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Comparative Studies on the Interaction of Proteinase-K with Nano-CuO and Copper Ions

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Abstract

The interaction of copper oxide nanoparticles and copper ions and proteinase K from Tritirachium album Limber has been investigated employing UV spectroscopy and kinetics measurements. The aim of this study was to evaluate the effect of nanoparticles of CuO for proteinase K. In this paper we compare the effect of copper oxide nanoparticles with the effect of copper ions on proteinase K stability and activity. Copper oxide nanoparticles had more inhibitory and destabilization effect than copper ions on proteinase K. We used thermodynamic parameter such as Tm and ΔG^0 and kinetic studies to emphases the effect of nanoparticles CuO and copper ions on stability and activity of proteinase-K.

1. Introduction

Living organisms are built of cells that are typically 10 μm across. However, the cell parts are much smaller and are in the sub-micron size domain. Even smaller are the proteins with a typical size of just 5 nm, which is comparable with the dimensions of smallest manmade nanoparticles. Proteins are the important part of the cell's language, machinery and structure, and understanding their functionalities is extremely important for further progress in organisms [1]. Enzymes are ubiquitous protein that use for natural biocatalysts. Practical use of enzymes has been realized in various industrial processes and products including laundry detergents, and is

being expanded in new fields: fine-chemical synthesis, pharmaceuticals, biosensors, polymerase chain reaction, and protein digestion in proteomic analysis [2]. The nanomaterials level is the most advanced at present, both in scientific knowledge and in commercial applications. A decade ago, nanoparticles were studied because of their size-dependent physical and chemical properties [3]. Now they have entered a commercial exploration period [4]. Nanotechnologies have already been used in hundreds of products across various industries such as electronics, healthcare, chemicals, cosmetics, materials, and energy [5]. The majority of commercial nanoparticles use in medicine. This application in medicine include: Bio detection of pathogens [6], Detection of proteins [7], tumour destruction via heating (hyperthermia) [8], Separation and purification of biological molecules and cells [9]. Data from some pulmonary toxicity studies in rats demonstrate that exposures to ultrafine/nanoparticles may produce enhanced toxicity when compared to fine sized (bulk) particle-types of similar chemical composition [10]. Metal oxide nanoparticles are often used as industrial catalysts and elevated levels of these particles have been clearly demonstrated at sites surrounding factories [11]. The metal oxide nanoparticles (NPs) of CuO and ZnO are widely used in industrial, cosmetic and medical applications [12]. CuO have been used in wood preservation and antimicrobial textiles[13]. As the production of nanoparticles of ZnO, TiO_2 and CuO is increasing, their toxicity to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus was studied [14]. All these studies substantially increased our knowledge on nanotoxicology and many of them focus on microorganism such as bacteria , alga and protozoa. However more studies are clearly needed to clarify both toxicological effects and their underlying mechanism of nanoparticles[15]. The aim of this study was to evaluate effect of NPs of CuO for Proteinase K. Proteinase K (E.C. 3.4.21.14) which is also known as endopeptidase K, is an extracellular endopeptidase from fungus Tritirachium album Limber [16]. Proteinase K is a monomeric protein with molecular weight of 29.9 kDa and belongs to the class of subtilisin-like serine proteases with the typical catalytic triad of Asp39–His69–Ser224 [17]. Proteinase K is a robust molecule and is more stable to chemical and thermal denaturants. Besides, the proteinase is highly active and stable in the pH from 3.0 to 11.0. Despite the intensive use in industries and research laboratories for isolation of DNA [18], the behavior of Proteinase K against of nanaoparticles such as Copper oxide nanoparticles (CuO) has not been studied to date. Such studies would help to establish the structure-function relationship of proteases in general and serine proteases specifically. In the present paper we studied thermal denaturation and activity of proteinase K in the presence of nano-CuO and Copper ions.

2. Materials and methods

2.1 Instruments and materials

Freeze-dried Proteinase K from *T. album* and Para nitro phenyl acetate (pNA) was purchased from Sigma Chemical Co. USA. Sodium phosphate, 0.05 M (pH = 7.4) was used as buffer. The absorption spectra of all liquids/solutions and kinetic analysis were recorded with a Model Ultrospec 4000, UV-

Visible spectrophotometer equipped with a thermostatic cell holder. A scanning electron microscope (SEM) was used to measure the size and shape of the nano-CuO. Nano-CuO (size10-14 nm) was used without further modification. It was suspended in deionized water and mixed ultrasonically for 3 times in 10 min before use. $CuSO_4$ was used as copper ions. Concentration of this material was 0 to 0.16 µm.

