J Nanostruct 8(2): 168-178, Spring 2018

RESEARCH PAPER

The Effect of TiO_2-Nanoparticle on the Activity and Stability of Trypsin in Aqueous Medium

Sakineh Sadeghi Kaji [1], *Behzad Shareghi* [1]*, *Aieh Bolouki* [1] *and Masoud Salavati-Niasari* [2]

[1] *Department of Biology, Faculty of Science, University of Shahrekord, Shahrekord, Iran*

[2] *Institute of Nano Science and Nano Technology, University of Kashan, Kashan, Iran*

ARTICLE INFO

Article History:
Received 05 February 2018
Accepted 10 March 2018
Published 01 April 2018

Keywords:
Inactivation
Kinetic parameters
Nano-TiO_2
Trypsin
Trypsin inhibitor

ABSTRACT

Trypsin (E.C.3.4.21.4) is a serine protease commonly used in proteomics for digestion of proteins. In the present study, the effect of nano-TiO_2 on the conformation and catalytic activity of trypsin were studied. The thermal denaturation of trypsin has been investigated in the presence and absence of nano-TiO_2 over the temperature range (293-373 K) at pH 3.0 and 7.25, using temperature scanning spectroscopy. In presence of nani-TiO_2, the ester lytic activity of trypsin is decreased. The result indicates that Nano-TiO_2 is a non-competitive inhibitor for enzyme trypsin. With the addition of TiO_2 to protein solution at pH 3.0, the maximum intensity of emission spectrum of trypsin is increased. But at pH 7.25, the maximum intensity of emission spectrum of trypsin is decreased. The result of fluorescence spectroscopy indicated that the structure of the Trp residue environments was altered. Increasing the concentration of nano-TiO_2 decreases the stability of trypsin to thermal denaturation.

How to cite this article
Sadeghi Kaji S, Shareghi K, Bolouki A, Salavati-Niasari M. The Effect of TiO_2-Nanoparticle on the Activity and Stability of Trypsin in Aqueous Medium. J Nanostruct, 2018; 8(2):168-178. DOI: 10.22052/JNS.2018.02.007

INTRODUCTION

Nanoparticles (NP) have many diverse applications in the life and physical sciences. Nanoparticles have been widely used in the immobilization of enzymes [1,27], immunoassay [2], bioseparation [3], biosensor [4], targeted drug delivery [,7,5], and environmental analysis [6,8]. The small size and the large surface areas confer very specific chemical, physical, and spectroscopic features. Metal nanoparticles are widely used for a variety of applications like Surface Enhanced Raman Spectroscopy (SERS), while semiconductors (quantum dots) are used for biomedical imaging. Metal oxide NPs are another class in which chemical properties can be manipulated, and their surface properties can be tailored by introduction of ligand functionality to provide versatility with regard to target specificity. NP-based probes have been used as labels in biological systems, with great potential for bio imaging, diagnostic and therapeutic purposes. Understanding the interactions of NP with biological molecules is important for the sensor applications [9]. Have unique physicochemical properties such as tiny size, large surface area, surface reactivity, charge, and shape and media interactions. As a result, the Properties of NPs differ substantially from their respective bulk materials of the same composition. However, certain novel properties of NPs could lead to adverse biological effects, with the potential to create toxicity [10]. More and more nanomaterials are being used in industrial production and daily life because of their unique characteristics [11]. For example, some of them

* *Corresponding Author Email: share.beh@sci.sku.ac.ir*

are added in sunscreens to absorb UV light and in toothpaste and paints to give them a white color which lasts for years. Some are used in the electronics industry. Besides, there are also many applications in medicine, sporting equipment, cosmetics, coatings, fuel cells, and other industries [12]. Nowadays, some nanomaterials are being tried as drug delivery products, and they can help to diagnose diseases as quantum dots that can allow the visualization of cancer cells within the body. With an increasing use of nanomaterials, their human exposure is inevitable. It is important to consider the hazards of these materials. The particle size of nanomaterials is smaller than cells and cellular organelles. It may penetrate these basic blocks, produce physical damage, or induce harmful inflammatory responses. The oxidative stress of nanoparticles can damage lipids, carbohydrates, proteins, and DNA in which lipid peroxidation is considered most dangerous, leading to alterations in cell membrane properties [13, 14]. Moreover, TiO_2 nanomaterials have attracted much more interest in medical fields due to its photo reactivity. It has already been reported that TiO_2 photo catalyst could ips to many respiratory and cardiovascular diseases, such as pneumonia, lung cancer, arteriosclerosis, and myocardial infarction [7]. Recent studies have shown that nanomaterials can cause genotoxicity and cytotoxicity in cultured human cells. There is evidence that nano-TiO_2 can cause inflammation, fibrosis, pulmonary damage, and even DNA damage [8, 15]. The goblet cell hyperplasia and Muc 5ac expression were induced in rats after a single intratracheal instillation of nano-TiO_2. Nano-TiO_2 might enter the human stratum corneum and interact with the immune system. Oxidative DNA damage and increases in the level of cellular nitric oxide were also observed in human bronchial epithelial cells after exposure to nano-TiO_2 [7, 16]. In recent years, semiconductor titanium dioxide (TiO_2) as photo catalyst has been applied to kill or suppress tumor cells, because TiO_2 particles can react with water molecules adsorbed on the surface of tumor cells to produce hydroxyl (OH) radicals with strong oxidation ability under ultraviolet (UV) light irradiation. Otherwise, TiO_2 particles can be easily engulfed by macrophage cells around blood vessel and fleetly eliminated from normal tissue [17]. The interactions of nanomaterials (often nano-TiO_2 or nano-SiO_2) with functional biomolecules such as proteins, enzymes and DNA are regarded as preconditions for their cytotoxicity and organ toxicity [18]. A large amount of epidemiological and experimental studies indicate that ultrafine particles have close relationship as a model enzyme in studies on enzymology, molecular biology, genetics, protein chemistry, and Immunology. The main objective of this work was to assess activity and structure of trypsin in the presence of TiO_2 NPs in solution.

