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Short Communication

Comparative Studies on the Interaction of Proteinase-K with Fe_2O_3, Fe_3O_4 and SiO_2 Nanoparticles

E. Yadollahi[1], B. Shareghi[1,*] and M. Salavati[2]

[1]Department of Biology, University of Shahrekord, P.O. Box: 115, Shahrekord, I. R. of Iran.
[2]Institute of Nano Science and Nano Technology, University of Kashan, P.O. Box 87317-51167, Kashan, I. R. Iran.

(*) Corresponding author: b_shareghi@yahoo.com
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Abstract:

The interaction of Fe_2O_3, Fe_3O_4 and SiO_2 nanoparticles with proteinase K activity was investigated using UV–vis spectroscopy. Proteinase K EC (3.4.21.14) is a member of serine protease family, which is produced from fungus Tritirachum album Limber. The effects of nanoparticles on proteinase K activity were studies at 40˚C in pH 7.0 using sodium phosphate as buffer. It was found that in the presence of nano-Fe_2O_3 and nano-Fe_3O_4, V_{max} was decreased but K_m was constant. This results indicated that nano-Fe_2O_3 and nano-Fe_3O_4 acted as noncompetitive inhibitors. In the presence of nano-SiO_2 the amount of K_m increased but V_{max} decreased, that showed nano-SiO_2 acted as a mixed inhibitor. The dissociation constant (K_i) value for binding nano-Fe_2O_3, nano-Fe_3O_4 to proteinase K was equal to 11µM and 8.5µM respectively that indicated the binding of nano-Fe_3O_4 to the enzyme was stronger than nano-Fe_2O_3. The K_I and K_i value for nano-SiO_2 was 22.5µM and 8µM respectively.

Keywords: *Proteinase-K, Nanoparticles, Experimental investigation*

1. INTRODUCTION

Serine proteases are present in virtually all organisms and can be extracellular or intracellular enzymes. These proteins exist as two families, the trypsin-like and the subtilisin-like families, that have been independently evolved with a common catalytic mechanism which has been widely investigated [1]. Both families play an important role in all the organisms through functioning in digestion, coagulation, blood, posttranslational processing of secreted proteins, neurotransmitters and hormones [2]. The functional importance of catalytic triad and oxyanion hole in catalysis has been clearly established [3, 4]. In this mechanism, Ser functions as the primary nucleophile and His plays a dual role as proton acceptor and donor at different steps in the reaction. The proposed role of aspartate in this mechanism is to bring histidine into the correct orientation to facilitate the nucleophilic attack by Serine. Though, the overall folding of various serine proteases may differ and they all follow the same mechanism of action by an identical stereochemistry of the catalytic triad [5].

Proteases are enzymes that catalyze the hydrolysis of peptide bonds.

The 279-residue serine protease, Proteinase K EC (3.4.21.14) from the fungus *Tritirachium album limber* belongs to the subtilisin family of enzymes [5, 6]. This family of enzymes has attracted intensive research interest from the industrial, academic, and agricultural communities. The academic interest is inspired by the ready amenability of subtilases to functional and structural investigation [7],

and by applications of proteinase K in biotechnology research such as the removal of DNases and RNases when isolating RNA and DNA from tissues or cell lines [6,8].

The agricultural and industrial applications of these enzymes include bio-control agents against parasites [9] and protein-degrading components in washing powders [10] respectively. Many of the properties of these enzymes involved in the catalysis, structure, stability to inactivation and substrate specificity and pH profile have been probed in detail by biochemical, protein-engineering, and structural studies [7, 9 and 11]. The native proteinase K contains two Ca^{2+} cations, which are considered to enhance the thermal stability of the enzyme and increase its resistance to proteolysis [12, 13, and 14].

The nanomaterials level is the most advanced at present, both in commercial applications and in scientific knowledge. A decade ago, nanoparticles were studied because of their size-dependent physical and chemical properties [15]. Nanotechnologies have already been used in a lot of products across various industries such as healthcare, electronics, materials, chemicals, cosmetics, and energy [16].

