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ORIGINAL RESEARCH ARTICLE

Experimental evaluation of kinetics and biochemical characteristics of MnO_2 nanoparticles as high throughput peroxidase-mimetic nanomaterials

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ABSTRACT

Despite the well-known concepts on the intrinsic peroxidase-like activity of MnO_2 nanoparticles, up to date, their biochemical and kinetics characteristics were not investigated, especially, the current information about their performances toward *n*-electron oxidation of 3, 3′-diaminobezedine for producing indamine polymers is on limitation. Therefore, herein, the MnO_2 nanoparticles were synthesized by a simple low-cost co-precipitation method and then characterized by XRD, SEM, and DLS analysis. Besides, their peroxidase-like activity was evaluated upon standard peroxidase enzyme assay, revealing high intrinsic peroxidase-like activity for the as-mentioned MnO_2 nanozymes. Considering their high intrinsic peroxidase-like activity, their optimal biochemical characteristics were quantified by probing the progress of *n*-electron irreversible oxidation of 3, 3′-diaminobezedine in the presence of MnO_2 nanozymes as peroxidase mimics. The maximal activity of the as-mentioned MnO_2 nanoparticles with high intrinsic peroxidase-like activity was observed when the pH and temperature of the reaction media were fixed over 3.0–6.0 and 23 °C–25 °C, in order, revealing very high pH and thermal stability of the as-prepared nanoparticles. The salt stability of these nanoparticles was also checked using NaCl as model salt, revealing that the nanozymatic activity was stable over a salt concentration as high as 3–7 M. In addition, the affinity constant (K_m) and maximum velocity of the nanozyme-catalyzed oxidation of 3, 3′-diaminobezedine were found to be 1.6 mM and 47 nM sec^{-1}, in turn.

Keywords: MnO_2 nanozyme; brown-colored polyDAB; pH stability of nanozymes; thermal stability of nanozymes; kinetics of nanozymes

1. Introduction

The fast development of nanoscience and material chemistry has increased interest in researching new and innovative synthesis methods to produce new nanomaterials with unique catalytic activity[1,2], unique optical properties[3], high active area[4], antibacterial properties[5], and high biocompatibility[6]. The new field of nanozyme-based catalysis, which has been introduced as an alternative to enzyme-based catalysis, is called nanozyme chemistry. On the other hand, nanozymes are known as nanomaterials with high enzyme-like activity and can be used to simulate enzymatic reactions in harsh environmental conditions (for example, higher temperature or wider pH range)[7–10]. As previously reported in the literature[11,12], native enzymes, for instance, native peroxidases or ureases suffer from several disadvantages and drawbacks such as low pH stability, low thermal stability, low recoverability, and no reusability. Commonly, to solve these difficulties

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and drawbacks of native enzymes, the development of enzyme immobilization protocols has been widely considered in the literature[13,14]. Although enzyme immobilization can enhance enzyme stability, however, the immobilized enzymes reveal very lower activity than the native enzymes due to the enzyme inactivation during the immobilization process[15]. Hence to solve these difficulties, the design and development of low-cost nanozymes with higher stability than the native enzymes along with high enzyme-like/mimic activity were considered as an interesting way for performing enzyme-catalyzed reactions in harsh conditions[16–18]. Recently, nanozyme-based systems had been used for several applications in the field of catalysis[19,20], biomedical imaging[21], tumor therapy[22,23], and sensing and detection[24–26].

Manganese dioxide (MnO_2) has been employed for the design and development of different catalytic processes, for example, electro-catalytical, chemical, and photocatalytic processes thanks to its low cost, high catalytic activity, nontoxicity, and high stability compared to other transition metal oxides[27,28]. However, the MnO_2 nanoparticles are known for their high enzyme-like activity with dual oxidase- and peroxidase-like activity which make them suitable nanozymes for sensing applications[29–33].

