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Anti-pepsin activity of silicon dioxide nanoparticles
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Anti-pepsin activity of silicon dioxide nanoparticles

Abstract

SiO_2NPs as an inhibitor of pepsin enzyme for treatment of gastro-esophageal reflux disease (GERD) were investigated. Silicon dioxide nanoparticles (pepsin coated SiO_2NPs) are among the safest nanoparticles that can be used inside the human body. The activity of pepsin before and after the addition of certain amounts of the NPs to the reaction mixture was measured spectrophotometrically. Furthermore, these experiments were repeated at different temperatures, different weights of NPs, and different ionic strengths. The kinetic parameters (K_m & V_{max}) of the pepsin-catalyzed reactions were calculated from the Lineweaver-Burk plots. The results showed that there is a significant reduction of pepsin activity by SiO_2NPs (V_{max} of free pepsin = 4.82 U and V_{max} of the immobilized pepsin = 2.90 U). The results also indicated that the presence of ionic strength causes remarkable reduction of pepsin activity. It can be concluded the best condition for inhibition of pepsin activity is by using a combinationof SiO_2NPs and high concentration NaCl at 37 ° C.

Keywords: GERD, SiO_2 nanoparticles, pepsin, enzyme inhibition.

Actividad antipepsina de nanopartículas de dióxido de silicio

Resumen

Se usaron nanopartículas de dióxido de silicio como inhibidores de la pepsina para el tratamiento del reflujo gastroesofágico (GERD). Estas nanopartículas (SiO_2NPs recubiertas de pepsina) son unas de las más seguras y pueden usarse en el cuerpo humano. Se midió a través de espectrofotometría la actividad de la pepsina antes y después de añadir cierta cantidad de NPs a la mezcla reactante. Adicionalmente, se repitieron estas pruebas a diferentes temperaturas, variando el peso de las NPs y la fuerza iónica. Se calcularon los parámetros cinéticos (K_m y V_{max}) de las reacciones catalizadas con pepsina a través de las gráficas de Lineweaver-Burk. Los resultados mostraron que, usando SiO_2NPs (V_{max} de pepsina libre = 4.82 U y V_{max} de pepsina inmovilizada = 2.90 U) y a través de la presencia de fuerza iónica, la actividad enzimática se reduce significativamente. Se concluye que la mejor condición para inhibir la actividad enzimática es usando una combinación de SiO_2NPs y una alta concentración de NaCl a 37 °C.

Palabras clave:GERD, nanopartículas de SiO_2, pepsina, inhibición enzimática.

Atividade antipepsina de nanopartículas de dióxido de silício

Resumo

Foram usadas nanopartículas de dióxido de silício como inibidores da pepsina para o tratamento do refluxo gastroesofágico (GERD). Estas nanopartículas (SiO_2NPs cobertas de pepsina) são uma das mais seguras e podem usar-se no corpo humano. Foi medida a atividade da pepsina mediante espectrofotometria antes e depois de agregar certa quantidade de NPs à mistura de reação. Adicionalmente, repetiram-se estas provas a diferentes temperaturas, variando o peso das NPs e a força iônica. Foram calculados os parâmetros cinéticos (K_m e V_{max}) das reações catalisadas com pepsina a través das gráficas de Lineweaver-Burk. Os resultados mostraram que, usando SiO_2NPs (V_{max} de pepsina livre = 4.82 U e V_{max} de pepsina imobilizada = 2.90 U) e a través da presença de força iônica, a atividade enzimática se reduze significativamente. Foi concluído que a melhor condição para inibir a atividade enzimática é usando uma combinação de SiO_2NPs e uma alta concentração de NaCl a 37 °C.

Palavras-Chave: GERD, nanopartículas de SiO2, pepsina, inibição enzimática.

Introduction

Gastro-esophageal reflux disease (GERD) evolves when reflux of stomach contents causes complications into the esophagus (*1*). GERD is a popular disease with a prevalence of 10% - 20% in the western countries (*2*) and some researchers reported up to 35.9% (*3*), but its risk factors and causes are not clearly known (*4*). The disease causes various symptoms, among which are heartburn and regurgitation, which affect up to 30% of the population and continue to increase (*5-7*). Pepsin, which is the most important substance in the gastric contents for the necrosis of the mucosal tissues, plays the main role in the formation of GERD and associated diseases (*8*).