MATERIALS AND METHODS

Instrument and materials

The kinetics studies of trypsin enzyme followed with a model ultrospec 4000, UV-Visible spectrophotometer equipped with a thermostatic cell holder. The fluorescence spectra of solutions were recorded with a Fluorescence spectrophotometer (Shimadzu RF-5301PC Fluorescence spectrophotometer). A scanning electron microscope (SEM) was used to measure the size and shape of the nano-TiO_2 (size of 21 nm). It was suspended in deionized water and mixed ultrasonically for 3 times in 10 min before use. N-α-benzoyl-L-arginine ethyl ester (BAEE), which was used as trypsin substrate, was dissolved in Tris-HCl buffer (pH 8.0). Bovine trypsin (purchased from sigma) was dissolved in Tris-HCl buffer (pH 8.0) and stored at less than 4 ˚C. Different buffer solutions and different pH was prepared to adjust the acidity of the solution.

Assay of enzyme activity

Standard trypsin activity assay

The standard trypsin assay mixture contained different concentrations (0.15 – 0.4 mM) N-α-benzoyl-L-arginine ethyl ester (BAEE) as substrate in 67 mM Tris–HCl buffer, pH 8.0. The reaction were initiated by the addition of 0.5 mg/ml trypsin solution. The ester lytic activity of trypsin was determined at 30°C. One unit of activity is defined as the amount of enzyme that hydrolyses 1.0 µmol of BAEE per minute at 30 ˚C.

Inactivation kinetics of trypsin by nano-TiO_2

The inactivation of trypsin by nano-TiO_2 was assayed as the decrease in trypsin activity over time of trypsin-nano-TiO_2 reaction at 523 nm and 30°C of 0.5 mg.ml^{-1} trypsin suspension in 67 mM Tris–HCl buffer (pH. 8.0) in the absence and presence of different concentrations(0-0.1671mg/mL) of nano-TiO_2. The rate of the decrease was proportional to the native enzyme concentration..

Fluorescence instrument

The intrinsic fluorescence measurements were performed on fluorescence spectrophotometer. The excitation wavelength of fluorescence spectrum was set at 280 nm and the emission spectra were recorded in the wavelength range of 300-450 nm. The bond widths of excitation and emission slits were set as 10 nm and 3 nm, respectively. All studies were carried out in quartz cells containing 0.5 mg/ml trypsin and different concentration nano-TiO_2 suspension. The fluorescence emission spectra of trypsin were measured at buffers 0.1 M glycin-Hcl (pH 3.0), 10 mM phosphate (pH 7.25).

Thermal stability of trypsin

Denaturation curves of trypsin(0.25 mg/ml) from 293 to 373 (K) at 280 nm at pHs 3.0, 8.0 and 10.0 were made using Pharmacia Biotech–34000 UV–Visible spectrophotometer having a cell holder whose temperature was regulated by an external thermostat. All measurements were monitored relative to control samples, which had the same composition except that nano-TiO_2 was omitted.

Calculation of Kinetics parameters (Vmax, Km and Ki)

In this work three important parameter, Km, Vmax and Ki were calculated for analysis of activity of trypsin. Km is the [S] at 1/2 Vmax. Km is a constant for a given enzyme. Km is an estimate of the equilibrium constant for substrate binding to enzyme. Small Km means tight binding and high Km means weak binding. Km is a measure of [S] required for effective catalysis to occur. Km= k-1+k2/K1. Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation: v = Vmax * [S]/ Km + [S]; Vmax is a constant for a given enzyme and it is the theoretical maximal rate of the reaction. To reach Vmax would require that all enzyme molecules have tightly bound substrate [19]. The inhibition constants Ki values in the presence of nano-TiO_2 were obtained from the Dixon plots according to Eq. 1 and secondary plots according to Eq. 2 and Eq. 3.

$$\frac{1}{Vo} = \frac{Km}{Vmax\,[S]\,Ki} I + \frac{1}{Vmax}\left(1 + \frac{Km}{[s]}\right) \quad (1)$$

$$\frac{Km}{Vmax'} = \frac{Km}{Vmax} + \frac{Km\,[I]}{Vma\,Ki} \quad (2)$$

$$\frac{1}{Vmax'} = \frac{1}{Vmax} + \frac{[I]}{Vmax\,Ki} \quad (3)$$

$$\left(Vmax' = Vmax_{app} = Vmax + \frac{Vmax\,Ki}{[I]}\right)$$

RESULTS AND DISCUSSION

Determination of particle size of nano-TiO_2

Fig. 1 shows the SEM picture of nano-TiO_2. From SEM measurement we observed that nano-TiO_2 particles have spherical in shape and the average particle diameter is about 21 nm. SEM image of nano-TiO_2 is presented at Fig. 1.