Considering the above-mentioned literature, since, the MnO_2 nanoparticles are well-known as nanozymes with high intrinsic peroxidase-mimic activity, there are several high-impact reports on their applications in the development of analytical and bioanalytical nanozyme-based sensors. Although the peroxidase-like activity of these nanozymes was evaluated in the literature and their potential application for sensing aims was also reported, however, based on our best knowledge, up to date, the biochemical features and kinetics properties of MnO_2 nanoparticles were not evaluated in any report, especially, toward enzyme-mediated oxidation of DAB. Hence, in this study, as a novel idea, the biochemical properties of an enzyme mimic (not a native enzyme) such as pH stability, thermal stability, and salt stability were investigated also, the kinetic features of these nanozymes were calculated toward irreversible oxidation of DAB.

2. Methods

2.1. Synthesis of MnO_2 nanozymes

To synthesize MnO_2 nanozymes, 150 mg $KMnO_4$ was dissolved in 15 mL of deionized water. Then, 150 µL of 30% hydrogen peroxide and 75 µL of 80% hydrazinium hydroxide were introduced into the solution followed by 5 min stirring. After 2 min, the brown precipitate was collected and then washed five times with deionized water.

2.2. Nanozyme activity assay

A mixture of 40 µL hydrogen peroxide, 0.5 mL of DAB (final concentration of 2.8 mM), and 40 µL of MnO_2-nanozymes (final concentration of 0.015 mg mL^{-1} in the mixture) was added to 2 mL of 0.4 M acetate buffer (pH = 4.0). After 30 min, the UV-Vis spectra against a reagent blank were recorded at 460 nm. It should be noted that the specific activity of the as-mentioned nanozymes (nM s^{-1}) was calculated using the absorption coefficient of the oxidation product at 460 nm (ε = 5500 M^{-1} cm^{-1}). It is notable that for calculating the nanozyme specific active (nM s^{-1}), the concentration of produced DAB-ox in the reaction media was calculated using Absorbance = εbc which ε and b are about 5500 M^{-1} cm^{-1} and 1.0 cm, in order, and c is represented to the concentration of DAB-ox. Afterward, the concentration (in nM) was divided by the reaction time to estimate the calculation of the nanozyme specific activity in nM s^{-1}. Thereafter, the relative activity of the MnO_2 nanozymes was calculated using Equation (1)[34]:

$$\text{Relative activity (\%)} = \text{activity}/(\text{maximum activity}) \times 100 \quad (1)$$

2.3. pH and temperature effect on the nanozymatic activity

The effect of pH on the nanozyme activity was determined by probing their activity over a pH range of 2.0–9.0. Afterward, the relative activity was calculated for each pH using Equation (1), and the plot of activity as a function of pH was used as an index for pH stability measurements. Besides, the thermal stability of the as-mentioned nanozymes was investigated by calculating their activity after incubation at different temperatures for 30 min.

2.4. Salt stability

The nanozyme stability against high salt concentrations as a serious problem of native enzymes was evaluated by recording their nanozymatic activity in reaction media with high salt concentration over 3–7 M. It is notable that NaCl was used as a model salt for this experiment.

2.5. Kinetics studies

The kinetics studies were performed by measuring the activity of the as-mentioned nanozymes as a function of DAB concentrations based on the Michaelis-Menten model. Afterward, the kinetic parameters, V_{max} and K_m were estimated by using the linear plot of Lineweaver-Burk.

2.6. Instrumentation for material characterization

The UV-Vis measurements were carried out using a CT Chorm Tech UV 3300 spectrophotometer. The SEM and TEM images were recorded using a TESCAN-Vega 3 from TESCAN Company (Czech Republic) and transmission electron microscope (Zeiss, model EL10C), in order. The DLS histogram was provided using a Shimadzu SALD-301V particle size analyzer. The XRD pattern was obtained using a Rigaku D/max-3C (Japan).

3. Results and discussion

3.1. Characterization of the as-prepared MnO_2 nanoparticles

The size and morphological properties of MnO_2 nanoparticles were determined using DLS and scanning electron microscopy imaging methods, respectively. Besides, to explore more precise on the evaluation of the morphology and size of the as-prepared nanoparticles, TEM image of the nanoparticles was also recorded. In addition, the crystalline properties of the as-prepared nanozymes were checked using XRD analysis.