Metal oxide nanoparticles, particularly the oxides of transition metals, are of interest because of their unique properties in medicine, biology, equipment, sporting, cosmetics, electronics, magnetic storage media, solar energy transformation, and other industries [13-.15].

Recently, magnetic nanoparticles have many uses, for example, in magnetic resonance imaging (MRI), a temperature increase of tumor therapy, cell labeling and sorting, DNA separation and drug delivery, etc. are used [17]. Anyway, some of studies are related to the effects of magnetic nanoparticles on amyloid aggregation of proteins. We showed that magnetic nanoparticles can attach selectively to the insulin fibrils leading to specific magnetization of fibrils, causing extraction of fibril/nanoparticle assemblies from the aqueous phase by a magnetic field [17, 18]. Iron oxide nanoparticles are mostly used in the magnetic material for high biocampability, strong paramagnetic properties, easy preparation and low toxicity [19].

Cationic proteins via domains positively charged from proteins located on the surface of silica nanoparticles (SiO_2) are connected. Changes in pH and ionic strength on the environment by controlling the intermediate layer leads to changes in protein tends to bind to the nanoparticles [20]. It is believed that immobilization of various enzymes onto nanoparticles will result in an accurate and much faster assay. Enzyme immobilization occurs by adsorption, however; maybe result showed loss partial of enzymatic activity. It is important, therefore, to characterize the unfolding of enzymes/proteins at surfaces in order to arrive at rational methodology for the development of nanoparticles-based sensors and protein microarrays [21]. Previous studies on the effect of nano-Fe_2O_3, nano-Fe_3O_4 [22] and nano-SiO_2 [23] on enzyme activity indicate their inhibitory effects. The aimed of this study was to investigate the effects of iron oxide nanoparticles (Fe_3O_4 and Fe_2O_3) and SiO_2 nanoparticle, on the kinetic activity of proteinase K.

2. MATERIALS AND METHODS

2.1. Instruments and Materials

Kinetics studies have been performed using UV-Vis spectrophotometer model pharmacia_4000 equipped with electronic control. Freeze-dried Proteinase K from *Tritirachum album Limber* was purchased from Sigma Chemical Co. USA that was dissolved in sodium phosphate buffer (25×10^3µM, pH 7.0) and stored at less than 40 ˚C. ρara nitro phenyl acetate (ρNA) (purchased from Sigma), which was used as Proteinase K substrate, was dissolved in deionized water. All of nanoparticles were suspended in deionized water and mixed

ultrasonically for 3 times in 10 min before use.

2.2. Standard Proteinase K Activity Assay

In this assay mixture contained 40 µg/ml of enzyme, different substrate concentrations and phosphate buffer (pH 7.0) .The enzyme activity was determined by measuring the concentration of product released. ρNA was used for substrate and amount of product were recorded at 425 nm[24]. In this study two important parameters, K_m and V_{max}, were calculated for analysis of activity of proteinase K.

2.3. Kinetics study of Proteinase K in the Presence and Absence of Nanoparticles

The activity of Proteinase K was monitored by UV-Visible spectrophotometer at 425 nm at pH 7.0 and 40˚C in the absence and presence of different amount of nanoparticles. All studies were carried out in quartz cells containing 40µg/ml proteinase K and different concentration (2.5, 5 and 10µM for Fe nanoparticles and 2, 4 and 8 µM for SiO_2 nanoparticles) nanoparticles suspension.