Although proton-pump inhibitors are known to alleviate symptoms in most patients, a significant portion of patients continue to present GERD (*9*). There is a wide range of specific inhibitors that can bind to the active site and effectively remove the activity of pepsin, one of the best known ones is pepstatin, a specific pepsin inhibitor, which at acidic pH, tightly binds to the catalytic site of both pepsin and its precursor pepsinogen (*10*). The best way to determine K_m (the concentration of substrate at $V_{max}/2$) and V_{max} (Maximum velocity of an enzyme-catalyzed reaction at definite conditions) values of the basic Michaelis-Menten equation is by taking the reciprocal of both sides of equation to give the double reciprocal equation or Lineweaver-Burk's equation.

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Where V_0 represents the initial velocity or the activity of the enzyme-catalyzed reaction. A plot of $1/V_0$ versus 1/[S] yields a straight line with an intercept of $1/V_{max}$ and a slope of K_m/V_{max} (*11*).

Use of NPs to inhibit the activity of papsin *in vitro* as a model for the treatment of GERD was performed and published previously (*12*). Silicon dioxide nanoparticles (SiO_2NPs), also known as silica NPs or nanosilica, are the basis for a great deal of biomedical research due to their stability, low toxicity, and ability to be functionalized with a range of molecules and polymers (*13*). SiO_2NPs have received an intensive attention by scientific community due to its broad applications in biomedical and biotechnological fields such as drug delivery, gene therapy and molecular imaging, cancer therapy, and enzyme immobilization (*14*). In addition, it is widely used in cosmetics, food, varnishes, papermaking, and drugs (*15*). Fruijtier-Polloth *et al.* (*16*) evaluated the toxic effects and safety of SiO_2NPs and concluded that they are as safe as conventional SiO_2. Amorphous SiO_2NPs are widely used in food products, for example, as thickeners, anticaking agents, carriers of fragrances and flavors, and additives (*17*). The aim of the present study is to optimize the process of pepsin inhibition by SiO_2NPs as a possible new treatment for GERD.

Materials and methods

Reagents

Pepsin (EC 3.4.23.1), MWt = 36,450 D, 99.5% purity, was supplied from BDH, England. Spherical Silicon dioxide nanoparticles (SiO_2NPs), particle diameter = 39.644 nm, 99.5% purity, was supplied from Nanjing nanotechnology, China. Lyophilized Hemoglobin (human red blood cells), 96% purity, was supplied from Lee Biosolutions, Missouri, USA. Trichloroacetic acid (TCA), 98% purity from Alpha Chemika, India. Hydrochloric acid (HCl, Analytical Grade, 35.4%) was supplied from Central Drug House, New Delhi, India.

Characterization of NPs

The SiO_2NPs used in the present study were visualized using SEM and TEM techniques to further confirmation for their shape and size. The TEM studies were performed by using a JEM-2010 instrument working with an acceleration voltage of 200 kV. SEM images were arried out by using a Hitachi S-4800 SEM at 20 kV.

Estimation of pepsin activity

The enzyme activity was determined by a kinetic method (*18*). The principle depends on the fact that pepsin cleaves peptides from hemoglobin which are soluble in trichloroacetic acid (TCA). The tyrosine and tryptophan content of these TCA-soluble peptides is determined by the measurement of the extinction at 280 nm. Briefly, pepsin is dissolved in 0.01 N HCl to obtain a concentration of 0.5 mg/mL. Just prior to assay there is another dilution in 0.01 N HCl to a concentration of 5-20µg/mL.

The steps of the method were as follows: 1 mL of hemoglobin substrate was pipetted into test tubes containing 0.2 mL of the diluted pepsin at 37 °C. After 10 min, the reaction was stopped by adding 2 mL of 5% TCA. The tubes containing the reaction mixture were removed from water bath after 5 min and clarified (filtrates should be clear).

E280 nm was read of filtrate and subtract E280 nm of the appropriate blank using spectrophotometer (Model 721-Taiwan) set at 280 nm and 37 °C. The method of estimation of pepsin activity was repeated at different temperatures (22, 27, 32 and 42 °C). A unit of pepsin enzyme was defined as an amount of enzyme which renders TCA soluble 0.001 E280 nm per minute at 37 °C, using a denatured hemoglobin substrate.