Kinetics study of trypsin in presence of nani-TiO_2

The activity of trypsin in presence of nano-TiO_2 is presented at Fig. 2. With the addition of nano-TiO_2 to trypsin, the activity of trypsin is reduced. Lineweaver-Burk plot for trypsin in presence and absence of TiO_2 NPs is presented at Fig. 3. Table 1 reports the catalytic parameters of the trypsin in presence of nano-TiO_2. The affinity of the enzyme for the substrate BAEE is no change in presence of nano-TiO_2 but the Maximum rate of trypsin activity is dicreased. The result indicates that

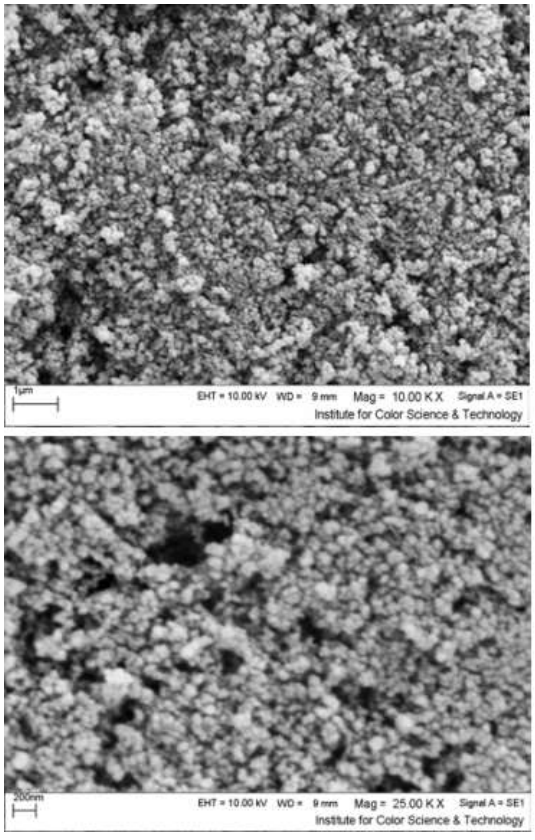

Fig. 1. SEM image of TiO2 NPs

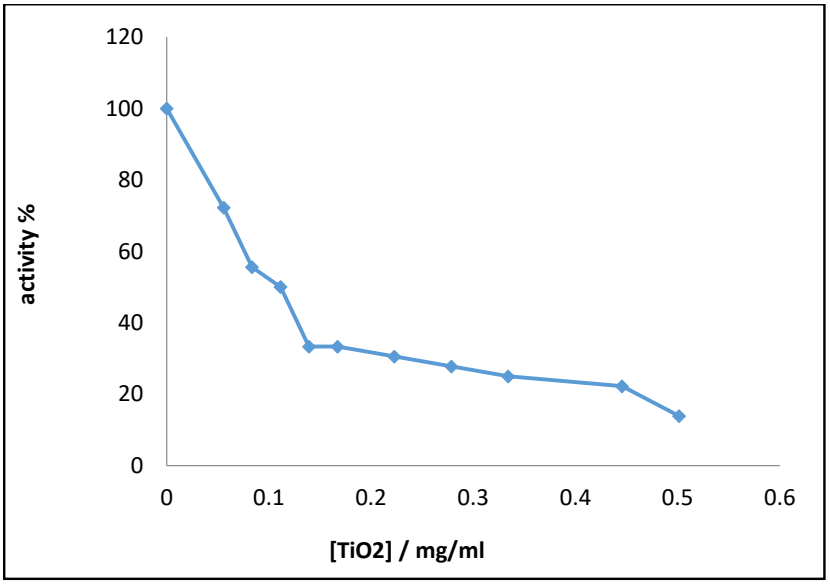

Fig. 2. The effect of TiO_2 NPs on the activity of trypsin.

Table 1. Catalytic properties of trypsin in presence of nano-TiO_2

$[TiO_2]$ ($mg.ml^{-1}$)	V_{max}	K_I	$K_m(\mu M)$	$K_{cat}(S^{-1})$
0	5	0.1	0.21	25
0.055	3.84	0.1	0.21	17
0.111	3.12	0.1	0.21	14
0.139	3.05	0.1	0.21	11
0.167	1	0.1	0.21	9

Nano-TiO_2 act as non-competitive inhibitor for enzyme trypsin. The Ki constant derived from the linear plot: Dixon plot(Fig. 4), $1/Vmax_{app}$ versus [I] (Fig. 5) and $Km/Vmax_{app}$ versus [I] (Fig. 6) and dixon plot(Fig. 5) is 0.1 mM. If an inhibitor binds not only to free enzyme but also to the enzyme substrate complex ES, inhibition is non-competitive. In this case, S (substrate) and I (inhibitor) do not mutually exclude each other and both can be bound to the enzyme at the same time [20]. In most instances, the structure of the inhibitor does not show a close similarity to that of substrate, which suggests that the binding of inhibitors is at an allosteric site, that is, at a site other than that of the substrate. The inhibition of the enzyme may result from a distortion of the three dimensional structure of the enzyme which is caused by the binding of the inhibitor. This distortion may be transmitted to the active site even though the inhibitor binds far from that site. In some cases two distinctly different conformers of the protein may exist, one binding substrate well and the other binding inhibitor well. In other instances the bound inhibitor may interfere with the catalytic action by partially overlapping the active site. In either case the ES complex reacts to give product in a normal way, but the ESI complex reacts more slowly or not at all. The investigation indicated that there is