2.4. Calculated K_i or Inhibitory Constant

K_i or inhibition constant calculated by secondary plots. In noncompetitive inhibition K_i measurement by drawing $1/V_{max}'$(V_{max} in presence of inhibitor) vs.[I], where is:

$$1/V_{max}' = 1/V_{max} + [I_0]/V_{max}K_i \quad (1)$$

In mixed inhibitor that:

$$E + I \rightleftharpoons EI \qquad K_i = [E][I]/[EI] \quad (2)$$
$$ES + I \rightleftharpoons ESI \qquad K_I = [ES][I]/[ESI] \quad (3)$$

K_I measurement by drawing $1/V_{max}'$ (V_{max} in presence of inhibitor) vs.[I] and K_i measurement by drawing K_m'/V_{max}' vs.[I]. Where is:

$$1/V_{max}' = 1/V_{max}(1+[I]/K_I) \quad (4)$$
$$K_m'/V_{max}' = K_m/V_{max}\,(1+[I]/K_i) \quad (5)$$

3. RESULTS

3.1. Determination of Particle Size of Nano-Fe_2O_3, Nano-Fe_3O_4, Nano-SiO_2

Fig.1 and Fig.2 show the SEM and TEM picture of nano-Fe_2O_3, respectively. From SEM measurement we observed that nano-Fe_2O_3 particles have flower in shape and the average particle diameter is about 30 nm. The average particle diameter of nano-Fe_3O_4 was about 45 nm. Figure 3 shows the SEM picture of nano-SiO_2. In shape the average particle diameter was about 11 nm.