Inhibition of pepsin activity by SiO_2NPs

To study the inhibition of pepsin by SiO_2NPs, a weight of 19.8 mg of SiO_2NPs was weighed by Sartorius balance (Model A200) dissolved in 10 mL of 0.01N HCl, and agitated by ultrasonic water bath (IsoLab, Germany) at 37 °C for 20 min. Five mg of pepsin were added to the SiO_2NPs-containing tubes and incubated at 37 °C for 30 min. These amounts of pepsin and NPs were calculated to produce pepsin monolayer on the SiO_2NPs. SiO_2NPs and pepsin surface areas were calculated in order to estimate theoretically the enough number of pepsin to cover the surface of one spherical SiO_2NP in one layer manner.

The calculations describe the pepsin and SiO_2NPs properties (radius, density, mass of one NP, and the volume of one NP) to calculate the surface areas of pepsin and SiO_2NPs which were 1.50 x 10^{-13} cm^2 and 4.94 x 10^{-11} cm^2, respectively. The number of pepsin molecules that can cover one SiO_2NP in a monolayer manner was obtained from the division of one SiO_2NPs surface area on one pepsin surface area ≈ 329 molecules of pepsin per SiO_2NP. This means that a 5 mg of pepsin were required to cover 19.8 mg of SiO_2NPs to obtain a monolayer of the adsorbed pepsin.

The method of pepsin activity estimation was repeated and the activity of the immobilized pepsin was estimated. To study the effect of weight on the pepsin activity the same method was repeated using different weights of SiO_2NPs (39.6 mg, 59.4 mg, 79.2 mg and 99.0 mg).

Effect of temperature on the inhibition of pepsin activity by SiO_2NPs

To study the temperature effect in the presence of SiO_2NPs, 19.8 mg of SiO_2NPs was dissolved in 10 mL of 0.01 N HCl, and agitated by ultrasonic water bath at different temperatures (22, 27, 32, 37 and 42 °C) for 20 min. Five milligrams of pepsin were added to each tube and incubated at 42 °C for 30 min. The method of estimation of pepsin activity was repeated and the pepsin activity was estimated after adding the SiO_2NPs.

Effect of a combination of ionic strength and SiO_2NPs on the inhibition of pepsin activity

To study the effect of the ionic strength in the presence of SiO_2NPs, 15 mg of SiO_2NPs and 1.3 mg of NaCl were dissolved in 10 mL of 0.01 N HCl, and agitated by ultrasonic water bath at 37 °C for 20 min. Five mg of pepsin were added to the tubes and incubated at 37 ° C for 30 min. The method of estimation of pepsin activity was repeated and the pepsin activity was calculated. The protocol of the research is presented in Figure 1.

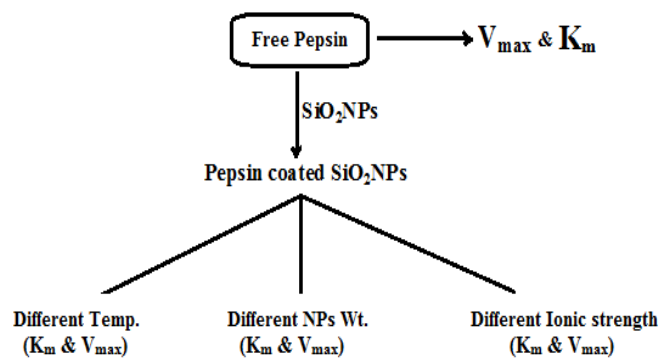

Figure 1. Workflow of the protocol of the research.

Results and discussion

The images of SiO_2NPs taken by SEM and TEM are presented in Figure 2. It is clear that the shape of SiO_2NPs is spherical with a particle size around 39 nm.

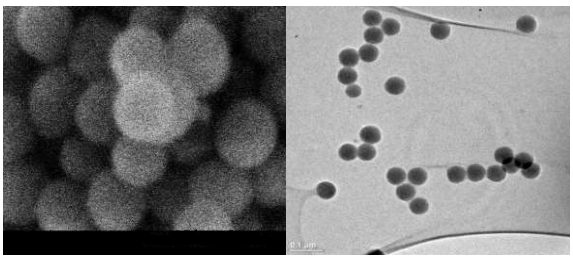

Figure 2. SEM (left) and TEM (right) images of the SiO_2NPs.

Effect of SiO_2NPs weight on the pepsin catalyzed reaction

The activity of the enzyme was measured by using seven different concentrations of hemoglobin (3.01, 6.21, 9.31, 12.41, 15.52, 18.62, and 21.72*$^{10-5}$M) and five different weights of added SiO_2NPs (19.8, 39.6, 59.4, 79.2 and 99 mg) to the 10 mL of the 0.01 N HCl to prepare SiO_2NPs solution. These experiments were used to examine the effect of SiO_2NPs weights on the activity of pepsin catalyzed reaction. The Lineweaver-Burk plots of the five experiments, were plotted in Figure 3.

