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Thermal Inactivation and Aggregation of Lysozyme in the Presence of Nano-TiO_2 and Nano-SiO_2 in Neutral pH

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Abstract

Protein aggregation is a problem in biotechnology. High temperature is one of the most important reasons to enhance enzyme inactivation and aggregation in industrial systems. This work focuses on the effect of TiO_2 and SiO_2 nanoparticles on refolding and reactivation of lysozyme. In the presence of TiO_2 and SiO_2 nanoparticles, after enzyme heat treatment at 98°C for 30 min, not only aggregates were observed, but the amount of those increased. Hence the residual activity of lysozyme (without additives) and even in the presence of TiO_2 and SiO_2 nanoparticles after heat treatment was very low (<5%). T_m of the aggregated lysozyme after this heat treatment was decreased with increasing concentrations of TiO_2 and SiO_2 nanoparticles from 0 to 0.02 mg/ml in neutral pH, Whether the T_m of natural enzyme was above 373 (K) or 100°C. these nanoparticles help enzyme denaturation and misfolding in heating.

1. Introduction

Usually, enzymes have to be in stable and active conformation for their production and their storage. In order to produce active enzymes and to use them in industrial processes, we need to prevent their thermal and physical denaturation. To avoid the denaturation of proteins, it is important to elucidate the mechanisms of enzyme degradation so that stabilization strategies can be specifically planed. High temperature and mechanical stresses are the main causes enhancing enzyme inactivation in industrial systems. Thermal denaturation of enzyme is a well-studied phenomenon [1]. Proteins fold into their unique native structure, even *in vitro*. However, they tend to form undesirable and uncontrollable aggregates during the unfolding and refolding processes, both in the laboratory and even in their natural environment in living cells. Protein aggregation is a major problem in the large-scale

production of recombinant proteins, as well as in living cells, where it may lead to the occurrence of fatal diseases [2]. Nanotechnology is a rapidly expanding field, encompassing the development of man-made materials in the 5-200 nanometer size range. Nanoparticles smaller than 100 nm have been expected to reveal the novel physical and chemical properties. Nanoparticles are being used as constituents for many applications in the various fields such as industrial production and daily life [3]. Recently proteins production and application have rapidly increased, both in biochemical research and pharmaceutical industries [4]. Activity and stability of proteins are required during their production, storage and use to enhance their industrial applications. Some of the environmental conditions such as extreme pH, high temperature and physical constraints can cause protein denaturation, leading to protein aggregation. Protein aggregates can be considered as inclusion bodies that have lost their native forms and functions [5, 6]. Various procedures have been reported to prevent thermal inactivation of enzymes in vitro. One of the most important methods to solving the aggregation problem is the utilization of aggregation suppressors that are small molecular additives. In this study Hen egg white lysozyme was used as a model protein because it's mechanism of folding and refolding has been studied [2]. Lysozyme is a 129-residue enzyme which hydrolyzes the cell walls of bacteria. Its native form contains four disulfide bonds and adopts mainly helical conformation (30% α helix, 6% β-sheet) [6-13]. The objective of this article is to examine the effects of nano-SiO_2 and nano-TiO_2 on lysozyme stability and activity during the course of denaturation. In previous research, it has been worked on Thermal stability of natural lysozyme [14, 15].

2. Materials and methods

2.1. Instrument and materials

The absorption spectra of all liquids/solutions were recorded with a Model ultrospec 4000, UV-visible equipped with a thermostatic cell holder. Hen egg white lysozyme (L-6876), MW= 14300 Da, pH_I=11.1 was purchased from Sigma Co and was used without further purification. Solutions, containing 0.1 $mg.mL^{-1}$ lysozyme in 50 mM sodium-phosphate buffer (pH 7.25) and different concentrations of additives were prepared. An electrolyte solution (1.5M NaCl) was prepared to adjust the ionic strength of the solution. *Micrococcus lysodeikticus* for the kinetics assay of lysozyme was obtained from bacterial cell culture. A scanning electron microscope (SEM) was used to measure the size and shape of the nano-TiO_2 and Nano-SiO_2. Nano-TiO_2 (size 21 nm) and Nano-SiO_2 (size 10 nm) was used without further modification. It was suspended in deionized water and mixed ultrasonically for 3 times in 10 min before use.