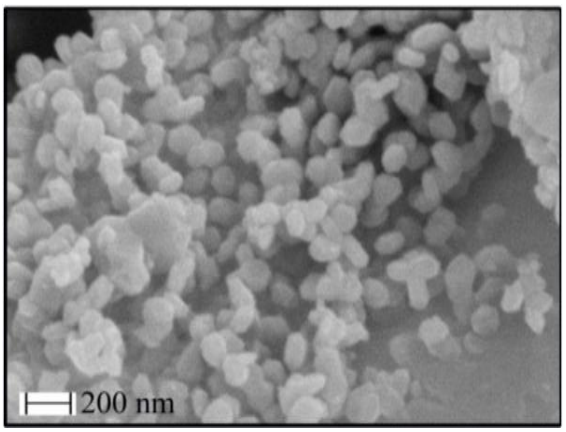

Figure 1*. SEM image of Fe_2O_3 nanoparticles.*

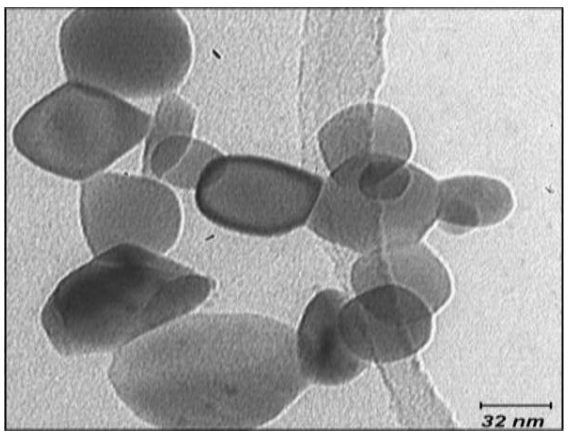

Figure 2. *TEM image of Fe_2O_3 nanoparticles.*

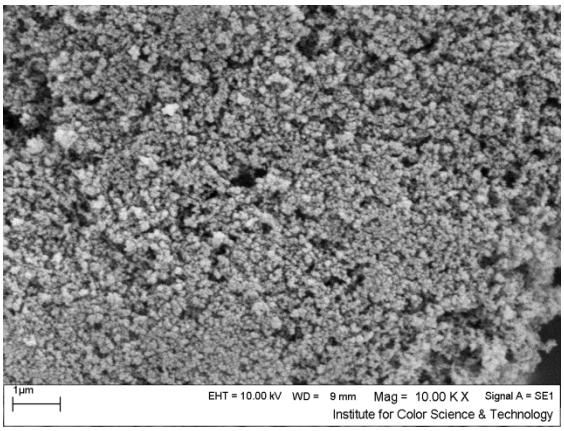

Figure 3*. SEM image of SiO_2 nanoparticles.*

3.2. Kinetics Study of Proteinase K in the Presence of Nano-Fe_2O_3, Nano-Fe_3O_4, and Nano-SiO_2

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. Para nitro phenyl acetate (ρNA) was used for substrate and amount of product were recorded at 425 nm [25]. In this work two important parameter, K_m and V_{max}, were calculated for analysis of activity of proteinase K. K_m is an estimate of the equilibrium constant for substrate binding to enzyme. Result showed Proteinase K had better activity on 40˚C and in 3×10^3µM of substrate enzyme arrived to V_{max}.

In Fig.4 effect of different concentration of nano-Fe_2O_3 was shown. As see in this figure, in presence of nano-Fe_2O_3, V_{max} was decreased and K_m was constant that indicated inhibition of enzyme by non-competitive mechanism. Kinetics data are tabulated on table 1.

In Fig.5, the secondary plot of enzyme in different concentration of nano-Fe_2O_3 is shown. As the intersection point of secondary plot of $1/V_{max}'$ vs.[I] and y axis is K_i, The K_i value in fig 6 is equal to 11µM.

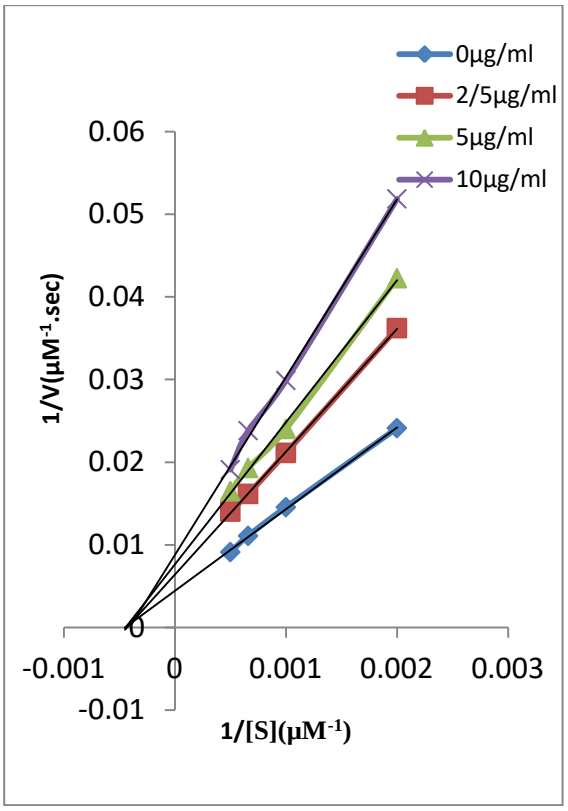

Figure 4*. Lineweaver-Burk plot of proteinase K at various nano-Fe_2O_3 concentrations at 40˚C and pH 7.0*

Table 1*. Kinetic parameter of V_{max} and K_m at various nano- Fe_2O_3concentrations at 40˚C and pH 7.0*

$[Fe_2O_3]$ µg/ml	V_{max} (µM.sec^{-1})	K_m (µM)	V_{max}/K_m ($\mu g^1.s^1$)×10^3
0	230	2300	0.1
2.5	150	2300	0.06
5	130	2300	0.05
10	110	2300	0.04

In the next part, activity of enzyme was measured in the presence of nano-Fe_3O_4. Condition of experiment was same with experiment in previous section. As shown in Fig.6. in presence of nano-Fe_3O_4, V_{max} was decreased and K_m was constant that indicate non-competitive mechanism. Kinetics data are tabulated on table 2.

The secondary plots of enzyme in different concentration of nano-Fe_3O_4 is shown in Fig.7.The K_i in this case is equal to 8.5µM.