3.1.1. SEM imaging

The morphological properties of the as-mentioned manganese dioxide nanozymes were investigated by SEM imaging method. In this regard, the SEM image of the prepared MnO_2 nanozymes was recorded. The results shown in **Figure 1**, revealed that the as-prepared MnO_2 nanozymes have uniform and small size particles. However, the SEM image cannot provide any useful information on their size distribution.

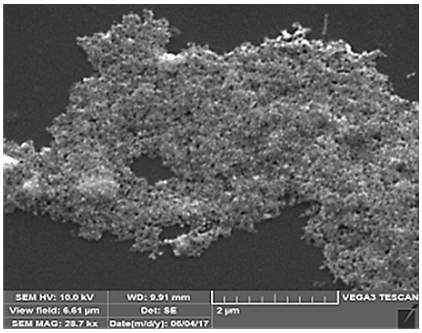


Figure 1. SEM image of the as-prepared MnO_2 nanozymes.

3.1.2. TEM imaging

Since the SEM imaging method cannot provide useful and reliable information about the as-mentioned MnO_2 nanozymes, to explore more precise on evaluation of the morphological properties and particle size of these nanozymes, the TEM imaging method was utilized for characterization of the as-prepared MnO_2 nanozymes. The results shown in **Figure 2** revealed that the as-prepared MnO_2 nanoparticles have a semi-spherical morphology along with an average size as small as 100 nm.

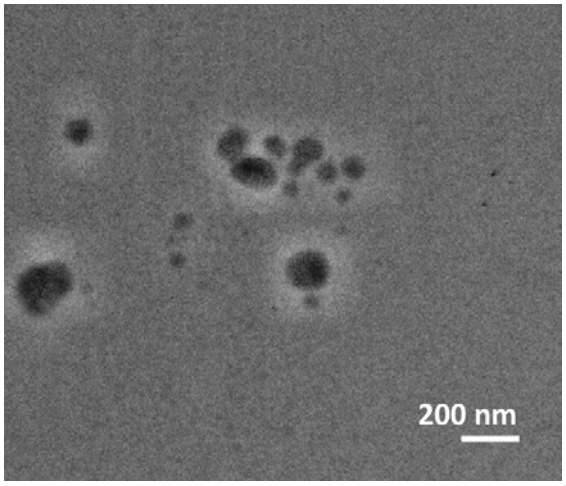


Figure 2. TEM images of the as-synthesized MnO_2 nanoparticles.

3.1.3. Particle size estimation using DLS analysis

For estimation of size distribution and accurate and precise calculation of the average size of the as-prepared nanozymes, the DLS analysis was performed using water as solvent. The histogram of the particle size as a function of frequency and undersize is shown in **Figure 3**. The results shown in **Figure 2** revealed that the as-prepared nanozymes have a size distribution over 64 nm–171 nm with an average size of 109 nm.

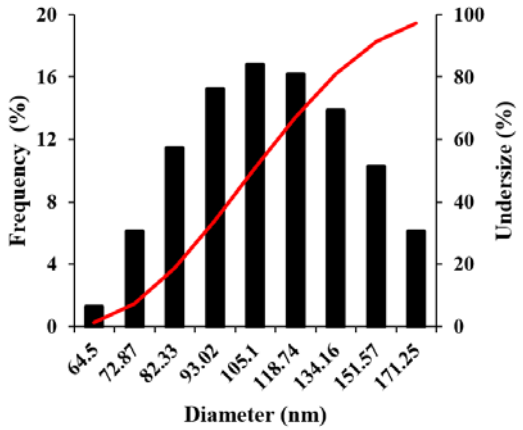


Figure 3. DLS results of the as-prepared MnO_2 nanozymes.