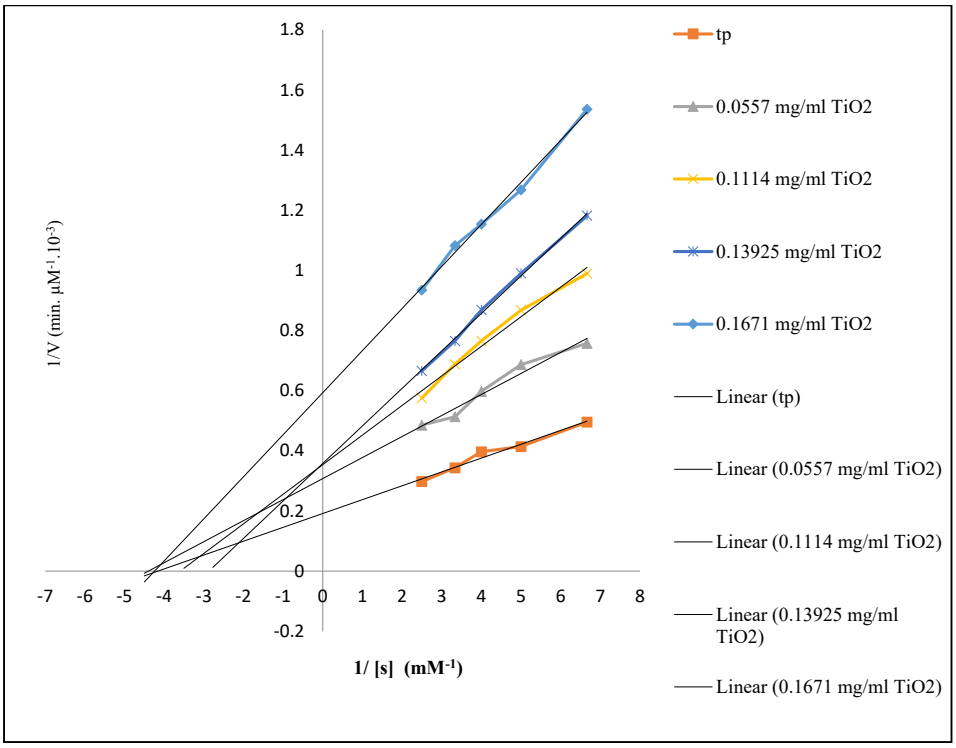

Fig. 3. Double reciprocal plots for trypsin in presence and absence of nano-TiO_2 at 30 ˚C, pH 8.0.

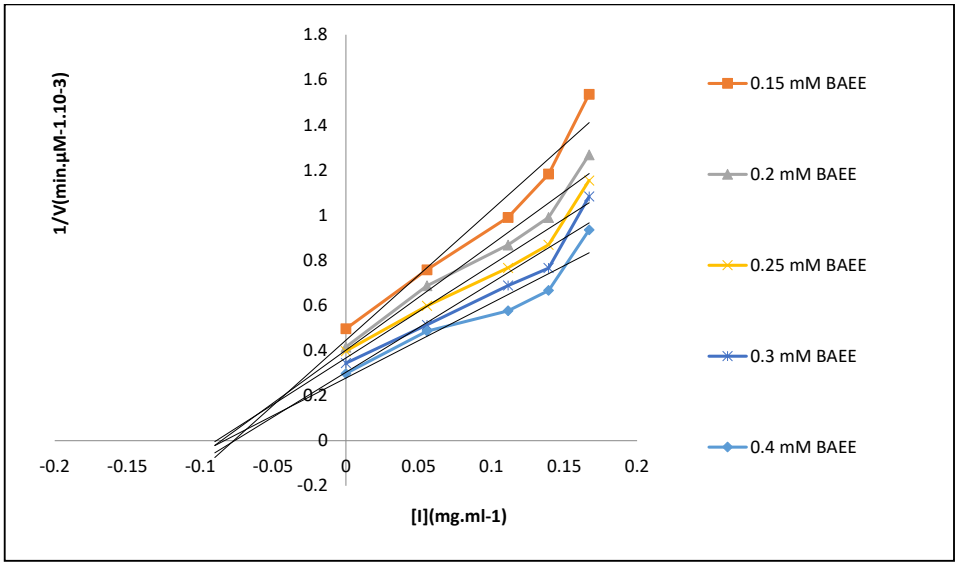

Fig. 4. Dixon plot for trypsin in presence of different concentration of nano-TiO_2 at 30 ˚C, pH 8.0.

an interaction between nano-TiO_2 and trypsin via ground state complex formation [21].

Fluorescence analysis of the trypsin nano-TiO_2 interaction

Fluorescence technique is excellent for the investigation of conformational changes of proteins. Tryptophan residues dominate the fluorescence because both their absorbance at the wavelength of excitation and their quantum yield of emission are considerably higher than the respective values of other amino acids. The large

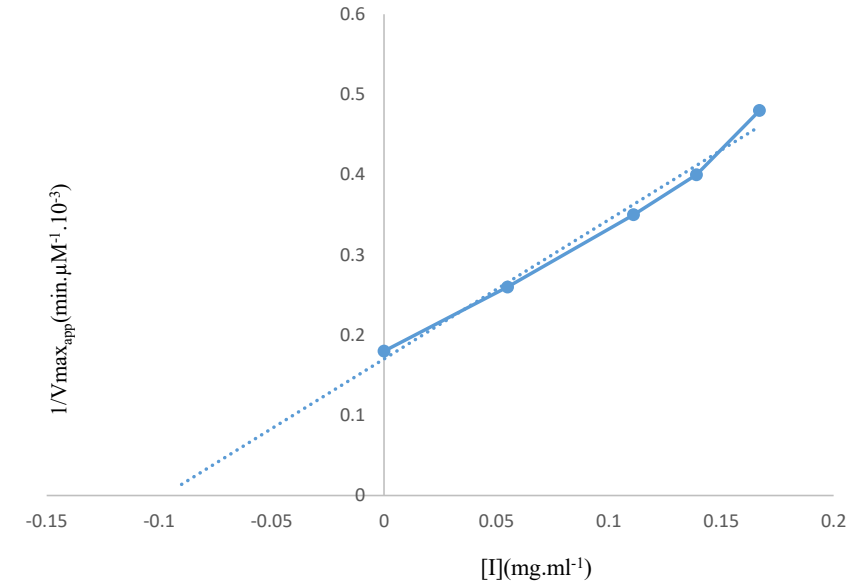

Fig. 5. (Secondry plot) effect different concentration nano-TiO_2 on the activity trypsin at 30 ˚C, pH 8.0.

