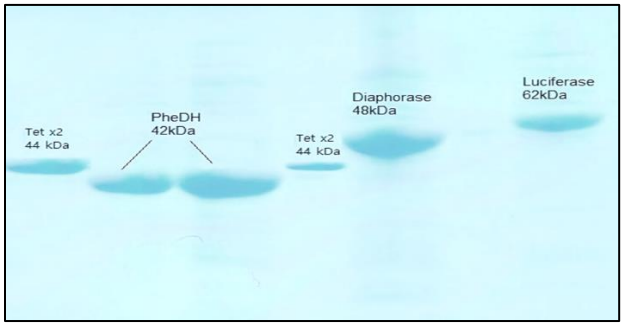

Figure 1. The SDS-PAGE analysis of TetX2 monooxygenase protein purification.

Changes in the tertiary structure of the enzyme in the presence and absence of HAuCl4 have been investigated using intrinsic fluorescence. TetX2 monooxygenase protein contains tryptophan residues in its structure that can be excited at a wavelength of 295 nm. The changes in the surrounding environment of the protein and their effects, such as oxidation or alterations in the protein structure, can be assessed by measuring the emission intensity of the amino acid tryptophan. As depicted in the t, the protein exhibits the highest emission in the absence of HAuCl4. As the concentration of HAuCl4 in the environment increases, the emission intensity decreases (Figure. 2).

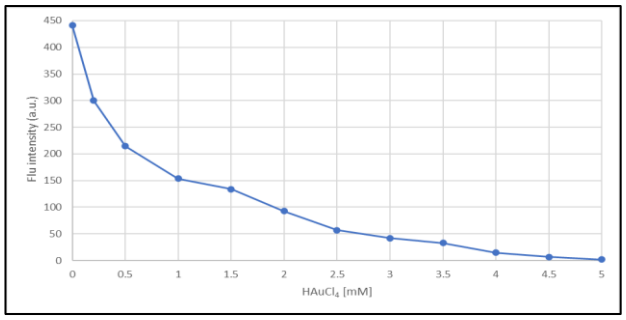

Figure 2. The diagram shows the fluorescence emission intensity of the TetX2 monooxygenase enzyme in the presence and absence of various concentrations of HAuCl4 at the maximum emission wavelength around 310 nm. Spectra were recorded at 25°C and pH 8.5. The concentration of proteins was 0.7 mg/ml. The excitation wavelength was 295 nm.

The size of the TetX2 monooxygenase enzyme in the presence of HAuCl4

The size of Tetx2 monooxygenase protein (0.7 mg/mL) is 828.7 d.nm at 25°C and pH 8.5. The size of Tetx2 monooxygenase protein at 25°C and pH 8.5 in the presence of HAuCl4 (0.2mM) is 276.8 d.nm . The size of HAuCl4 at 25°C and pH 8.5 is 227d. nm (Figure. 3).