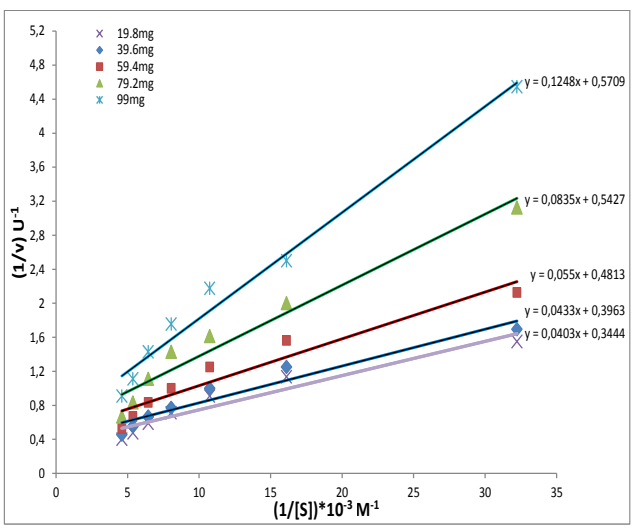

Figure 3. Lineweaver-Burke lines of pepsin catalyzed reaction after adding different amounts of SiO_2NPs.

The results in Figure 3 revealed that the presence of SiO_2NPs causes reduction in the V_{max} of pepsin catalyzed reaction. The values of V_{max} and K_m are shown in Table 1.

Table 1. Effect of weight of SiO_2NPs at 37 °C on the activity of pepsin coated SiO_2NPs.

Wt. of SiO_2NPs (mg)	Slope	Intercept	V_{max} (U)	K_m x 10^{-5} (M)
0	0.034	0.208	4.82	19.210
19.8	0.040	0.344	2.90	11.702
39.6	0.040	0.396	2.52	10.177
59.4	0.055	0.481	2.08	11.427
79.2	0.083	0.543	1.84	15.202
99.0	0.125	0.571	1.75	21.860

The data in Table 1 demonstrate the effect of weight of SiO_2NPs on pepsin activity. These weights were selected from the calculations of the amount of SiO_2NPs needed to be coated with a monolayer of pepsin molecules. The first weight (19.8 mg) of SiO_2NPs in the reaction mixture represents a NPs coated with a monolayer of pepsin molecules. The results showed that V_{max} of free pepsin (4.82 U) decreases to 2.90 U when the enzyme was immobilized on the surface of SiO_2NPs. The activity continued to decrease as the weight of the added NPs increases until becoming 1.75 U when the weight added is 99 mg. It is clear that the SiO_2NPs has remarkable inhibitory effect on pepsin activity. Most of the reduction in the pepsin enzyme activity is due to the change in the secondary structures of the whole enzyme and particularly in the active site structure. These changes are caused by the adsorption forces between the surface of the NPs and various chemical groups of the pepsin molecules. The attractive forces are strong enough to hold molecules on the NPs surface and modify the H-bonding that constitute the secondary structure of the pepsin molecules. Therefore, the change in the secondary structure is the most probable cause for the decrease in the activity of the immobilized forces.

Studies indicate that the activity of lysozyme adsorbed onto SiO_2NPs is lower than that of the free protein, and the fraction of activity lost correlates well with the decrease in α-helix content (*19*). Binding of proteins on planar surfaces often induces significant changes in the secondary structure (*20*). However, a study of a variety of nanoparticle surfaces and proteins indicates that perturbation of protein structure still occurs to varying extents. The proteins show a rapid conformational change at both secondary and tertiary structure levels (*20, 21*). Numerous studies have found that activity reduction is related to the loss of α-helical content when proteins are adsorbed onto NPs regardless of an increase in the β-sheet (*20*). Binding of proteins to planar surfaces often induces significant changes in secondary structure; the high curvature of NPs can help proteins to retain their original structure (*22*). Several *in vivo* and *in vitro* studies of the toxicity of SiO_2NPs have been performed and found that they are safe and can be employed in food production (*23*). These findings and the findings of the present research encourages the use of SiO_2NPs as an inhibitor of pepsin for the treatment of GERD in vivo.