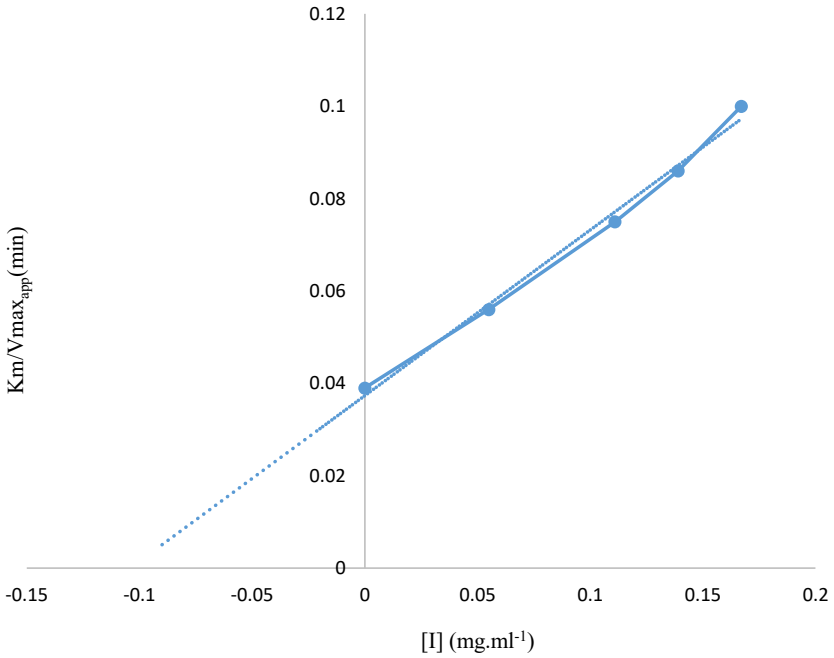

Fig. 6. (Secondary plot) effect different concentration nano-TiO_2 on the activity trypsin at 30 ˚C, pH 8.0.

changes in the fluorescence emission may lead to the changes in protein conformation. In protein containing tryptophan residues, both shifts in wavelength and changes in intensity are observed upon unfolding [22]. Since trypsin has four tryptophan residues in its molecular structure, excitation has been carried out at 280 nm with the emission range of 300–450 nm. The effect of increasing concentration of colloidal TiO_2 NPs on the fluorescence emission spectrum of trypsin is presented at Fig. 7 and Fig. 8 at pH 3.0 and 7.25 at different temperature of 30˚C, 45˚C, 75˚C. with the addition of TiO_2 to protein solution at pH 3.0, the maximum intensity of emission spectrum of

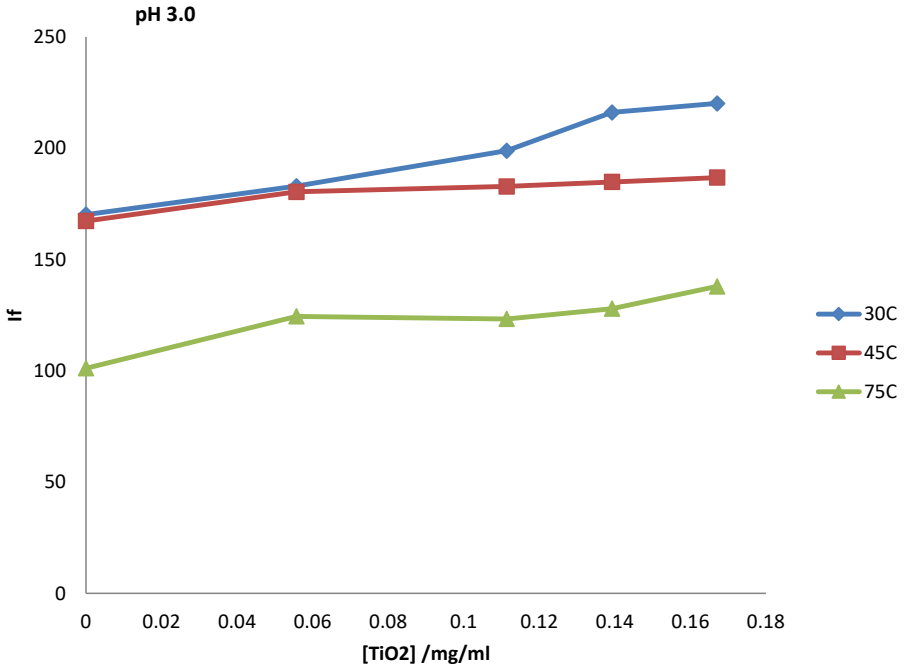

Fig. 7. The emission intensity of trypsin in presence and absence of nano-TiO_2 at pH 3.0 and temperatures of 30 ˚C, 45 ˚C, 75 ˚C