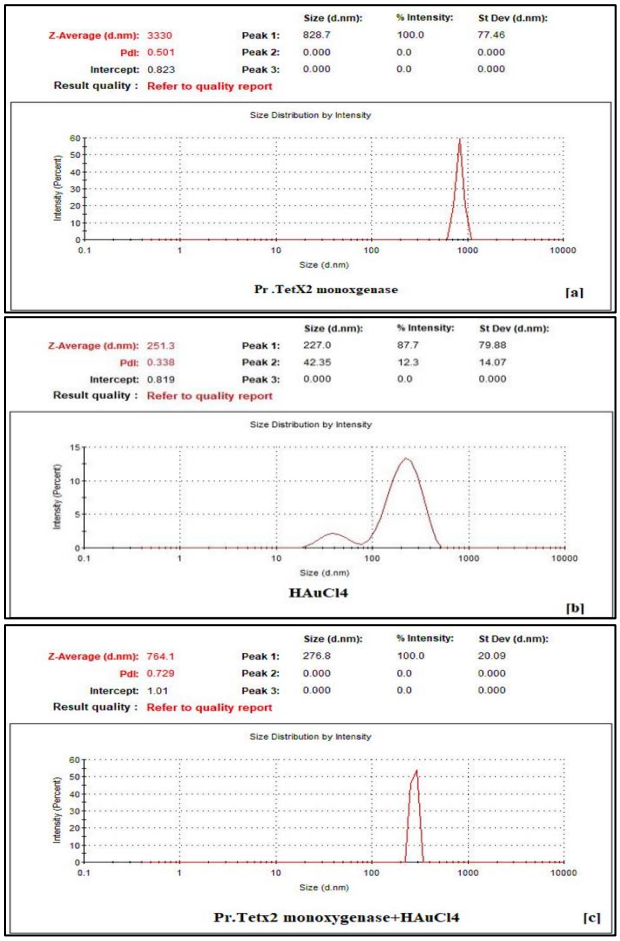

Figure 3. a) shows the size of TetX2 monooxygenase (0.7 mg/ml), **b)** HAuCl4 (0.2 mM), and **c)** TetX2 monooxygenase enzyme (0.7 mg/ml) in the presence of HAuCl4 (0.2 mM) by dynamic light scattering at room temperature (25°C) and pH 8.5.

Determination of Kinetic Properties

The affinity of the TetX2 monooxygenase enzyme (0.7 mg/ml) to the substrate and the enzyme in the presence of HAuCl4 (0.2 mM) was measured at various concentrations of tetracycline using the Michaelis–Menten and Lineweaver-Burk diagram (Figure. 4). The calculation of Km and Vmax values for tetracycline revealed that the Km and Vmax of the enzyme in the absence of HAuCl4 were higher than when the enzyme was in the presence of HAuCl4 (Table. 1).

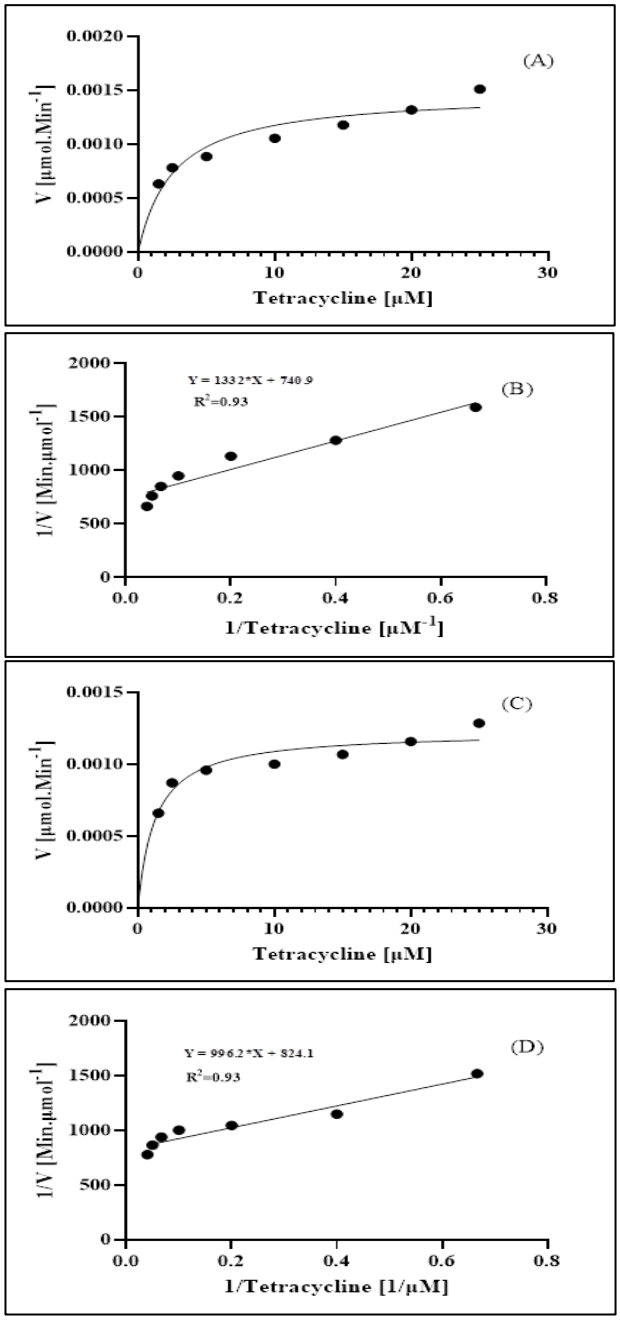

Figure 4. A) Michaelis-Menton and **B)** Lineweaver-Burk plot of native TetX2 monooxygenase (0.7mg/ml) enzyme at different concentrations of Tetracycline. **C)** Michael Menton and **D)** Line weaver-Burk plot of native TetX2 monooxygenase (0.7mg/ml) enzyme in the presence of HAuCl4 (0.2mM) at different concentrations of Tetracycline.