2.2. Thermal aggregation of lysozyme Heat

Induced aggregates of enzyme were quantified as follow. All stock solutions for nanoparticles and lysozyme were dissolved in deionized water. After 30 min heat treatment in 98°C, the samples were centrifuged at 15000 g for 30 min. Then the absorption spectra of all solutions in 280 nm were recorded with a model Ultrospec 4000, UV-Visible spectrophotometer equipped with a thermostatic cell holder. So the concentration of soluble lysozyme in the supernatants was determined by using an extinction coefficient of 2.63 cm^{-1} per $mg.mL^{-1}$.

2.3. Thermal inactivation kinetics of lysozyme

Catalytic activity of enzyme was determined by measuring the decrease in absorbance at 450 nm and 35°C of 2 $mg.mL^{-1}$ *Micrococcus lysodeikticus*

suspension in 50 mM sodium-phosphate buffer, pH 7.25 in the absence and presence of different concentrations of each nanoparticle. The rate of the decrease was proportional to the native enzyme concentration.

2.4. Calculation of thermodynamic parameters on thermal inactivation

All studies were carried out in quartz cells containing 0.1 $mg.mL^{-1}$ lysozyme and different concentration nanoparticles suspension obtained from supernatants that were heated in 98°C for 30 min. The UV-Vis spectrum of the aggregated enzyme in the wavelength was set at 280 nm was and the absorbance in the absence and presence of different amounts of each nanoparticle were recorded. In other hand, the midpoint temperature of thermal unfolding (T_m), enthalpy change ($\Delta H°$) and activation entropy change ($T\Delta S°$) were determined.

3. Results

3.1 Determination of particle size of nano-TiO_2 and nano-SiO_2

In Fig.1 nano-TiO_2 was found to be spherical in shape and the average particle diameter was about 21 nm. In Fig.2 nano-SiO_2 was found to be spherical in shape and the average particle diameter was about 11 nm.

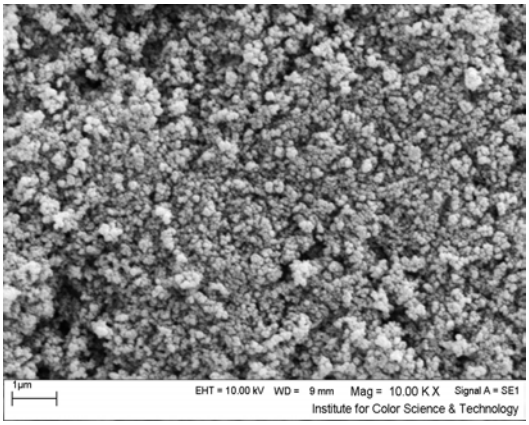

Fig. 1. SEM image of nano- TiO_2.

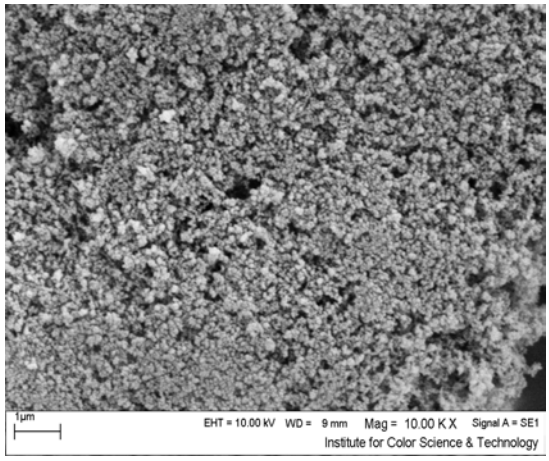

Fig. 2. SEM image of nano-SiO_2.