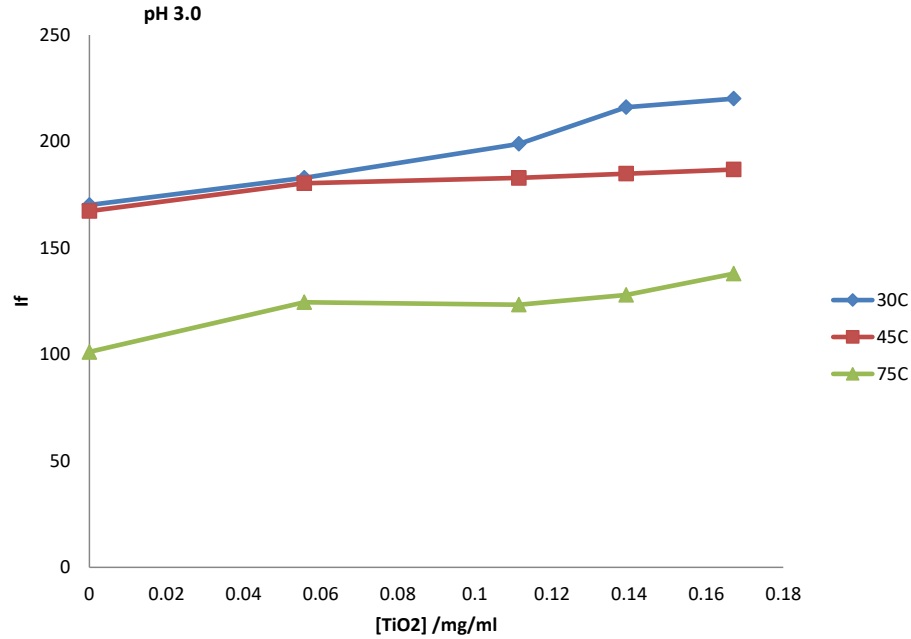

Fig. 8. The emission intensity of trypsin in presence and absence of nano-TiO2 at pH 7.25 and temperatures of 30 ˚C, 45 ˚C, 75 ˚C

trypsin is increased at all temperature. But, with the addition of TiO_2 to protein solution at pH 7.25, the maximum intensity of emission spectrum of trypsin is decreased at all temperature, which resulted in the quenching of its fluorescence emission. Obviously, nano-TiO_2 particle surfaces carry lots of negative charges at pH 7.25 because its isoelectric point is at pH 6 [23]. In contrast,

trypsin carries lots of positive charges in a neutral media (the isoelectric point is at pH 10.1) [23]. At first, the adsorption of trypsin occurred by the electrostatic attraction when trypsin is mixed into nano-TiO_2 particle suspension at pH 7.25. When the distance between trypsin and TiO_2 is short enough, the hydrogen bond will form between TiO_2 and the polar side chains of amino acid residues. Thus, the combination of noncovalent electrostatic interactions and hydrogen bonds led to the firm binding of trypsin on TiO_2 particle. Trypsin and nano- TiO_2 particle carries lots of positive charges at pH 3.0. Then, the hydrogen bond between TiO_2 and the polar side chains of amino acid residues led to the firm binding of trypsin on TiO_2 particle at pH 3.0. The result of fluorescence spectroscopy indicated that the structure of the Trp residue environments was altered [24].

Thermal stability of trypsin

Denaturation data were analyzed by assuming two-state mechanism between the folded and unfolded states [25, 26]. Apparent fraction of denatured form, F_D, was calculated by normalizing denaturation curves using the following Eq. 4:

$$F_D = \frac{(Y - Y_N)}{Y_D - Y_N)} \quad (4)$$

Where Y indicates observed variable parameter at a given denaturant concentration. Y_N and Y_D are the variable characteristics of the native and denatured states, respectively and these values were obtained by linear extrapolation of pre- and post-transition regions. The apparent equilibrium constant, K_D, was calculated using values of F_D. The calculation for K_D values between the native and denatured states of protein at a given denaturant concentration is given by Eq. 5:

$$K_D = \frac{F_D}{(1 - F_D)} = \frac{(Y_N - Y)}{(Y - Y_D)} \quad (5)$$

Gibbs free-energy changes (ΔG_D) were calculated from K_D values using Eq. 6:

$$\Delta G_D = -RTlnK_D = -RTln\left[\frac{F_D}{(1-F_D)}\right] = -RTln[\frac{(Y_N-Y)}{(Y-Y_D)}]$$

Where R is the gas constant (1.987cal/deg/mol)

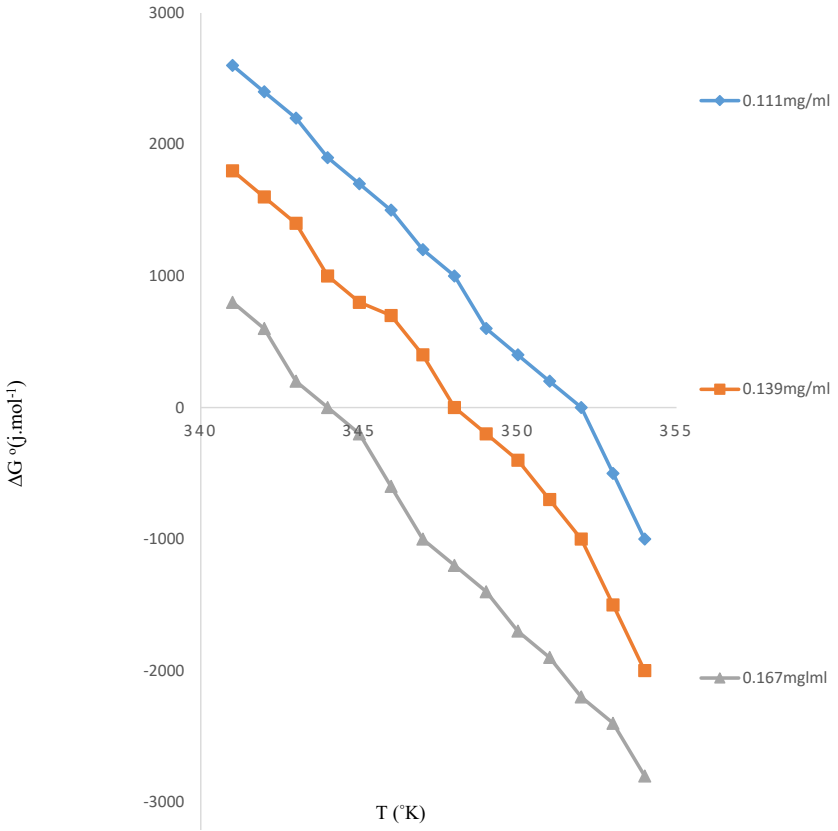

Fig. 9. Variation of Gib,s free energy vs. T (oK) at pH 3.0 .