Table 1. Kinetic Properties of Native and TetX2 monooxygenase in the Presence of HAuCl4 with Different Concentration of Tetracycline Substrate

Different concentration of Tetracycline	Km(µM)	V_{max} (µM.min^{-1})
TetX2 monooxygenase	1.8	1.34×10^{-3}
TetX2 monooxygenase+$HAuCl_4$	1.2	1.21×10^{-3}

Thermal Stability of the Native enzyme and TetX2 monooxygenase in the Presence of HAuCl4

The analysis of the results showed that the thermal stability of the enzyme in its natural state is higher compared to the enzyme in the presence of HAuCl4, and the presence of HAuCl4 decreased the enzyme's thermal stability (Figure. 5).

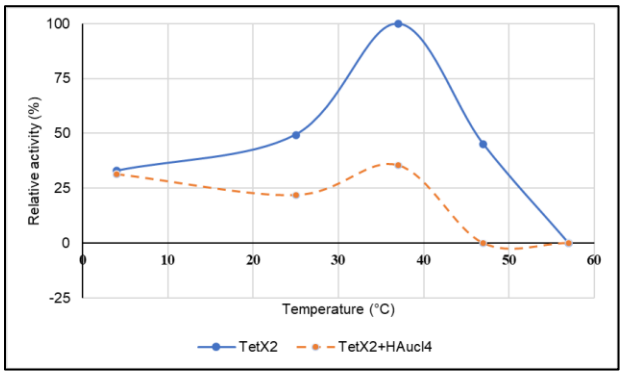

Figure 5. Comparison of the thermal stability between native TetX2 monooxygenase and TetX2 monooxygenase in the presence of HAuCl4.

Remaining Activity of the Native enzyme and TetX2 monooxygenase in the Presence of HAuCl4

The analysis of the results revealed that the remaining activity of the enzyme is higher in its normal state compared to when it is in the presence of HAuCl4. Furthermore, the presence of HAuCl4 was found to decrease the residual activity of the enzyme (Figure. 6).