2.2. Spectrophotometric determination of the nano-CuO-proteinase K

All studies were carried out in quartz cells containing 0.2mg/ml proteinase K and different concentration nano-CuO suspension. After 1 hour the solid with adsorbed proteinase K was separated by centrifugation of a suspension at 12,000 rpm (twice, 10 min). The absorbance of the supernatant was measured at 280 nm against water by UV–Vis spectrophotometry. Simultaneously, a reagent blank without proteinase K was performed according to the same procedure. With the above procedures, a series of suspensions was prepared, initially containing 0.2 mg/ml proteinase K, different concentration nano-CuO.

2.3. Thermodynamic analysis

The thermodynamic analysis of small proteins has shown that the behavior of these proteins can usually be described as equilibrium between two states, folded and unfolded, with a very cooperative transition between them [19]. Protein stability is defined, therefore, as the difference in free energy between the folded (native) and the unfolded state. This difference in free energy, which under physiological conditions is small and in favour of the folded (native) state, arises from the difference between two large numbers: the factor stabilizing the folded state and those stabilizing the unfolded state [20].

The standard Gibbs energy, ΔG^0_D, is shown by $\Delta G^0_D = -RT \ln K_{eq}$, where R is the gas constant and T is the absolute temperature, K_{eq} is equilibrium constant and obtain from: $K_{eq} = [F_d/(1-F_d)]$ and denaturation fraction(F_d) is: $F_d = [(Y_N - Y_{obs}) / (Y_N - Y_D)]$, which Y_N is absorbtion on native state and Y_D is related to absorbtion on denaturate state. These results can be used to determined T_m at which $\Delta G^0 = 0$, ΔS^0_m and ΔH^0_m. The standard-state entropy of transfer, ΔS^0_m , is obtained from the slope denaturation curves at T_m. The standard enthalpy obtained from $\Delta H^0_m = T_m \Delta S^0_m$ [21].

2.4. Enzyme kinetic

Para nitro phenyl acetate (pNA) was used for substrate and amount of product were recorded at 425 nm [22]. In this work two important parameter, K_m and V_{max}, were calculated for analysis of activity of proteinase K. K_m is the [S] at 1/2 V_{max}. K_m is a constant for a given enzyme. K_m is an estimate of the equilibrium constant for substrate binding to enzyme. Small K_m means tight binding and high K_m means weak binding. K_m is a measure of [S] required for effective catalysis to occur. $K_m = k_{-1}+k_2/K_1$. Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation: $v = V_{max} * [S]/ K_m + [S]$; V_{max} is a constant for a given enzyme and it is the theoretical maximal rate of the reaction. To reach V_{max} would require that all enzyme molecules have tightly bound substrate [23].

3. Results and discussion

3.1. Determination of particle size of nano-CuO

Fig.1. shows the SEM picture of nano-CuO. From SEM measurement we observed that nano-CuO particles have flower in shape and the average particle diameter is about 10-14 nm.

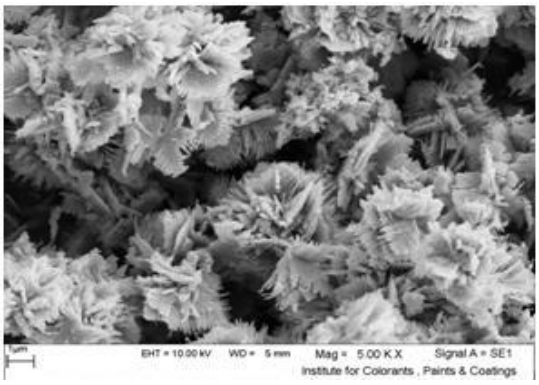

Fig. 1. SEM image of nano-CuO.

3.2. Thermodynamic analysis of Proteinase K in the presence of nano-CuO and Cu^{2+}

In this section of work, enzyme dissolved in dilute water and stock of enzyme frozen at -20 C^0. The absorbance of the enzyme sample in the absence and presence of nano-CuO and Cu^{2+} were recorded at rang temperature 30-100 ^{0}C. Enzyme didn't show any conformational change below 0.008 µm of NPs. But by adding 0.008 µM of nano-CuO to enzyme sample conformational changes were observed and by increases of concentration of NPs significant change were observed in structure of enzyme that reflect on denaturation fraction plot. Between 0.008 and 0.014µM of NPs, there is an decreases in T_m. As shown in Fig. 2 by increasing the concentration of nano-CuO to enzyme the ΔG^0_D curves show high degree of conformational change on Proteinase K. In previous work on lyzozyme, it has shown that the stability of Lysozyme were decreased by increasing of nano-TiO_2 and nano-SiO_2 nanopaticles [24, 25].