3.1.4. Crystalline characteristics

The crystalline properties of the synthesized MnO_2 nanozymes were investigated using XRD analysis. The results of this analysis are shown in **Figure 4**. As seen in this figure, the results of X-ray diffraction analysis indicate the presence of two characteristic peaks of MnO_2 at the diffraction angles of 23.66 and 60.11 which are assigned to (101) and (312) plans of MnO_2, in order. Notably, the XRD pattern of the as-prepared MnO_2 nanozymes is in good agreement with the reported XRD patterns for MnO_2 nanoparticles[33].

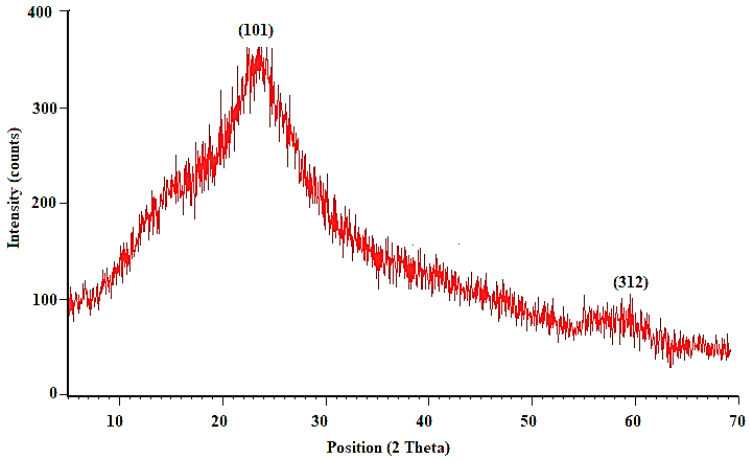


Figure 4. The XRD pattern of the as-prepared MnO_2 nanozymes.

3.2. Investigation of nanozymatic behavior of the as-prepared MnO_2 nanoparticles

The peroxidase-like activity of MnO_2 nanoparticles was investigated using DAB as a peroxidase substrate and its brown-colored oxidation product (i.e., polyDAB) as an analytical probe system (**Figure 5**). As seen in **Figure 5**, in the presence of DAB, the synthesized MnO_2 nanozymes catalyze the oxidation process of DAB with hydrogen peroxide to form its corresponding brown-colored indamine polymer (polyDAB) with a maximum absorbance at 460 nm. In fact, during the oxidation of DAB, MnO_2 nanozymes act on hydrogen peroxide molecules and produce active hydroxyl radicals[24,26,31,33]. Then the generated radicals react with DAB molecules to produce the DAB cation (DAB^+). The DAB^+ then reacts with a DAB molecule to produce a DAB dimer ($(DAB)_2$). By proceeding with this cycle, finally, an indamine polymer was produced as the final product of DAB oxidation, as reported[24,26]. It is notable that the schematic representation of the *n*-electron irreversible oxidation of DAB over MnO_2 nanoparticles is shown in **Scheme 1**.

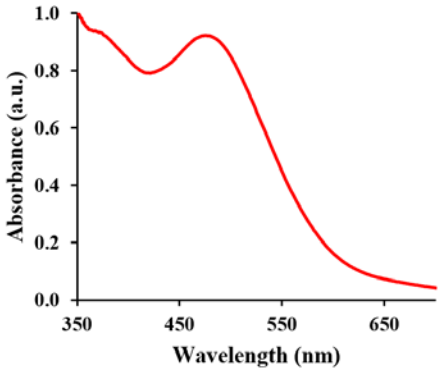
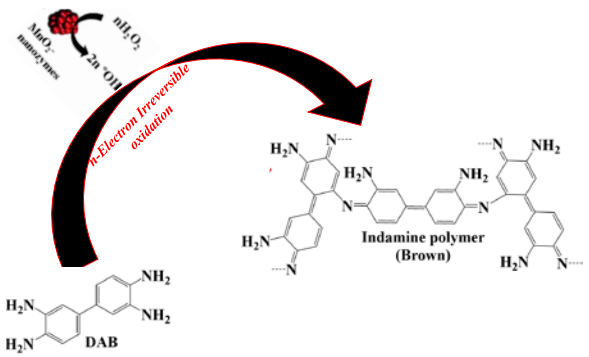


Figure 5. The UV-Visible spectrum of oxidation product of MnO_2 nanozymes-mediated oxidation of DAB.



