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Original Research Article

Biochemical Characterization of Protease and Its Impact By Nano Particles in Sera of Iraqi Patients with Burns

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Abstract

Proteases have great medical and pharmaceutical importance due to their key role in biological processes and in the life-cycle of many pathogens. The present study aims to characterization of protease and evaluate their impact by Gold Nano Particles and Nickel Nano Particles. A total of 30 patients with burn and 25 healthy individual with matches were included in this study. Gold nanoparticle and nickel nanoparticle were prepared using Pulsed Laser Ablation in Liquid method .The activity of serum protease was determined using casein as substrate. The results indicated increased protease activity in sera of burn patients and inhibition effects of both gold and nickel nanoparticles on protease activity. Thermodynamically favorability of the reaction can depend on the temperature. We conclude that nanoparticles such as gold and nickel can be used as treatment of burns through their role in homeostasis due to their inhibition impact on protease activity.

Key words: Burn, protease, gold nanoparticle, nickel nanoparticle, immunoglobulin

التوصيف الكيميائي الحياتي للبروتيز وتأثره بالدقائق النانوية في امصال دم العراقيين المصابين بالحروق

الخلاصة

الانزيمات المحللة للبروتينات لها أهمية طبية وصيدلانية كبيرة نظراً لدورها الهام في العمليات البيولوجية وفي دورة حياة كثير من الكائنات الممرضة. تهدف الدراسة الحالية إلى توصيف البروتيز وتقييم تأثره بالدقائق النانوية للذهب والنيكل. تضمنت الدراسة جمع (٣٠) عينة من المصابين باالحروق و(٢٥) عينة من الاصحاء بنفس المدى العمري والجنس لغرض المقارنة. أعدت جسيمات متناهية الصغر من الذهب والنيكل بإستعمال طريقة التبخر ألانفجاري في السائل. قيست فعالية البروتيز بإستعمال الكازئين بوصفها مادةأساس. أظهرت النتائج ازدياد فعالية البروتيز في امصال دم المصابين بالحروق وتأثير مثبط لكل من دقائق الذهب والنيكل النانوية على فعالية الأنزيم. بينت الديناميكة الحرارية ان أفضلية التفاعل يمكن أن تعتمد على درجة الحرارة. نستنتج أن الدقائق النانوية مثل الذهب والنيكل يمكن إستعمالها في علاج الحروق من خلال دورها في التوازن بسبب التأثير التثبيطي لها على فعالية البروتيز.

Introduction

A burn is a type of damage to skin caused by heat, chemicals, electricity , radiation or friction, [1]. Burns are one of the most destructive of all injuries and a major global crowd health turn [2-4].They are the fourth most popular type of hit worldwide, following traffic accidents, falls, and interpersonal violence [5,6]. Burns result in about two million physician visits per year [7]. Approximately 90 percent of burns occur in low to middle income countries, regions that generally lack the substantial infra-structure [8,9].Burn injuries encompass the partial or complete devastation of the integumentary system: the skin. The layers of the skin are ruined and these results in local and systemic disorders. When the skin is damaged by a burn, it may result in compromised immunity, hypothermia, increased fluid loss, infection, changes in appearance, function, and body image [10].Inflammation is a typical and early response of a burns tissue. It results in an increase in the number of immune cells in the area of damage or infection which remove damaged or dead cells and initiate the healing process which involve conversion of an inactive proteolytic enzyme into an active enzyme[11].These enzymes have great medical and pharmaceutical importance due to their key role in biological processes and in the life-cycle of many pathogens. Proteases are widely applied enzymes in several sectors of industry and biotechnology, furthermore, numerous research applications require their use, including production of Klenow fragments, peptide synthesis, digestion of unfavorable proteins during nucleic acid purification, cell culturing and tissue dissociation, preparation of recombinant antibody fragments for research, diagnostics and therapy, exploration of the structure-function relationships by structural studies, removal of affinity tags from fusion proteins in recombinant protein techniques, peptide sequencing and proteolytic digestion of proteins in proteomics [12]. Gold nanoparticles (GNPs) with controlled geometrical, optical, and surface chemical properties are the subject of intense studies and applications in biology and medicine. To date, the ever increasing variety of published examples has included genomics and biosensorics, immunoassays , clinical chemistry, targeted delivery of drugs, and antigens, photo thermolysis of cancer cells and tumors and optical bioimaging of cells and tissues with state-of-the-art nanophotonic detection systems [13].Nickel nanoparticles (NNPs) has become one of the interesting materials in research communities due to the varied promising applications in the field of catalysis [14-16] , and magnetism [17].Biomedical applications of Nickel nanoparticles can be categorized according to their application inside*(in vivo)* or outside the body (*in vitro*). The main use *in vitro* applications, is in diagnostic, separation, and selection, while *in vivo* applications, it could be further separated in therapeutic and diagnostic applications (nuclear magnetic resonance [NMR] imaging) [18-20].