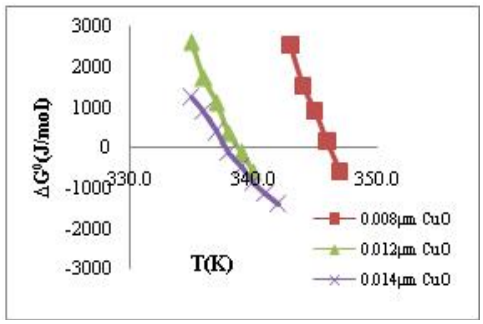

Fig. 2. The effect of nano-CuO on ΔG^0_D at different temperatures at pH=7.4.

Thermodynamic data of T_m, ΔS^0_m and ΔH^0_m at various concentration of nano-CuO are tabulated in Table 1. As shown in this table, increasing the concentration of NPs, decrease T_m and ΔS^0_m and ΔH^0_m. Hence enzyme show lower stability in the presence of nano-CuO.ΔS^0_m and ΔH^0_m can be shown using: $\Delta S^0_m = S^0_m(D)-S^0_m(N)$, $\Delta H^0_m = H^0_m(D)- H^0_m(N)$. As the denatured state of proteins is random coil, thus decreasing ΔS^0_m and ΔH^0_m in the presence of nano-CuO imply unfolding of native conformation of enzayme [21].

Table 1. Thermodynamic data of proteinase K at various nano-CuO concentrations at pH=7.4.

[Nano-CuO]µM	T_m(K)	ΔS^0_m(J/mol.K)	ΔH^0_m(J/mol)
0.008	346	740.8	256316.8
0.012	340	716.2	243508
0.014	338	393.9	133138.2

In order to clarify the respective roles of particle size of nanoparticles, bulk formations of metal ions as well as $CuSO_4$ were used for compare their effects. As discussed in the introduction section, we compare the effect of nano-CuO with Cu^{2+}. Experiment carried out in the same condition. Result show Cu^{2+} had a little change on Proteinase K with compare to nano-CuO particles in same concentration. As show in Fig 3 in the presence of Cu^{2+}, T_m of enzyme were decreased but this reduction was lower than nano-CuO. As see in this figure

significant change was occurred at 0.008 and 0.016μM of Cu^{2+}.

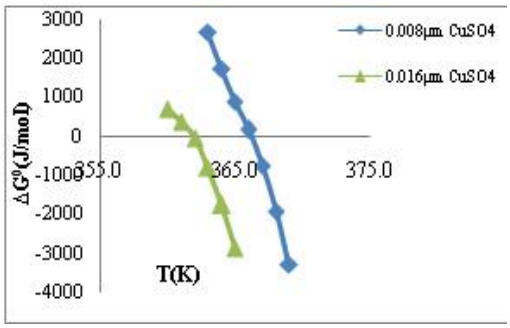

Fig. 3. The effect of Cu^{2+} on ΔG^0_D at different temperatures at pH=7.4

Thermodynamic data of T_m, ΔS^0_m and ΔH^0_m at various concentration of CU^{2+} are tabulated in Table 2. As see, in presence of Cu^{2+}, ΔS^0_m and ΔH^0_m decreased that indicate stability of Proteinase K was reduced. Comparing the amounts on table 1 and 2 realized that nano-CuO was clearly more affected on enzyme stability than the ions form.

Table 2. Thermodynamic data of proteinase K at various Cu^{2+} concentrations at pH=7.4

$[Cu^{2+}]\mu M$	$T_m(K)$	$\Delta S_m{}^0$(J/mol.K)	$\Delta H_m{}^0$(J/mol)
0.008	368	898.5	330648
0.016	363	711	258093

Proteinase K has positive charges at pH=7[16]. The positive charge side chain of surface of enzyme may be interaction with CuO and bind to the CuO by electrostatic interaction. When more interactions formed between them other interaction such as hydrogen bond occurs. Hydrogen bond formed between nano-CuO and the polar side chains of amino acid residues, hence conformational changes occurred in proteinase K and substantial many interaction such as noncovalent interaction occurred [23]. Thus many of inner area go expose-surface. Upon this variation, enzyme loses stability probably due to local structural deformation. More effect of nano- CuO might be due to its size and form strong interaction with enzyme[25, 26].