Scheme 1. Schematic representation of DAB oxidation over MnO_2 nanozymes.

3.3. pH stability

The effect of pH on the nanozyme activity was determined by probing their activity over a pH range of 2.0–9.0. In fact, this experiment can provide insights into the stability of these nanozymes against environmental pH changes. The results are shown in **Figure 6**. According to these results, the maximum nanozyme activity of these nanozymes was estimated over a wide pH range of 3.0–6.0. It should be noted that at harsh acidic conditions (pH = 2) and harsh basic conditions (pH = 9.0), the as-mentioned nanozymes saved 82% and 71% of their maximal activity, in turn, pointing to their high pH stability. Compared to the native peroxidase enzymes, the as-prepared MnO_2 nanozymes showed a very wider pH working range. The native peroxidase enzyme reveals a narrow pH range with an optimal pH of 7.0 using pyrogallol as the peroxidase substrate, as reported. It is notable that at pH = 4.0, the native peroxidase shows only about 30% of its maximal activity[35]. In fact, upon using the as-prepared MnO_2 nanozymes instead of the native peroxidase enzymes, the enzyme-catalyzed reactions can be performed in hash conditions (for example, pH = 3.0) and in a wide pH range. The activity of the as-prepared nanozymes over pH = 3.0–6.0 was found to be independent of the pH variations which is its significant advantage against the native enzymes.

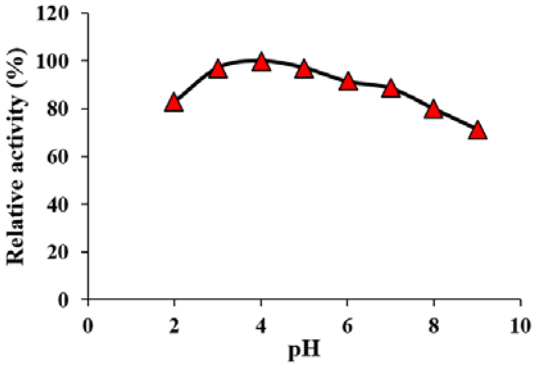


Figure 6. The pH stability of the as-prepared MnO_2 nanozymes.

3.4. Thermal stability

The thermal stability of the as-mentioned nanozymes was evaluated by measuring the relative activity of nanozymes over the temperature range of 23 ℃–40 ℃. The results can obtain useful information about both the optimal temperature range of MnO_2 nanozymes and their stability against environmental temperature variations. The results are shown in **Figure 7**, according to this figure, the maximum nanozyme activity was estimated at a temperature range of 23 ℃–25 ℃ and then it was decreased by increasing the temperature. As a significant advantage from a practical point of view, considering the temperature effect on the nanozyme activity, the as-prepared nanozymes can be utilized for proceeding with the peroxidase-mediated reactions in ambient conditions without needing the complex instruments for temperature controlling. In contrast, the native enzyme shows its maximal activity at 50 ℃, at 30 ℃ and 55 ℃ can save only 40% and 80% of its maximal activity, in order, as reported[35]. Hence, it can be concluded that the native enzyme is characteristically dependent on the temperature of the reaction media while the as-prepared nanozymes can be applied for catalyzing the enzyme-mediated reaction in ambient temperature (23 ℃–25 ℃).

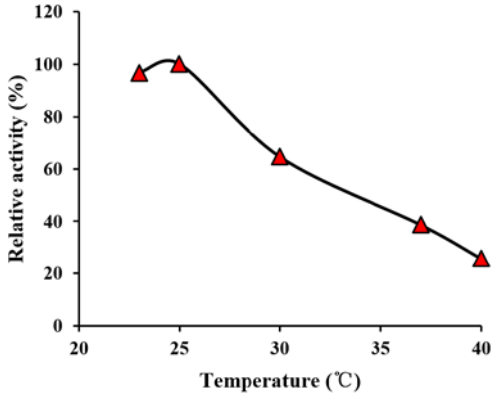


Figure 7. The Thermal stability of the as-prepared MnO_2 nanozymes.