The present study aims to characterize of protease kinetically and thermos-dynamically and evaluate the GNPs and NNPs effect on protease activity as example of inflammatory proteins in sera of patients with burns.

Materials and Methods

A total of 30 patients with burn attending Al-Kindy Hospital in Baghdad city and Medical City Hospital burns specialist were participated in this study. We obtained general information about each patient, including age, sex, etiology, location of burns, degree burn. As a control of 25 healthy individual with matches were included in this study. Five ml were collected from healthy donors and patients. The blood sample was centrifuged at 3000 rpm for 5 min after allowing the blood to clot at room temperature. Serum separated and transferred into test tube, and stored at -20^{o}C until being used. Gold nanoparticle and nickel nanoparticle were prepared using Pulsed Laser Ablation in Liquid method[21].Structure and nanosize measurement of nanoparticles samples

were identified by the Scanning Electron Microscope (SEM), Atomic Force Microscope (AFM). Absorbance spectra of NPs solution was measured by UV-VIS double beam spectrophotometers The activity of serum protease was determined using casein as substrate according to assay method of Isshaya, et al [22],with modification [23].

Thermodynamic parameters of protease were determined using Arrhenius plots (1/T ×1000 vs. log of activity) of casein. Energy of activation (Ea)was calculated from the curve slope, other thermodynamic parameters, such as, free energy change (ΔG), entropy change (ΔS), enthalpy change (ΔH) were calculated using the following equations: [24]

$$\textbf{Log Vmax = log A- Ea /2.303 R} \times \textbf{1/T}$$

$$slope = \frac{\text{Ea}}{2.303\text{R}}$$

Enthalpy change ΔH was calculated from the following equation:

$$\textbf{ΔH*= Ea –RT}$$

The Free Gibbs energy ΔG was calculated from the following equation:

$$\textbf{ΔG* = -RT Lin Vmax + RT Lin (KT/ h)}$$

Change of entropy was calculated from the following equation:

$$\textbf{ΔS*=(ΔH*-ΔG*)/T}$$

The statistical software (SPSS v 19; Chicago, IL, USA) was used. The data were analyzed using unpaired t-test and person correlation coefficients. Differences were considered significant when $P< 0.05$.

Results and Discussion

An interesting aspect of metal NPs is that their optical properties depend strongly on the particle size and shape. As an example, bulk gold looks yellowish in reflected light, while thin gold films look blue in transmission. This blue color steadily changes to orange as the particle size is decreased to ~ 3 nm. These effects are the result of changes in the Surface Plasmon Resonance (SPR) [25]. Figures(1a,and 1b) show the Surface Plasmon Resonance absorption spectrum of colloidal solutions of GNPs, and NNPs respectively. The pulse energy at the target surface was 700 mJ/Pulse for GNPs, which shows the absorbance peaks with broad band around 530nm, 465nm, and 195nm(Figure 1:a). We observed a faint pink coloration of the solution after several pulses of the experiment, as shown in optical picture. In the absorption spectrum of the solution, the surface plasm on related peak could be clearly distinguished. The peaks were around 195-530 nm These peaks were consistent with the presence of small 20-100 nm particles in the colloid, which confirmed by AFM and SEM, as compared to other research, which showed the presence of small 3-30 nm particles in the colloid [26].(Figure 1:b) which shows the absorbance peaks that occur at around 195 nm is the characteristic of NNPs[27]. The pulses energy at the target surface was 700 mJ/Pulse, The NNPs was transparent tends to faint black in color as shown in optical picture.