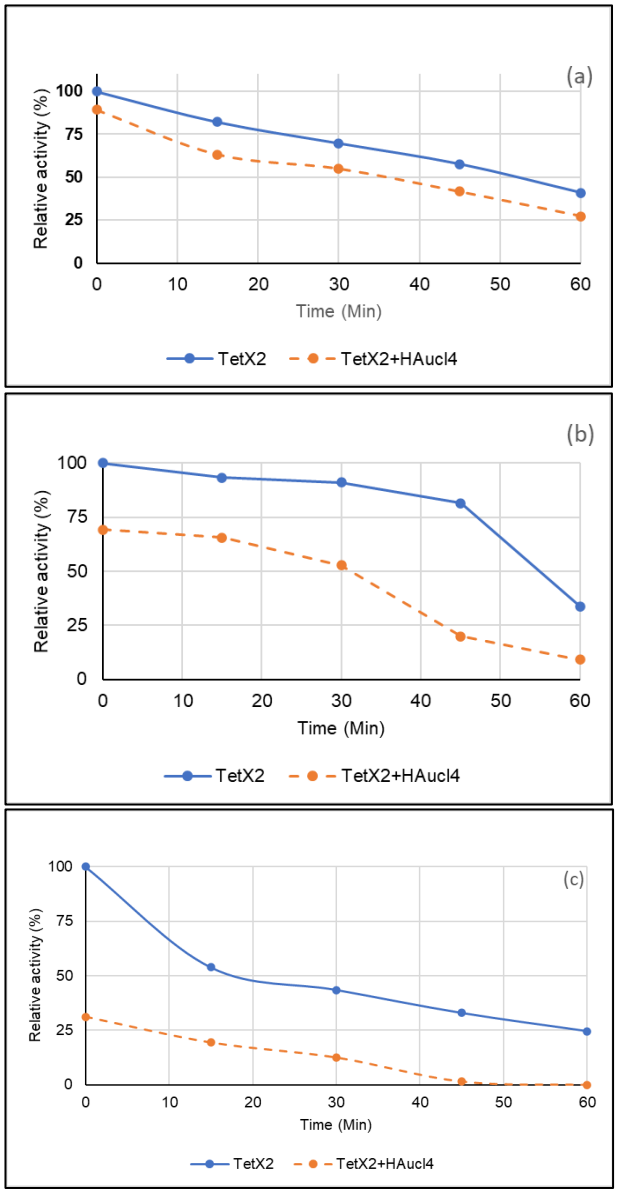

Figure 6. Comparison of the remaining activity of the native TetX2 monooxygenase enzyme (0.7 mg/ml) in the presence and absence of HAuCl4 (0.2 mM) was conducted over 60 minutes at pH 8.5 and temperatures of a) 4°C, b) 25°C, and c) 37°C.

DISCUSSIONS

Tetracyclines are broad-spectrum antibiotics that are effective against a wide range of Gram-positive and Gram-negative bacteria [14, 15]. Tetracyclines are typically bacteriostatic agents that do not kill bacteria but inhibit bacterial

growth by halting protein synthesis. Therefore, the mode of operation of tetracycline includes the absorption of tetracyclines by bacteria and the binding of tetracycline to ribosomes [1, 16]. The indiscriminate use of tetracycline antibiotics in industries, such as food production, has caused serious harm to consumers of products containing this substance. Therefore, rapid detection of this antibiotic is a crucial step in enhancing the quality and safety of food products. There are many methods to detect tetracycline, such as microbiology assay [17], HPLC, ELISA, CE, and LC/MS [18-20]. However, these methods have disadvantages, such as long duration, complexity, and expensive tools for detection [21,22]. The potential of TetX2 monooxygenase enzyme can offer an effective approach for specific, rapid, and accurate diagnosis in identifying tetracycline antibiotics [28, 29]. It should be noted that diagnostic methods based on enzyme reactions are sensitive to environmental factors such as temperature and pH, and their performance may be affected [4]. Therefore, the utilization of analytes to enhance the efficiency and performance of enzymes is highly significant. Due to special properties such as easy preparation, suitable chemical stability, good water solubility, and low toxicity, gold nanoparticles have been widely studied to create nano biosensors [23-25].

Study of the Kinetic Parameters

Investigation of the kinetic properties of TetX2 monooxygenase enzyme with the specific tetracycline substrate revealed that the Km and Vmax of the enzyme decrease in the presence of HAuCl4. In the research conducted by Shahrashob et al. regarding the effect of HAuCl4 on the activity of the enzyme phenylalanine dehydrogenase, it has been shown that HAuCl4 reduces the activity of the enzyme [5].

Intrinsic Fluorescence of TetX2 monooxygenase enzyme

Fluorescence is a valuable technique for studying the tertiary structure of proteins because tryptophan amino acids in proteins are responsive to environmental changes. As observed, elevating the concentration of HAuCl4 in the presence of TetX2 monooxygenase enzyme decreases the intensity of fluorescence emission at a wavelength of 310 nm compared to the native enzyme after excitation at a wavelength of 295 nm (Figure. 2). These results are consistent with Shahrashob's findings on the impact of HAuCl4 on the intrinsic fluorescence of phenylalanine dehydrogenase enzym[5]. In another study, the impact of gold nanoparticles (AuNP) on the superoxide dismutase enzyme was investigated. The study revealed that increasing the concentration of gold nanoparticles enhances the fluorescence of the SOD enzyme [26]. The examination of the enzyme's size in the presence and absence of HAuCl4 revealed that the native enzyme is larger than the protein in the presence of HAuCl4. This suggests that the tertiary structure of the protein undergoes changes in the presence of HAuCl4.

Thermal Stability and Remaining Activity of TetX2 monooxygenase

The results of the thermal stability study of the TetX2 monooxygenase enzyme in the presence and absence of HAuCl4 indicate that the native enzyme is more stable at various temperatures compared to the TetX2 monooxygenase enzyme in the presence of HAuCl4. Also, the remaining activity of the native enzyme and TetX2 monooxygenase in the presence of HAuCl4 at temperatures of 4, 25, and 37 °C, and pH 8.5 for 60 minutes, indicated that the enzyme exhibits higher activity in the absence of HAuCl4 compared to TetX2 monooxygenase in its presence.