Effect of temperature on the interaction of SiO_2NPs with pepsin

The activity of the immobilized enzyme was measured at different temperatures (22, 27, 32, 37, and 42 °C) by using 19.8 mg of SiO_2NPs and the same concentrations of hemoglobin. These experiments were used to examine the effect of temperature on the activity of pepsin catalyzed reaction in the presence of SiO_2NPs. The Lineweaver-Burk lines of the immobilized pepsin activity at five different temperatures are presented in Figure 4.

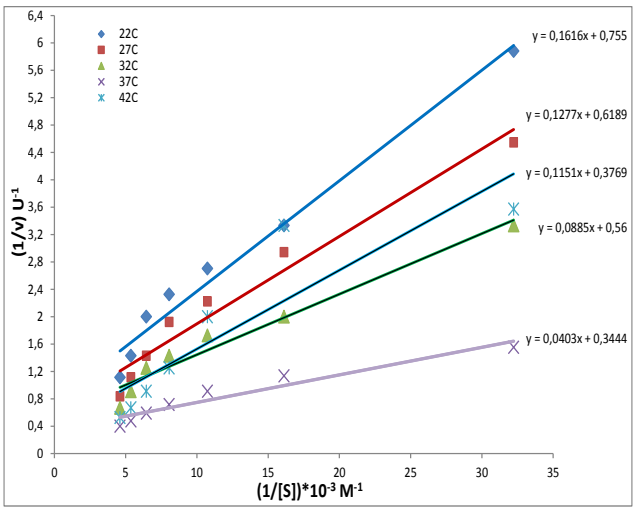

Figure 4. Lineweaver-Burk lines of the SiO_2-immobilized pepsin catalyzed reaction at (22, 27, 32, 37, and 42 °C).

The values of V_{max} and K_m of the pepsin catalyzed reaction at different temperatures in the presence of SiO_2NPs are listed in Table 2. The results revealed that the V_{max} at 37 °C is 2.91 U and then is reduced as temperature decreases. Whereas the K_m at 37 °C equal to 11.72 x 10^{-5} M then increases as temperature decreases. It means that the change in temperature may cause a change in the form of active site and thus change V_{max}.

Table 2. Maximum velocities and Michaelis constants of the SiO_2NPs-immobilized pepsin at different temperatures.

Temp. (°C)	Equation	V_{max} (U)	K_m x 10^{-5} (M)
22	y = 0.162x + 0.755	1.33	21.40
27	y = 0.128x + 0.620	1.62	20.63
32	y = 0.089x + 0.560	1.79	15.80
37	y = 0.040x + 0.344	2.91	11.72
42	y = 0.115x + 0.380	2.65	30.54

The results in Table 2 revealed that K_m and V_{max} change when the temperature changes. V_{max} increases as the temperature increases until reaching the optimum temperature and then tends to decrease when the temperature reaches 42 °C, while K_m decreases as temperature increases. However, at 42 °C, the K_m increases again indicating that there are conformational changes in the immobilized pepsin or changes in the interaction between the pepsin molecules and the SiO_2NPs.

In a previous study, it is found that the interaction between SiO_2NPs and three different enzymes are dependent on the functional groups on the surface of NPs (*24*). These findings indicated the presence of weak electrostatic interactions between the protein molecules and the surface of NPs. When nanomaterials are in contact with a biological environment, the proteins can immediately bind to the surface of the NPs, which creates protein coronas (*25*).

The bio-distribution of the nanomaterial is affected by this protein coating, which aids in understanding the mechanisms of protein coronas formations on nanomaterial surfaces including the effect of the nanomaterial surface properties (*26*). Weak protein-NPs interactions were studied previously in a low binding regime as a model for the soft protein corona around NPs in complex biological fluids. Noncovalent and reversible interactions between protein and SiO_2NPs showed significant alteration in conformation and enzymatic activity in a NP-size dependent manner. These facts indicated the presence of very weak interactions between protein and SiO_2NPs (*27*). Changes of environment temperature can alter the intramolecular attractive forces (hydrogen bonding, dipole-dipole interaction, hydrophobic interaction etc.) of the protein (e.g. enzyme). This can alter the active site of the enzyme rendering it inactive (*28*).

Effect of SiO_2NPs and ionic strength

The pepsin activity was measured at various conditions, i.e., free pepsin, in the presence of 130 mg of NaCl, immobilized pepsin on SiO_2NPs, and immobilized pepsin in the presence of 130 mg NaCl. All experiments were carried out at 37 °C. These experiments were carried out to explore the effect of the combination of ionic strength and SiO_2NPs on the activity of pepsin. The Lineweaver-Burk plots of the four experiments were plotted in Figure 5.