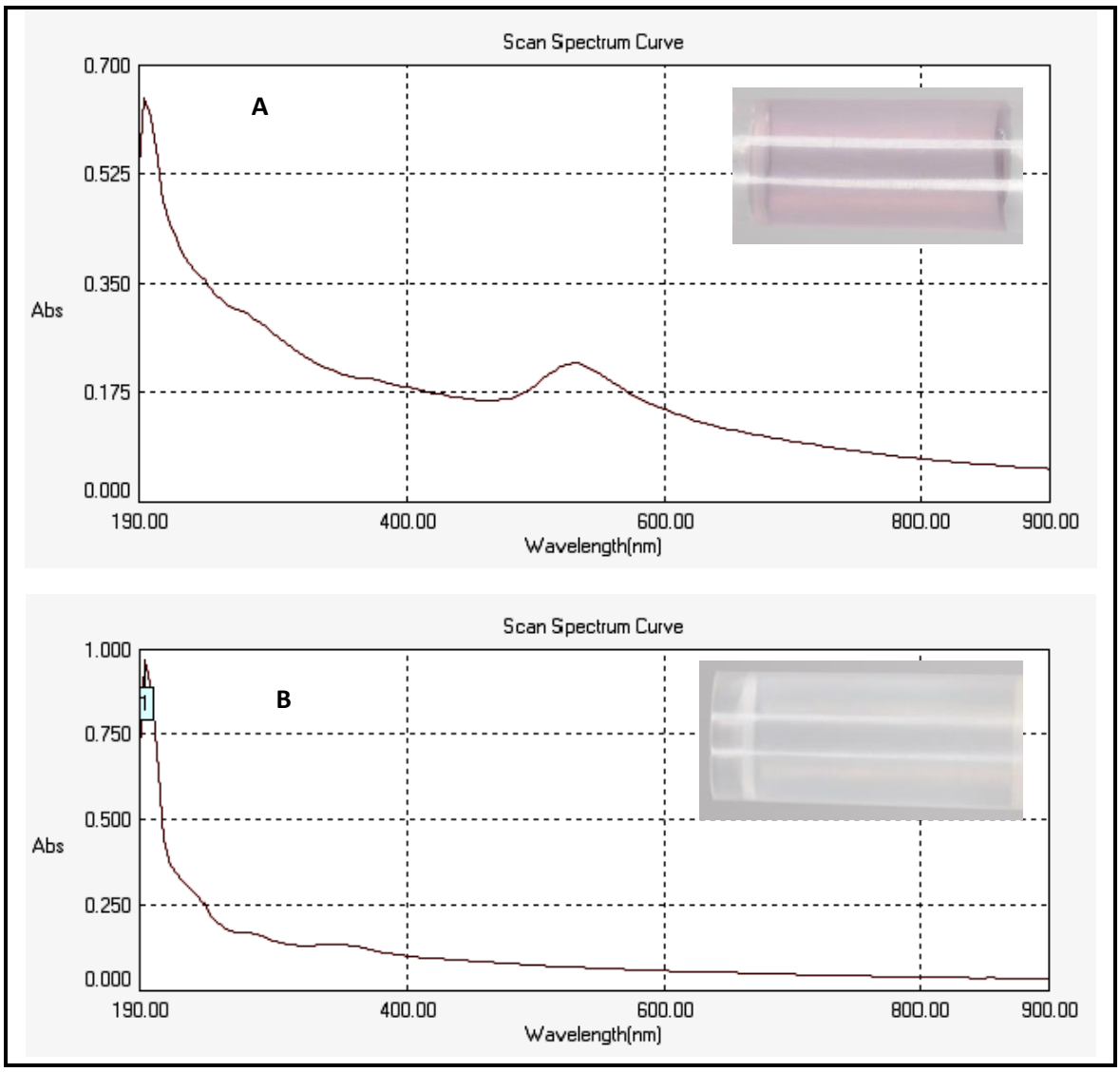

Figure 1:UV-Vis Spectrum and Optical Picture of GNP(a) and NNPs(b)