3.5. Salt stability

The nanozyme stability against high salt concentrations as a serious problem of native enzymes was evaluated by recording their nanozymatic activity in reaction media with high salt concentration over 3–7 M of NaCl. It is notable that since 1 M is a moderate salt concentration and the native enzymes such as natural proteases are active in this concentration[36], we checked the salt stability at a very high salt concentration over 3–7 M to prove the higher salt stability of the nanoparticles. The results shown in **Figure 8** revealed that the as-mentioned nanozymes can save their maximal activity over a wide range of high salt concentrations over 3–7 M of NaCl. Based on the above results, it can be concluded that the as-prepared MnO_2 nanozymes can be used for catalyzing the peroxidase-mediated oxidation reactions at high salt concentrations without any decrease in catalytic efficiency and nanozymatic activity instead of the unstable native peroxidase.

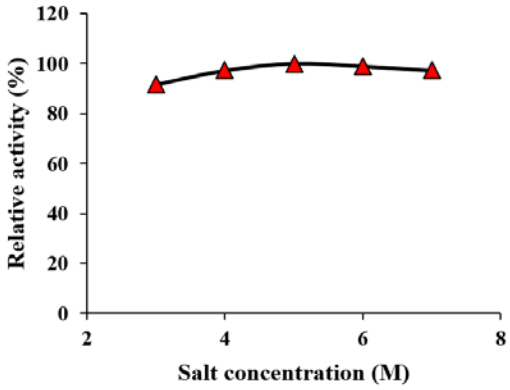


Figure 8. The salt stability of the as-prepared MnO_2 nanozymes.

3.6. Kinetics studies

Kinetic studies were carried out to estimate the kinetic parameters (i.e., K_m and V_{max}) of the as-prepared MnO_2 nanozyme as peroxidase-like nanoenzyme toward *n*-electron irreversible oxidation of 3, 3′-diaminobezedine. It is well known that the V_{max} value reflects the intrinsic properties of the enzyme/nanozyme and is defined as the highest possible rate of the enzyme/nanozyme-catalyzed reaction (i.e., catalytic efficiency) when all enzyme molecules or all nanozyme particles are saturated with the substrate[34,37]. The higher value of V_{max} is assigned to the higher catalytic efficiency of the enzyme/nanozyme. In contrast, the affinity of the substrate of an enzyme/nanozyme to interact with its active site is represented by the K_m value, the lower values indicate a higher affinity of the substrate for binding to the enzyme/nanozyme[34,37]. The estimation of the kinetic parameters of MnO_2 nanozymes was performed by measuring the initial velocity of the nanozyme-mediated reaction as a function of the DAB concentration. The Michaelis-Menten saturation curve and the

Lineweaver-Burk linear plot for the as-mentioned nanozymes were shown in **Figure 9**. As seen in **Figure 9A**, the reaction rate was increased by increasing the DAB concentration and then reached a saturation state after a certain substrate concentration. Besides, Lineweaver-Burk linear plot (**Figure 9B**) provided a K_m as low as 1.6 mM and a V_{max} as high as 47 nM sec^{-1} for the MnO_2 nanozymes toward irreversible oxidation of DAB to produce brown-colored polyDAB. Considering the high value of the V_{max}, it can be concluded that the as-prepared nanozymes can catalyze the oxidation reaction of DAB with excellent catalytic efficiency. Besides, the low value of the K_m is pointed to the high affinity of the enzyme-substrate (here, DAB) for binding to the active nodes of the as-prepared nanozymes. It is notable that the kinetic performances of the as-prepared MnO_2 nanozymes were compared with those of the native peroxidase enzyme. As reported in the literature, the V_{max} value of native horseradish peroxidase is about 35 nM sec^{-1}[38] (enzyme substrate = TMB) which is about 1.4-fold lower than the V_{max} of the MnO_2 nanozymes (enzyme substrate = DAB), exhibited that the catalytic efficiency of the as-prepared nanozymes is higher than that of the native enzyme. Besides, the K_m of native enzyme was found to be as high as 3.6 mM[38] which is 2.3-fold higher than that of the MnO_2 nanozymes, revealing that the substrate affinity toward MnO_2 nanozymes is 2.3-order higher than the substrate affinity toward native enzyme.