3.2. Heat-induced aggregation of lysozyme

Hen egg white lysozyme (pH_I=11.1) was used as a nice system to investigate the in vitro fibrillation process. Under neutral pH lysozyme tends to form irreversible aggregates during heating [6, 16]. Proteins tend to form inactive aggregates at high temperatures [2]. Fig. 3 shows the effects of nanoparticles on the thermal aggregation of lysozyme. As previously describe, after incubation at 98°C for 30 min, the amount of aggregates was determined by measuring the enzyme concentration of the supernatant. With increasing concentration of nano-SiO_2, the amount of aggregates increased. In the other hand, nano-SiO_2 did not prevent the thermal aggregation of lysozyme, while nano-TiO_2 for primary concentration did, while by increasing the nano-TiO_2 concentration after 0.01 $mg.mL^{-1}$, the amount of aggregates increased(such as nano-SiO_2).

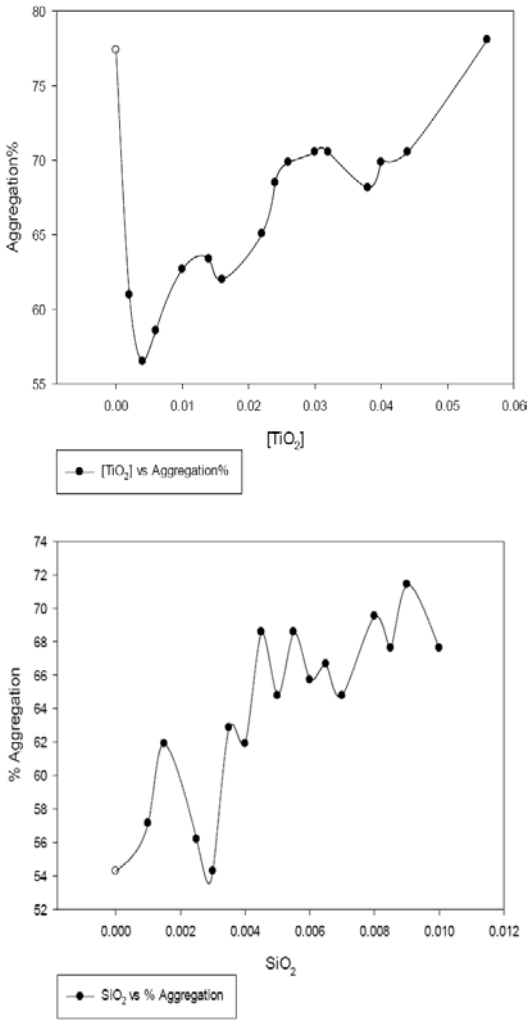

Fig 3. Amount of heat induced aggregates of lysozyme in presence of nano-TiO_2 and nano-SiO_2.

3.3. Thermal stability of aggregated lysozyme

In this study, values of the Gibbs free energy, ΔG° and Tm for denatured lysozyme are reported in the range 20-100°C and pH 7.25. Thermal denaturation curves for aggregated lysozyme in the presence and absence of nanoparticles are shown in Fig. 4. The fraction of the denatured protein, F_d, was calculated using the relation:

$$Fd = \frac{Yobs - YN}{YD - YN}$$

Where Y_N and Y_D are respectively the optical properties of the native anddenatured molecules under the conditions in which Y_{obs} has been determined. By assuming a two-state mechanism (folded state ↔ unfolded state), the difference in free energy between the folded and unfolded conformation, $\Delta G°_D$, can then calculated using:

$\Delta G°_D = -RTLn[F_d/(1- F_d)] = -RTLn[Y_{obs} - Y_N / Y_D - Y_{obs}]$

Where R is the gas constant and T is the absolute temperature. This result can be used to determine T_m at which $\Delta G°_D = 0$.BThe entropy change in midpoint temperature (ΔS_m°) was obtained from the slope of the $\Delta G°_D$ plot in fig.3. So ΔH_m° is enthalpy change in midpoint temperature and was calculated using:

$\Delta H_m^\circ = T_m \Delta S_m^\circ$

The free energy of denaturation, ΔG_D°, as a function of temperature for lysozyme in the presence and absence of nano-TiO_2 and nano-SiO_2 are shown in Fig.5. It is clear that increasing the concentration of each nanoparticle in this study, decreases the stability of lysozyme to thermal denaturation and the curves shift to lower temperatures [17]. Thermodynamic at data of T_m, $\Delta S°_m$ and $\Delta H°_m$ at different concentrations of nano-TiO_2 and nano-SiO_2 at pH 7.25 are listed in table.1&2.

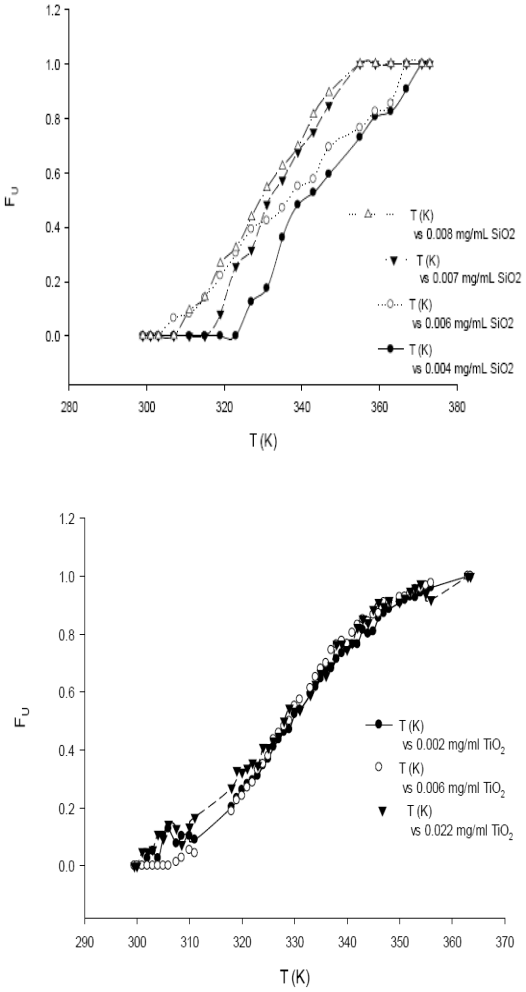

Fig 4. Thermal denaturation curve of lysozyme in the presence of nano-TiO_2 and nano-SiO_2 at pH 7.25.

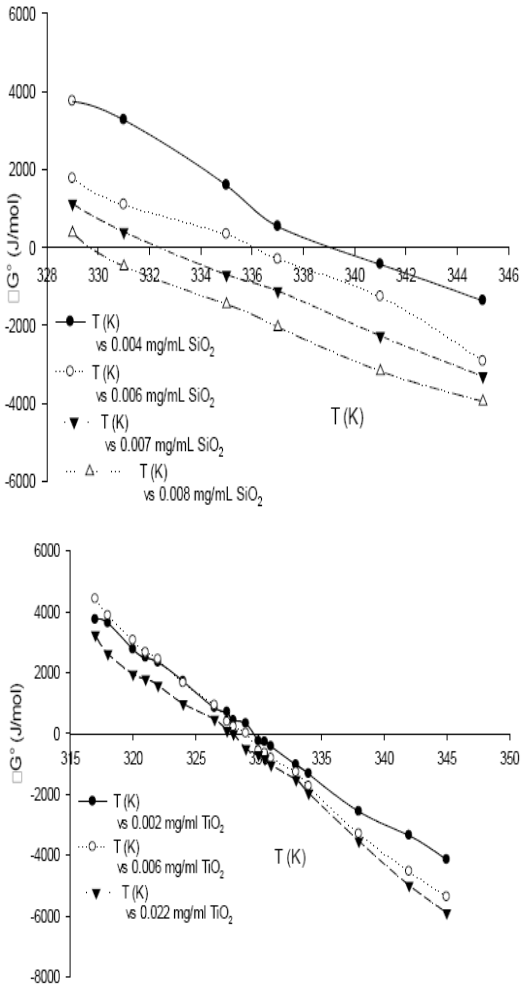

Fig. 5 ΔG_D° curves for lysozyme as a function of temperature at pH 7.25.