Table 2. Tm, ΔSm°(J/mol) and ΔHm° (J/mol) values at various nano-TiO_2 concentrations at pH 3.0.

ΔH_m° (J/mol)	ΔS_m°(J/mol)	T_m (°K)	[nano-TiO_2] mg/ml
123200	350	352	0.111
104400	300	348	0.139
68800	200	344	0.167

Table 3. Tm, ΔSm°(J/mol) and ΔHm° (J/mol) values at various nano-TiO_2 concentrations at pH 7.25.

ΔH_m° (J/mol)	ΔS_m°(J/mol)	T_m (°K)	[nano-TiO_2]/Mg/ml
80541.5	247.06	326	0.111
77568.8	239.41	324	0.139
75852.3	236.3	321	0.167

and T is the absolute temperature [27]. The free energy of denaturation, ΔGD°, as a function of temperature for trypsin in the presence e of nano-TiO_2 at pH 3.0 and pH 7.25 is presented in Fig. 9 and Fig. 10, respectively. These results can be used to determine Tm at which $\Delta G_D^\circ = 0$. Tm, ΔSm◦ and ΔHm◦ parameters of thermal denaturation in presence of nano-TiO_2 at pH 3.0 and pH 7.25 are presented in table 2 and 3, respectively. The result indicates that increasing the concentration of TiO2 decreases Tm of trypsin. Monovalent binding is often weak and non-specific but a combination of many non-covalent bonds may alter the conformation and function of the protein. Inevitably, the function of protein would be affected if bond with any chemical substance. TiO_2 has strong effect of binding and –OH can be generated on the surface of particles in aqueous condition [28]. The binding effect and -OH collectively might change the polarity of trypsin and the structure of active center, leading to the decrease of trypsin activity. Similar to the small molecule–protein interaction, the interaction of trypsin with nano-TiO_2 particle may be mainly due to the monovalent combination of the electrostatic interaction with hydrogen bonds [11].

CONCLUSION

At this paper, the effect of nano-TiO_2 on the activity and conformation of trypsin has been studies. The activity of trypsin in presence of nano-TiO_2 is decreased. The result indicates clearly that TiO_2 NPs is acted as a non-competitive inhibitor for trypsin. The result of fluorescence spectroscopy indicated that the structure of the Trp residue environments was altered in presence of TiO_2 NPs. this may indicate that an interaction have to occur in a domain near of tryptophan residues The effect of nano-TiO_2 on trypsin stability has also been studied. Increasing the concentration of nano-TiO_2 decreases the stability of trypsin to thermal denaturation. This result indicates TiO_2 NPs effect on the activity and structure of trypsin.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

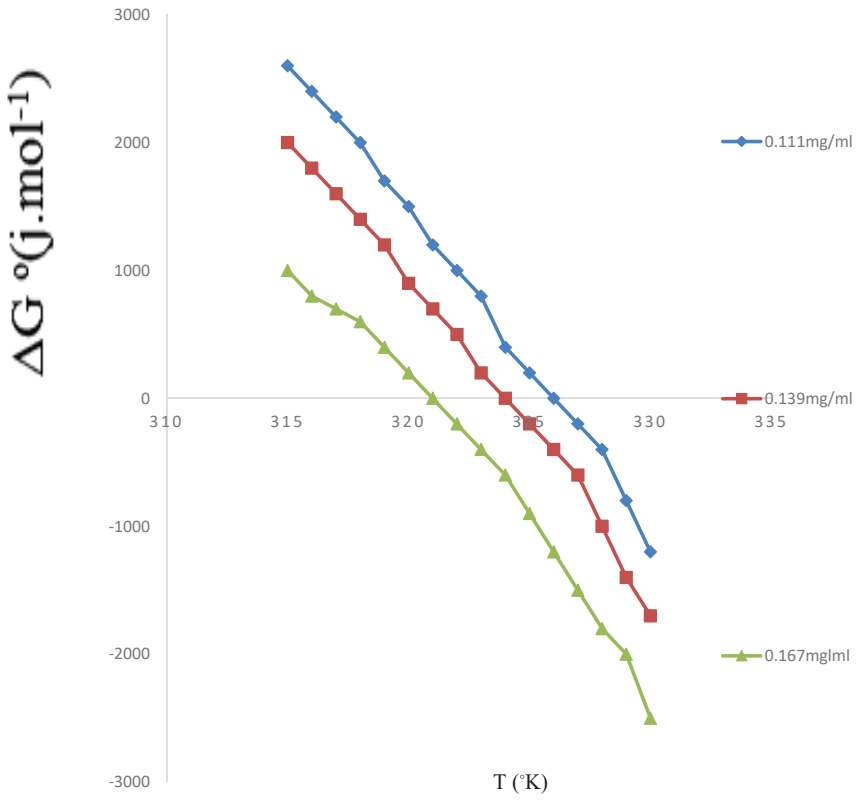

Fig. 10. Variation of Gib,s free energy vs. T (oK) at pH 7.25 .