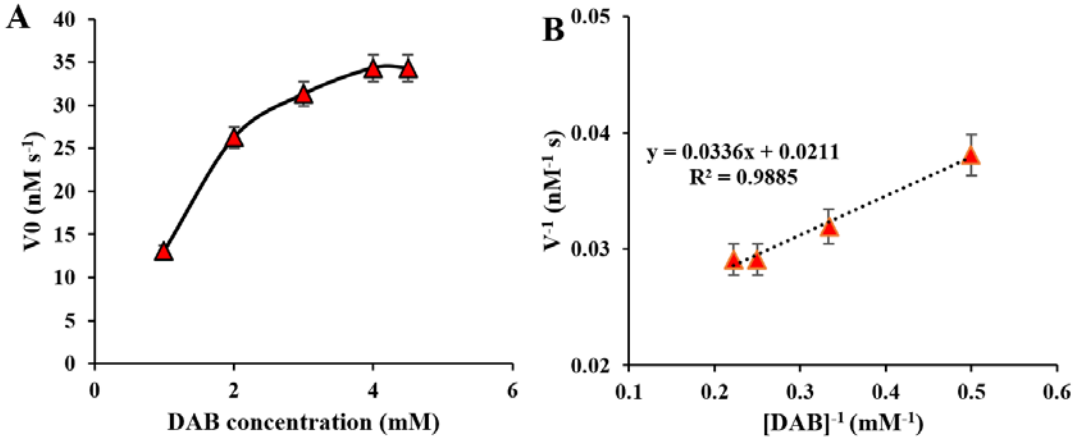


Figure 9. (A) Steady-state Michaelis-Menten saturation curve; **(B)** Lineweaver-Burk linear plot for the nanozyme-mediated oxidation of DAB over the as-prepared MnO_2 nanozymes.

4. Conclusions

Despite the well-known concepts on the intrinsic peroxidase-like activity of MnO_2 nanoparticles, up to date, their biochemical and kinetics characteristics were not investigated, especially, the current information about their performances toward *n*-electron oxidation of 3, 3′-diaminobezedine for producing indamine polymers is on limitation. Therefore, herein, the MnO_2 nanoparticles were synthesized by a simple low-cost co-precipitation method and then characterized by XRD, SEM, and DLS analysis. Besides, their peroxidase-like activity was evaluated upon standard peroxidase enzyme assay, revealing high intrinsic peroxidase-like activity for the as-mentioned MnO_2 nanozymes. Considering their high intrinsic peroxidase-like activity, their optimal biochemical characteristics were quantified by probing the progress of *n*-electron irreversible oxidation of 3, 3′-diaminobezedine in the presence of MnO_2 nanozymes as peroxidase mimics. The maximal activity of the as-mentioned MnO_2 nanoparticles with high intrinsic peroxidase-like activity was observed when the pH and temperature of the reaction media were fixed over 3.0–6.0 and 23 °C–25 °C, in order, revealing very high pH and thermal stability of the as-prepared nanoparticles. The salt stability of these nanoparticles was also checked using NaCl as model salt, revealing that the nanozymatic activity was stable over a salt concentration as high as 3–7 M. In addition, the affinity constant (K_m) and maximum velocity of the nanozyme-catalyzed oxidation of 3, 3′-diaminobezedine were found to be 1.6 mM and 47 nM sec^{-1}, in turn.

Acknowledgments

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Conflict of interest

The author declares no conflict of interest.

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