Table 1. Thermodynamic data, at various nano-SiO_2 concentrations in phosphate buffer.

$[SiO_2]$ mg/ml	T_m (K)	ΔS°_m (J/mol.K)	ΔH°_m (J/mol)
0.004	340	334.28	113655.2
0.006	337	278.53	94201.61
0.007	332	276.61	90506.52
0.008	330	267.94	88420.2

Table 2. Thermodynamic data, at various nano-TiO_2 concentrations in phosphate buffer.

$[TiO_2]$ mg/ml	T_m (K)	$\Delta S°_m$ (J/mol.K)	$\Delta H°_m$ (J/mol)
0.002	329.6	322.24	106210.3
0.006	329.1	318.42	104792
0.022	327.8	315.52	103427.5

3.3. Residual activity of lysozyme after heat treatment

Conformational changes of protein may result in the changes in biological activity. The recovery of enzymatic activity after heat treatment is another standard, used to estimate the effect of additives because it is the most creditable measure of whether additives prevent irreversible misfolding as well as aggregation [2]. According to this, the residual activity of lysozyme after 30 min heat treatment, was very low (<5%), also in the presence of nano-TiO_2 and nano-SiO_2, heat inactivation was observed. Adsorption onto silica and titanium nanoparticles were shown to result in a greater loss of α helix content of lysozyme and as α helix content decrease, enzyme activity and stability decreases [14, 18]. Plot of lysozyme activity at various nano-TiO_2 and nano-SiO_2 concentrations at 308 K, pH 7.25 in phosphate buffer are shown at Fig. 6. Table 3 is about V_{max} values of lysozyme at various concentrations of nano-TiO_2 and nano-SiO_2 at pH 7.25 in phosphate buffer.

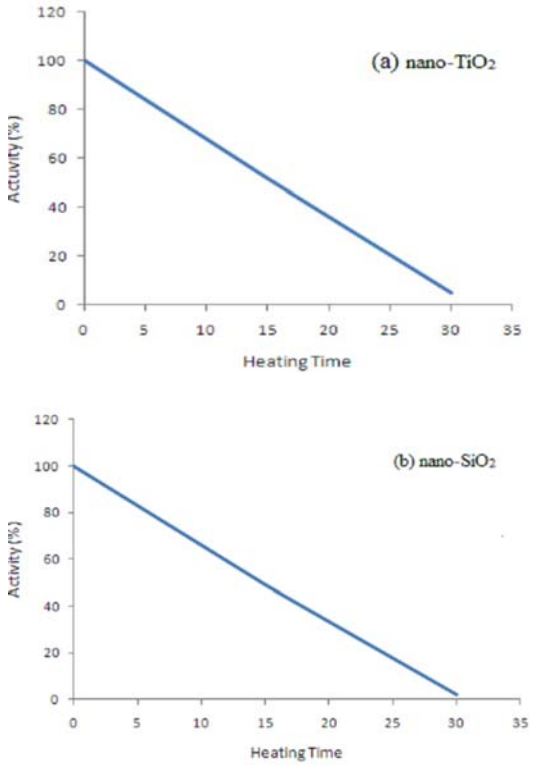

Fig. 6. Plot of lysozyme activity at various nano-TiO_2 and nano-SiO_2 concentrations at 308 K, pH 7.25.

Table. 3 V_{max} values of lysozyme at various concentrations of nano-TiO_2 and nano-SiO_2 at pH 7.25 in phosphate buffer.