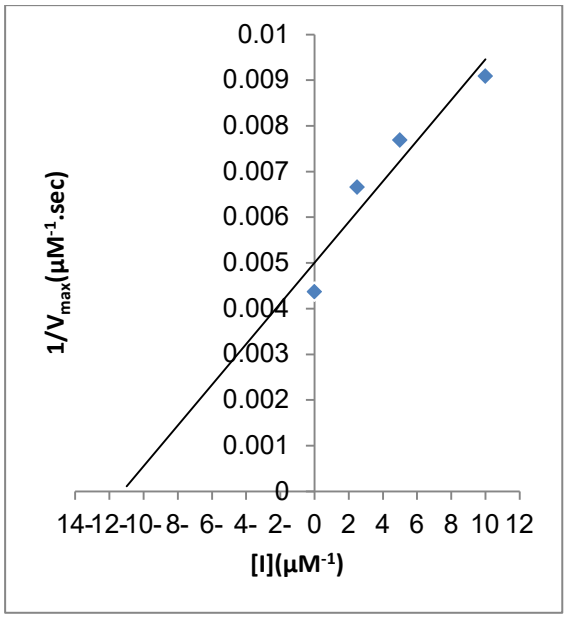

***Figure 5**. Secondary plot of proteinase K at various nano-Fe_2O_3 concentrations at 40˚C and pH 7.0*

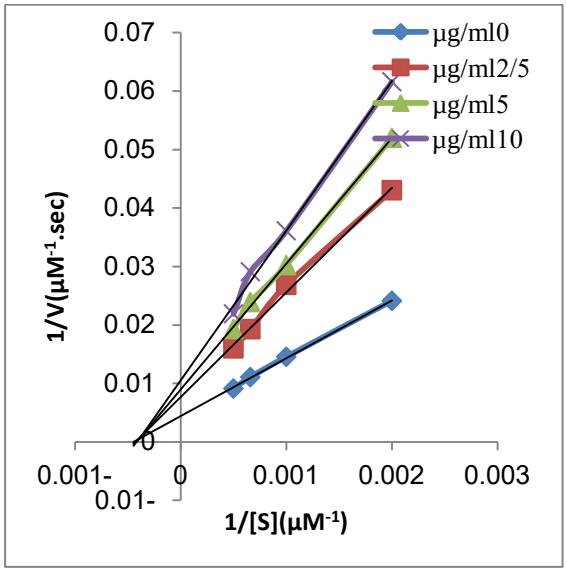

***Figure 6**. Lineweaver-Burk plot of proteinase K at various nano-Fe_3O_4 concentrations at 40˚C and pH 7.0*

***Table 2**. Kinetic parameter of V_{max} and K_m at various nano- Fe_3O_4 concentrations at 40˚C and pH 7.0*

[Fe_3O_4] µg/ml	V_{max} (µM.sec^{-1})	K_m (µM)	V_{max}/K_m ($\mu g^1.s^1$)×10^3
0	230	2300	0.1
2.5	130	2300	0.05
5	110	2300	0.04
10	90	2300	0.03

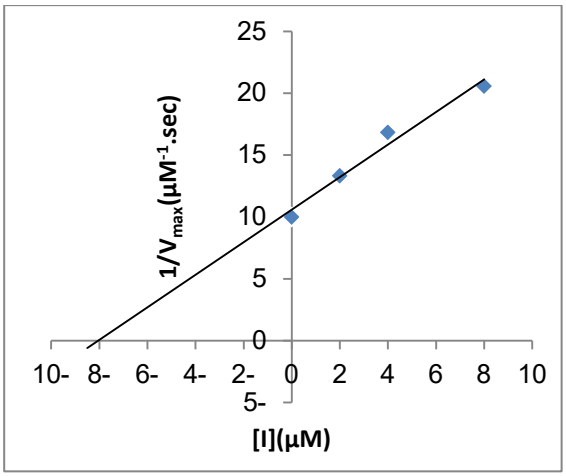

***Figure 7**. Secondary plot of proteinase K at various nano-Fe_3O_4 concentrations at 40˚C and pH 7.0*

In the other part of work, activity of enzyme was measured of nano-SiO_2. Condition of experiment was same with experiment in previous section (Fig.8).