Figure 2 show the two Dimension of AFM images and the corresponding size distribution of GNPs and NNPs, respectively by laser ablation of metal immersed in DDW at 400pulse for GNPs and 200 pulse for NNPs with a same pulse energy. Morphological analysis is carried out using AFM, which produces topological images of surfaces at a very high magnification and facilitates the observation of the atomic structure of crystals. The origin of the surface morphology of the irregularly shaped particles sizes and the size distribution broaden can be explained by absorption with defects and thermally induced pressure pulses which cause cracking [28, 29].

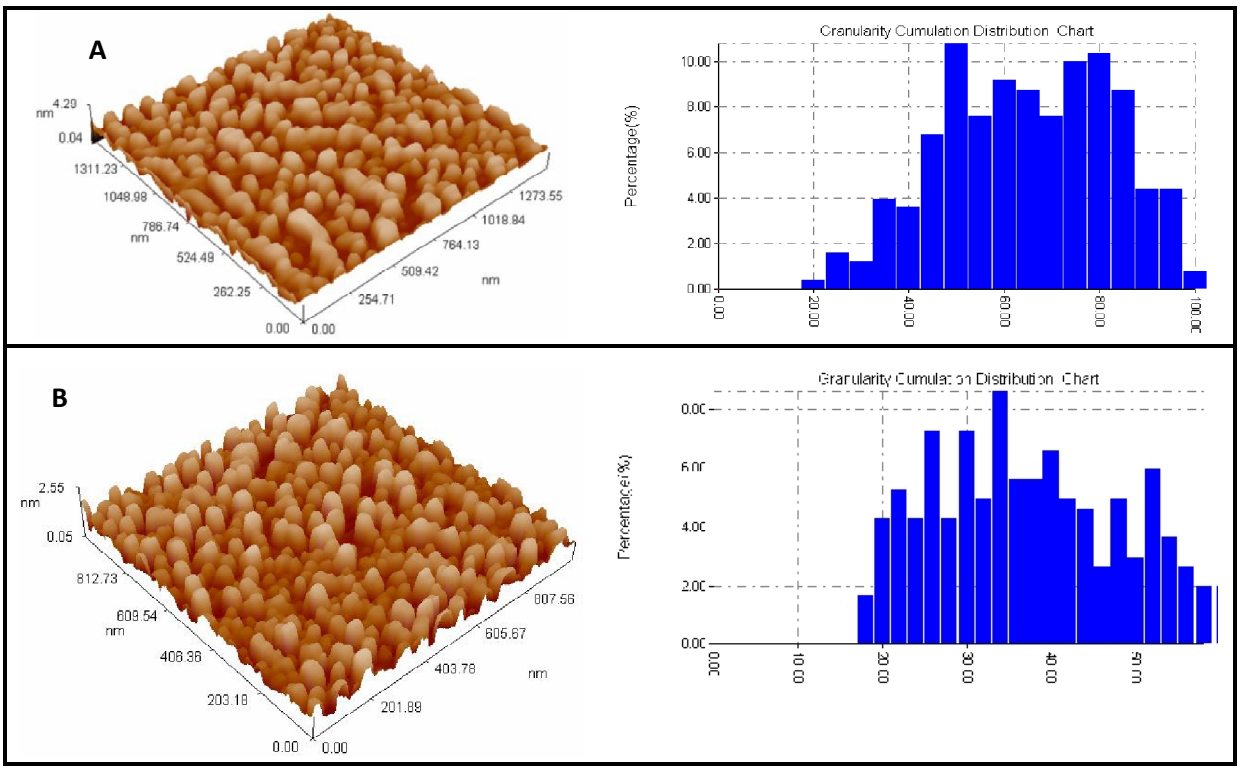

Figure 2:AFM Image and Size Distributions of GNPs(a) and NNPs (b)

Figure (3a&b) shows the SEM images for GNPs and NNPs(700mJ/pulse).The NPs produced were calculated to have the average diameters range of 20-100 nm (inagreement with AFM) obtained by laser ablation of metal plate immersed in DDW with laser shots of 400 pulse and wave length 1064 nm of Nd:YAG laser.