$[TiO_2]$ mg/ml	V_{max}	$[SiO_2]$ mg/ml	V_{max}
0.002	0.013	0.002	0.005
0.006	0.012	0.006	0.005
0.014	0.010	0.010	0.005
0.02	0.010	0.014	0.004

4. Discussion

The aggregation of proteins is a fundamental issue in many fields, since it is related to industrial technologies and clinical pathologies, such as Alzheimer disease, Creutzfeld-Jacob disease, and other neurodegenerative or systemic diseases. Moreover, the importance of a proper control and design of a specific protein assembly is strengthened by the growing interest in protein assemblies as new biomaterials, with potential application in food texture or cellular scaffolds [25]. Intermolecular interactions are probably induced or enhanced by the tendency to minimize the contacts with the solvent (hydrophobic effect); from this point of view an irreversible aggregation is competitive with the refolding of the single molecule [26]. All proteins have an inherent tendency to aggregate unless they are maintained in a highly regulated environment [19]. Protein aggregation leads to low yields of biologically functional protein [20]. At a fixed high temperature, the degree of the inactivation is linearly related to the time course of heating [21]. In this study, silica and titanium nanoparticles are chosen as a model system since these are extensively used in other investigation [8]. The thermal aggregation of lysozyme is considered to follow two steps of an irreversible reaction at high temperatures:

$$U \rightarrow A \qquad \text{Eq. (1)}$$

$$A + An \rightarrow An + 1 \qquad \text{Eq. (2)}$$

Where, U reveals the unfolded molecule that can refold after heat treatment, A reveals the irreversibly denatured molecule and A_n represents the insoluble aggregates. Under Eqn (1), nano-SiO_2 and nano-TiO_2 do not prevent the interaction shown in Eqn (2) [16, 22]. So nanoparticles, such as nano-SiO_2 and nano-TiO_2, decreased the solubility of enzyme and increased the aggregation [23, 24]. Moreover, after heat treatment at 98°C for 30 min at 280 nm, more aggregates were observed in the presence of very low concentration of each nanoparticle, also lower than 5% of the molecules were activated. So because of improving the Eqn (2), the irreversibly denatured molecule (A), was decreased and the insoluble aggregate was increased. Such a behavior may be due to the fact that the protein, being highly positively charged, may be unstable [27]. In the pH range of 4 to 9, lysozyme is positively charged. Since the silica and titanium nanoparticles are negatively charge (especially silica), the electrostatic interaction between the protein and nanoparticle surface should promote unfolding in parallel heating. So the thermal denaturation of many globular proteins, like lysozyme, consists in a thermal inactivation of the enzymatic properties. The structure of the lysozyme aggregates is mainly made of weak and non-covalent interactions with some dimers and trimers linked by disulfide bonds. Once an enzyme has been denatured, the exposed hydrophobic surfaces tend to avoid interaction with the aqueous solvent. Then unfolded enzymes became insoluble and formed aggregates because of the amphiphilicity of the surface of the disordered enzym. The aggregates formed during inactivation are made up of 77% of monomers, 18% of dimers and 5% of trimers. Most of the interactions in the aggregates are non-covalent bonds, but dimers and trimers are linked by disulfide bonds. Stirring does not induce cleavage of enzyme molecules during inactivation but inactivated lysozyme aggregates were irreversible. The irreversibility was shown whatever the initial lysozyme concentration, the size of the aggregate and the denaturant parameter — stirring speed or temperature — stopped [1]. The secondary conformational changes of lysozyme upon adsorption lead to a loss of α helical content with a corresponding increase in β-sheet and random coil. The rapid unfolding of

lysozyme upon adsorption onto nanoparticles surface is believed to be due to the rapid decrease in the free energy of adsorbed protein molecule [14, 18].

5. Conclusion In this study, conformational changes induced by temperature on lysozyme dissolved in different environments were analyzed in relation to protein unfolding and self-assembling. An enhanced sensitivity of protein conformations to the temperature variation was revealed with a reduced thermal stability of the aggregated state of lysozyme. An enhancement of intermolecular interactions was observed upon increase of nano-particle concentration.

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