CONCLUSIONS

TetX2 monooxygenase is a FAD-dependent monooxygenase that degrades a wide range of tetracycline analogs. The catalytic ability of this enzyme in the selective hydroxylation of tetracycline to 11 a-hydroxytetracycline through the participation of NADPH as a cofactor and O2 as an electron acceptor can be used as an indicator for quick, accurate, and specific identification of tetracycline antibiotics. This can help prevent damage to the environment, public health, and the food industry. Therefore, it is very important to use analytes to enhance the efficiency of enzyme activity. Gold particles have attracted the attention of researchers in recent years due to their low toxicity, bio-binding ability, and the creation of detectable spectra. The aim of this research was to investigate the effect of HAuCl4 on the activity and structure of the TetX2 monooxygenase enzyme. The results showed that the tertiary structure of the TetX2 monooxygenase enzyme likely changes in the presence of HAuCl4. The investigation of kinetic parameters, such as Km and Vmax, as well as indicators like thermal stability and remaining activity, revealed that the enzyme's activity decreases in the presence of HAuCl4 compared to the native enzyme. These results can be used in the future to utilize the TetX2 monooxygenase enzyme as a novel platform for synthesizing gold nanoclusters on the TetX2 protein substrate. Utilizing the characteristics of TetX2 monooxygenase enzyme and the unique properties of HAuCl4 can be beneficial in designing biosensors for detecting tetracycline antibiotics.

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REFERENCES

[1] F. Granados-Chinchilla, C. Rodríguez, J Anal Methods Chem. (2017) 1315497.
[2] M. Besharati, et al., Biotechnol Appl Biochem. (2022) 69:41–50.
[3] W. Yang, et al., J Biol Chem. (2004) 279:52346–52352.
[4] C. Cháfer-Pericás, A. Maquieira, R. Puchades, TrAC Trends Anal Chem. (2010) 29:1038–1049.
[5] M. Shahrashoob, et al., Biomacromol J. (2021) 7:18–24.
[6] M. Shamsipur, A. Pashabadi, F. Molaabasi, RSC Adv. (2015) 5:61725–61734.
[7] F. Molaabasi, et al., RSC Adv. (2015) 5:33123–33135.
[8] M. Shamsipur, et al., Microchim Acta. (2015) 182:1131–1141.
[9] F. Ataei, S. Hosseinkhani, K. Khajeh, J Biotechnol. (2009) 144:83–88.
[10] M. Mortazavi, S. Hosseinkhani, Protein Eng Des Sel. (2011) 24:893–903.
[11] Z. Amini-Bayat, et al., BBA Proteins Proteom. (2012) 1824:350–358.
[12] S. Hosseinkhani, Cell Mol Life Sci. (2011) 68:1167–1182.
[13] E. Samadi, et al., Future Med Educ J. (2020) 7(4):308–14.
[14] W. Yang, Tetx2 A Tetracycline Inactivating Enzyme. (2003).
[15] A.S. Schmidt, et al., Appl Environ Microbiol. (2001) 67:5675–5682.
[16] S. Giguère, Antimicrobial Therapy Vet Med. (2013) 66(7): 1–10.
[17] G. Kebede, et al., Afr J Basic Appl Sci. (2014) 6:87–97.
[18] F. Cacciola, P. Dugo, L. Mondello, TrAC Trends Anal Chem. (2017) 96:116–123.
[19] H. Sun, et al., J Chromatogr A. (2012) 1237:1–23.
[20] A.G. Frenich, et al., J Chromatogr A. (2004) 1048:199–206.
[21] A. Alshannaq, J.-H. Yu, Int J Environ Res Public Health. (2017) 14:632.

[22] F. Yeni, et al., Food Control. (2014) 40:359–367.
[23] L.-Y. Chen, et al., Biosens Bioelectron. (2013) 43:38–44.
[24] Y. Wang, J.-T. Chen, X.-P. Yan, Anal Chem. (2013) 85:2529–2535.
[25] C.L. Liu, et al., Angew Chem Int Ed. (2011) 50:7056–7060.
[26] M. Eghtedari, et al., Iran J Biotechnol. (2021) 19: e2741.
[27] S. Zomorodimanesh, et al., Biomacromol J . (2019) 1;5(1):35-46.
[28] S. Zomorodimanesh, et al., Food Bioscience. (2024) 1;62:105086.
[29] S. Zomorodimanesh, et al., International Journal of Biological Macromolecules. (2024) 1;283:137777.