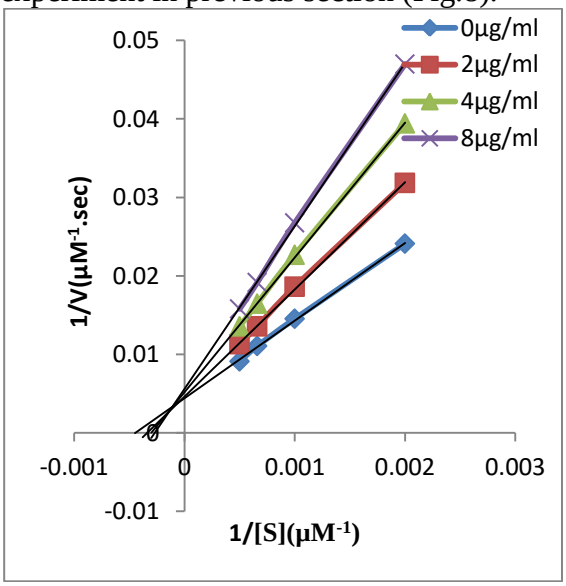

***Figure 8**. Lineweaver-Burk plot of proteinase K at various nano-SiO_2 concentrations at 40˚C and pH 7.0*

As it could be seen in the presence of nano-SiO_2 amount of K_m increased and V_{max} decreased that show inhibitory mechanism of nano-SiO_2 was mixed-inhibition. Kinetics data are tabulated on Table 3. In this type of inhibition K_I and K_i are different and determined respectively by $1/V_{max}'$vs.[I] and K_m'/V_{max}'vs.[I].

***Table 3**. Kinetic parameter of V_{max} and K_m at various nano- SiO_2 concentrations at 40˚C and pH 7.0*

$[SiO_2]$ µg/ml	V_{max} (µM.sec^{-1})	K_m (µM)	V_{max}/K_m (µg^1.s^1)×10^3
0	230	2300	0.1
2	210	2800	0.07
4	190	3200	0.05
8	170	3500	0.04

In Figs. 9 and 10, the secondary plots of enzyme in different concentration of nano-SiO_2 are shown. The corresponding values for K_I and K_i are 22.5 and 8µM respectively.

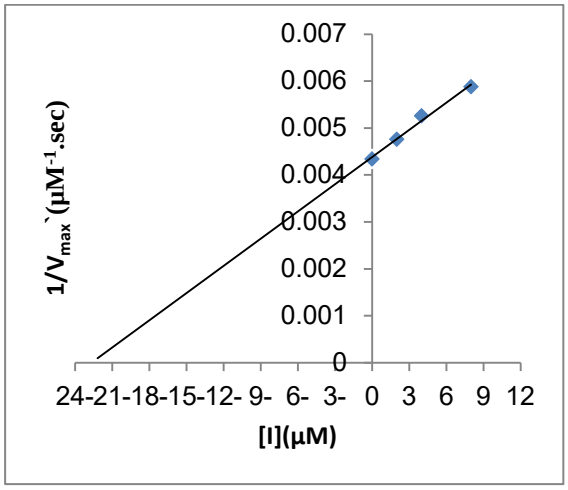

***Figure 9**. Secondary plot ($1/V_{max}$´ vs.[I]) of proteinase K at various nano-SiO_2 concentrations at 40˚C and pH 7.0*