3.3 Kinetics study of proteinase K in the presence of nano-CuO and Cu^{2+}

Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation. The best way to analyze enzyme kinetic data is to fit the data directly to the Michaelis-Menten equation using nonlinear regression. Before nonlinear regression was available, investigators had to transform curved data into straight lines so they could analyze with linear regression. One way to do this is with a Lineweaver-Burk plot. Ignoring experimental error, a plot of 1/V vs. 1/S will be linear, with a Y-intercept of $1/V_{max}$ and a slope equal to K_m/V_{max}. The X-intercept equals $-1/K_m$ [27].

We measure enzyme velocity at many different concentrations of substrate and temperature. Para nitro phenyl acetate (pNA) was used as substrate and p- nitro phenol is product that amount of its measure at 425 nm. Result show Proteinase K had better activity on $40C^0$ and in 3mM of substrate enzyme arrived to V_{max}. In Fig. 4 effect of different concentration of NPs was shown. As see in this figure in presence of NPs, V_{max} and K_m were decreased that indicate NPs was inhibited enzyme by un-competitive mechanism. kinetics data are tabulated on Table 3.

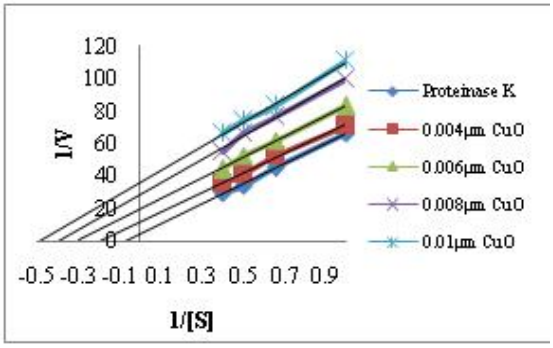

Fig. 4. Lineweaver-Burk plot of proteinase K at various nano-CuO concentrations at 313 K, pH = 7.4.

The next part of work, activity of enzyme was measured against of different concentration of Copper ions(Cu^{2+}). Condition of experiment was same with experiment in previous section. As shown in Fig 5, at lower

concentration of Cu^{2+} has a small positive effect on activity of Proteinase K but at high concentration of Cu^{2+} the activity of enzyme were decreased.

Table 3. Kinetic parameter of V_{max} and K_m at various nano-CuO concentrations at 313 K

Concentration of NPs	K_m	V_{max}	V_{max}/K_m
0 µM	10	0.21	0.131
0.004 µM	5	0.08	0.101
0.006 µM	3.5	0.05	0.010
0.008 µM	2.7	0.03	0.098
0.010 µM	2	0.02	0.10

As see on table 4 in the presence of high concentration of Cu^{2+}amount of K_m and V_{max} was decreased that show inhibitory mechanism of Cu^{2+} was mixed- inhibition. kinetics data are tabulated on tTable 4.

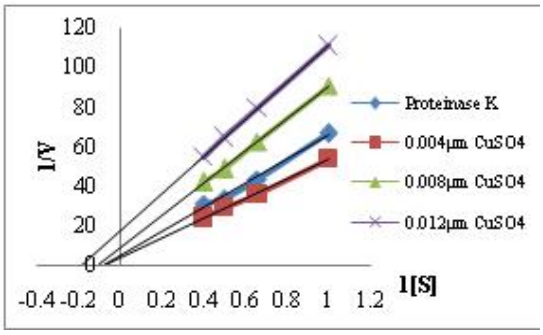

Fig. 5. Lineweaver-Burk plot of proteinase K at various Cu^{2+} centrations at 313 K, pH=7.4

Table 4. Kinetic parameter of V_{max} and K_m at various Cu^{2+} concentrations at 313 K

Concentration of Cu^{2+}	K_m	V_{max}	V_{max}/K_m
0µM	10	0.19	0.083
0.004 µM	10	0.22	0.061
0.008 µM	7.6	0.11	0.049
0.012 µM	4.5	0.05	0.010

In the presence of NPs and Cu^{2+}.hyrogen bond net work of enzyme was affected. Depending on the occupied site by NPs and Cu^{2+}, the hydrogen bonds of the catalytic tired severely disturbed and its ability to carry out the enzymatic function were affected [26].

Conclusion

To our knowledge this is the first study on the effects of CuO nanoparticle on stability and activity of proteinase K. NPs of CuO remarkably more toxic to proteinase K. As see in result section CuO nanoparicle had inhibitory effect on activity of proteinase K and decreased its stability by reduction of T_m. Thus nano CuO might have some toxic effect on biomolecules. Hence we suggest that prior of use of nanoparticle on various industry, different aspect of interaction of nano particles with various proteins must be considered.

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