REFERENCE

1. Yong Y, Bai Y, Li Y, Lin L, Cui Y, Xia C. Preparation and application of polymer-grafted magnetic nanoparticles for lipase immobilization. J. Magn. Magn. Mater., 2008; 320: 2350-2355
2. Handy R.D, von der Kammer F, Lead J.R, Hassellov M, Owen R and Crane M. The ecotoxicology and chemistry of manufactured nanoparticles. Ecotoxicology. 2008; 17:287-314.
3.. Rekha K, Nirmala M, Nair M.G and Anukaliani A. Structural, optical, photocatalytic and antibacterial activity of zinco xide and manganese doped zinc oxide nanoparticle. Phys. B., 2010;405: 3180-3185.
4.. Kasemets K, Ivask A, Dubourguier H.C and Kahru A. Toxicity of nanoparticles of ZnO, CuO and TiO_2 to yeast Saccharomyces cerevisiae. Toxicol. In Vitro., 2009;12: 1645-1654.
5. Elsabahy M, Wooley KL. Design of polymeric nanoparticles for biomedical delivery applications. Chem Soc Rev., 2012; 41:2545–61.
6. Wei H, Zhuo RX, Zhang XZ. Design and development of polymeric micelles with cleavable links for intracellular drug delivery. Prog Polym Sci., 2013; 38:503–35.
7. Zhu M.Z, Feng W.Y, Wang B, Wang T.C, Gu Y.Q, Wang M, Wang Y, Ouyang H, Zhao Y.L, Chai Z.F. The Design of Stable, Well-Defined Polymer-Magnetite Nanoparticle Systems for Biomedical Applications. Toxicology. 2008; 247:102-111.
8. Dunford R, Salinaro A, Cai L, Serpone N, Horikoshi S, Hidaka H, Knowland. Chemical oxidation and DNA damage caused by inorganic sunscreen ingredients. FEBS letters., 1997; 418: 87-90.
9. You C.C, De M, Rotello V.M. Monolayer-protected nanoparticle protein interactions, Curr. Opin. Chem. Biol., 2005; 9, 639-646.
10. Wang H, Wick R.L, Xing B, Environmental Pollution. Toxicity of nanoparticulate and bulk ZnO, Al_2O_3 and TiO_2 to the nematode Caenorhabditis elegans. 2009; 157: 1171-1177.
11. Xu Z, Liu XW, Ma YS, Gao HW. Interaction of nano-TiO_2 with lysozyme: insights into the enzyme toxicity of nanosized particles. Environ Sci Pollut Res., 2009;17:798–806.
12. Masciangioli T and Zhang W.X. Environmental technologies at the nanoscale. Environmental Science and Technology., 2003; 37: 102A–108A.
13. Bystrzejewska-Piotrowska G, Golimowski J, Urban P.L. Nanoparticles: heir potential toxicity, waste and environmental management. Waste Management., 2009; 29: 2587-2595.
14. Heinlaan M, Ivask A, Blinova I, Dubourguier H.C, Kahru A. Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus. Chemosphere., 2008; 71:1308 – 1316.
15. Donaldson K, Stone V, Seaton A, MacNee W. Ambient particle inhalation and the cardiovascular system: Potential mechanisms. Environ Health Persp., 2001; 109:523–527.
16. Wang J.J, Sanderson B.J.S, Wang H. Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid

cells. Mutation Research/Genetic Toxicology and Environmental Mutagenesis., 2007; 628: 99-106.
17. Wang J, Wu J, Zhang Z. H, Zhang X. D, Wang L, Xu L, Guo B. D, Li H. and Tong J. Sonocatalytic damage of Bovine Serum Albumin (BSA) in the presence of nanometer Titanium dioxide (TiO_2) catalyst. Chinese Chemical Letters., 2005; 16:1105
18. Xu Z, Wang SL, Gao HW. Effects of nano-sized silicon dioxide on the structures and activities of three functional proteins. J Hazard Mater. 2010;180:375–383.
19. Sadeghi kaji S., Shareghi B., Salavati M. Urease Activity Protection with EDTA against Nanoparticles (Fe_2O_3 and Fe3O4) Inactivation. Journal of Nanostructures., 2014; 4:217-226.
20. Lehninger A.L, Nelson D. L and Cox M. Lehninger principles of biochemistry. New York; H Freeman
and Company.2005.
21. Farhadian S, Shareghi B, Salavati M, Amooaghaei R. Spectroscopic studies on the interaction of Nano-TiO_2 with lysozyme. JNS., 2012; 1:95-103.
22. Lakowicz J.R, Weber G. Quenching of fluorescence by oxygen. A probe for structural fluctuations in macromolecules. Biochemistry., 1973; 12:4161-4170.
23. Oliva F.Y, Avalle L.B, Camara O.R, De Pauli C.P. Adsorption of humanserum albumin (HAS) onto colloidal TiO_2 particles, Part I Journal of colloid and interface science., 2003; 261:299-311.
24. Shareghi B, Hashemian A, Farhadian S, Salavati-Niasari M, Saffar B, Moshtaghi H. Thermal Inactivation and Aggregation of Lysozyme in the Presence of Nano-TiO_2 and Nano-SiO_2 in Neutral pH. JNS., 2013; 3 :281-288.
25. Privalov P.L. Stability of proteins: small globular proteins. Adv protein chem., 1979; 33: 167-241.
26. Shareghi B, Arabi M. Anti-fertility effect of nicotine. Thermal Iranian Journal of Science & Technology., 2008; 32 (323-330).
27. Shareghi B, Farhadian S, Zamani N, Salavati-Niasari M, Moshtaghi H, Gholamrezaei S. Investigation the activity and stability of lysozyme on presence of magnetic nanoparticles. Journal of Industrial and Engineering Chemistry., 2015; 21: 862-867.