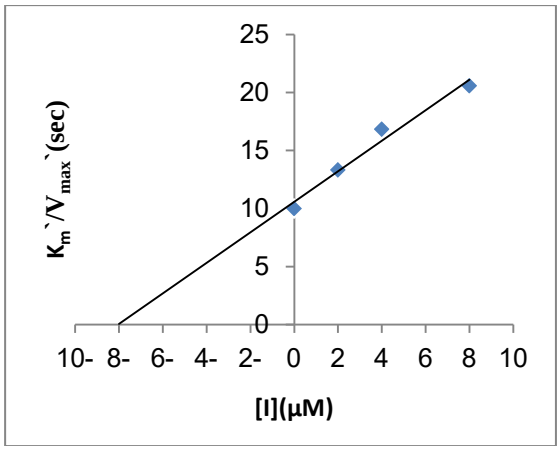

***Figure 10**. Secondary plot (K_m´/V_{max}´ vs.[I]) of proteinase K at various nano-SiO_2 concentrations at 40˚C and pH 7.0*

4. DISCUSSION

In this study, the effect of iron oxide (Fe_3O_4 and Fe_2O_3) and SiO_2 nanoparticles on the kinetic activity of proteinase K was investigated. The results showed a reduced activity in the presence of different concentrations of nanoparticles that could be due to enzyme binding. Furthermore, the change in the structure of the enzyme had been found resulting in a decrease in enzyme activity [26]. Proteinase K had hydrogen net in their active site, that it was very important for enzyme activity [27]. In this paper we showed all of nanoparticles had inhibitory effects on enzyme activity, which could be a result of disturbed hydrogen net in proteinase K active site.

The experimental results showed that with increase concentration of nanoparticles the enzyme activity decreased. In presence of nano-Fe_2O_3 and nano-Fe_3O_4 V_{max} parameter decreased while K_m is constant, thus it indicated noncompetitive inhibitor [28].

It could be concluded from the result that nano-Fe_3O_4 had more inhibitory effect on enzyme activity and decreased V_{max}. K_i was an indication of EI complex dissociation constant that for nano-Fe_3O_4 was lower compared to nano-Fe_2O_3 and showed more inhibitory effect. Probably reason for this result was bigger size of Fe_3O_4 than Fe_2O_3. It should we noted that pI for proteinase K and Fe nanoparticles is 8.3 and 5.1 respectively [29]. Thus at pH 7.0 the enzyme has positive charge and Fe nanoparticles have negative charge. So that bounding between them could probably result in a change in structure and activity of the enzyme.

The inhibitory effects of nano-SiO_2 on Proteinase K activity has been shown in this study. Nano-SiO_2 change K_m and V_{max} of enzyme altogether, where V_{max} decreases and K_m increases that this change point to mixed inhibitory that:

$$K_{m'} = K_m ((1+I_0/K_I)/ (1+I_0/K_I) \quad (6)$$

$$V_{max'} = V_{max}/ (1+I_0/K_I) \quad (7)$$

Furthermore, regarding enzyme secondary plot in presence of nano-SiO_2 that indicated K_I (ESI complex dissociation constant) is bigger than K_i (EI complex dissociation constant) [25, 28]. As a result inhibitory pattern is between competitive and noncompetitive inhibition. The inhibitory effect of nano-SiO_2 could be because of bond formation between nano-SiO_2 and enzyme. Nano-SiO_2 has pI=4.3 and it has been negative charge in pH 7.0, where it can bond with enzyme and change its structure. Conformational changes of protein may result in the changes in biological activity. The binding of nanoparticles may change the micro-environment of the catalytic triad Asp39–His69–Ser224 for the intruding molecule in the vicinity of them from the over-all perspective. Consequently, it may influence the enzyme activity.

5. CONCLUSION

The purpose of the current study was to determine change in the activity of proteinase K in the presence of Fe_3O_4, Fe_2O_3 and SiO_2 nanoparticles. To authors' information, few investigations have been performed on the proteinase K enzyme activity in presence of nanoparticles. As it could be seen in the results section, nano-Fe_2O_3 and nano-Fe_3O_4 had noncompetetive inhibitory effect on activity of Proteinase K, while nano-SiO_2 had mixed inhibitory effects. Further studies should be performed in order to investigate the effect of such nanoparticles on the activity of enzyme at different pHs, Temperatures and substrates.

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