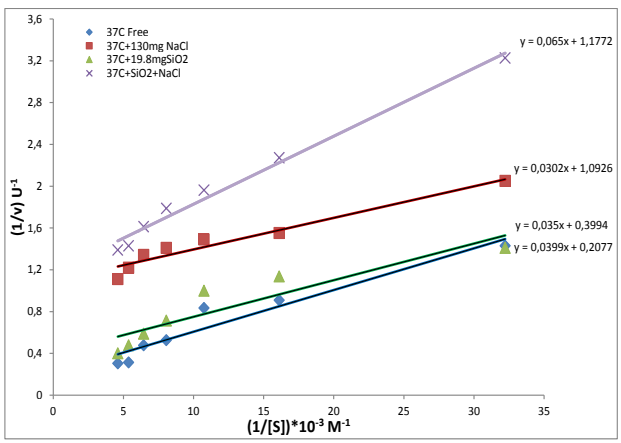

Figure 5. Lineweaver-Burke lines of pepsin catalyzed reaction at 37 °C after adding 130 mg NaCl, 19.8 mg SiO_2NPs and in the presence of both (130 mg NaCl and 19.8 mg SiO_2NPs).

Table 3. Comparison of free pepsin, pepsin with 130 mg NaCl, 19.8 mg SiO_2NPs, and both (19.8 mg SiO_2NPs + 130 mg NaCl).

Wt (mg)	Slope	Intercept	V_{max} (U)	Km x 10^{-5} (M)
Free	0.040	0.208	4.82	19.21
19.8 mg SiO_2NPs	0.035	0.399	2.50	8.76
130 mg NaCl	0.030	1.093	0.92	2.76
19.8mg SiO_2+130 mg NaCl	0.065	1.177	0.85	5.52

In Table 3, the comparison of free pepsin activity, immobilized pepsin on SiO_2NPs, and immobilized pepsin in the presence of 130 mg NaCl is shown. The results showed the V_{max} of free enzyme equal to 4.82 U and then became 2.50 U when 19.8 gm of SiO_2NPs were added. Then V_{max} decreased and became 0.92 U when 130 mg of NaCl were added to the enzyme reaction mixture, and then it became equal to 0.85 U when NaCl and SiO_2NPs were added together to the reaction mixture. It can be easily noticed that the effect of the combination of the ionic strength and SiO_2NPs causes high reduction of pepsin activity.

Most of the reduction in the enzyme activity is due to the changes in the secondary structures of the whole enzyme and particularly in the three dimensional structure of the active site, as previously mentioned in the Effect of SiO_2NPs weight on the pepsin catalyzed reaction section. Furthermore, a study by Wu *et al*. (*20*) showed that both β-lactoglobulin and lysozyme unfolded to a greater extent at lower surface concentration on SiO_2NPs. The proteolytic activity of pepsin is affected by the conditions of the dissolution medium. There is a significant reduction in the activity of pepsin after adding different concentrations of surfactants salts (*29*). Salts can form weak bonds with the charged functional groups on the protein surface.

Depending on the nature of ions, the balance among the forces are changed. However, higher concentration of salt can lead to salting out effect or decreased solubility of protein (*30*). There have been many interesting studies done about the inhibition of various enzyme activities upon adsorption on the surface of different nanoparticles (*31, 32*). Using advanced techniques such as circular dichroism and fluoroscopy, it is found that the most affective factors responsible for the reduction in the enzyme activity, after adsorption on the nanoparticle .surface, is the change in the secondary and tertiary structures of the enzyme especially around 18 the active site (*32, 33*). The same explanation can be generalized for the pepsin-SiO_2NPs system and it can be concluded that the reduction in the pepsin activity is due to the perturbation in the secondary and tertiary protein structures.

Conclusions

The results of the present study revealed that the SiO_2NPs has an ability to inhibit the pepsin activity. The results also indicates that the presence of high ionic strength causes remarkable reduction of pepsin activity. The increase in the amount of SiO_2NPs in the reaction medium leads to more reduction in the pepsin activity. Furthermore, the optimum temperature for the NPs to inhibit the reaction is 37 °C. Therefore, the best conditions of inhibition of pepsin enzyme is by using higher amounts of SiO_2NPs in the presence of NaCl at 37 °C.

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