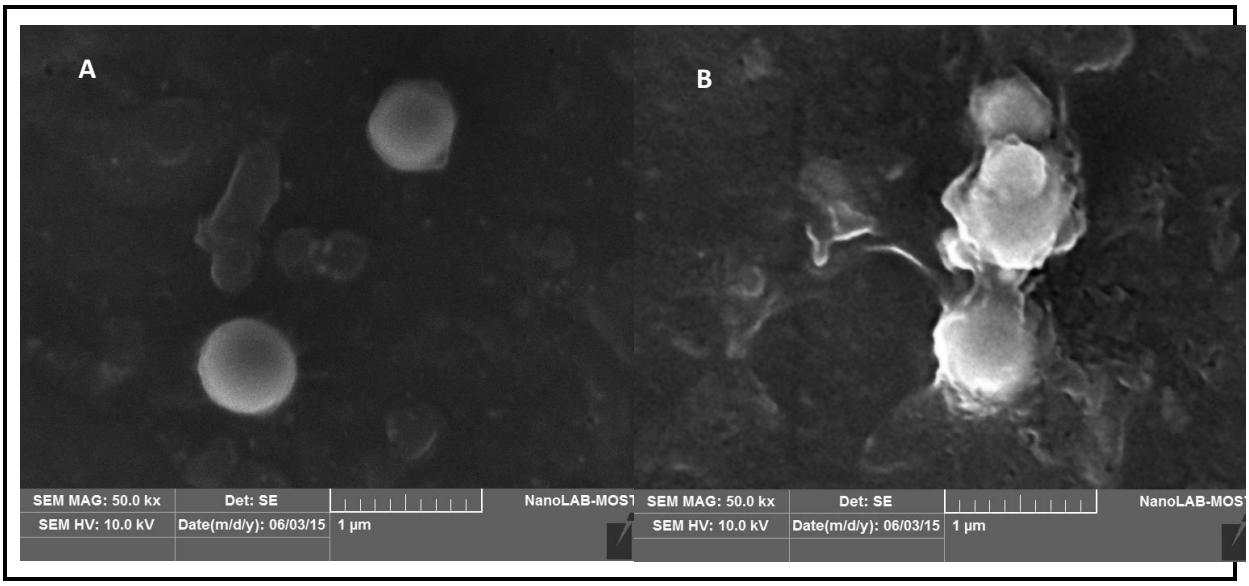

Figure 3:SEM Images of GNPs(a) and NNPs(b) (700 mJ/Pulse)

Protease activity was measured according to the modified method of Isshaya *et al*.The results presented in Table 1showed a highly significant (P>0.001) increased in protease activity concentrations in sera of burns patients in compared to their concentrations in control group. This is the first study to our knowledge in evaluating protease activity in sera of burns patients.

Table 1:Mean values of protease activity in sera of control and patients with burns

Groups	Range U/L	Mean U/L	Std. Deviation	Std. Error	P value
Control	(28-1024)	405.0400	322.80696	72.18183	0.001
Patient	(124-1021)	739.3867	247.00711	45.09712	

This results was agreement with Mittl and Grutter who reported that decomposition of proteins by protease in inflammatory conditions such as burns were increased [30]. Also, this result comes in line with the fact thatProtease-activated receptor participates in various biological activities, such as homeostasis, inflammation, pain perception, and melanosome transfer in the skin [31].

The relationship between the substrate concentrations and the velocity of enzymatic catalyzed reaction was studied using different concentrations of casein as a substrate as shown in Figure 4a. The results demonstrated that the increase in the enzymatic activity is directly proportional to substrate concentrations in the range of concentrations (0.5 -1%). The decreasing in protease activity after optimum point may refer to inhibition of enzyme by the substrate. The turnover kinetic parameters (Km and V max) were determined using Line weaver-Burk plot, where Km equals 0.2857% and Vmax equals 76.9230 U/L as shown in figure 4b.

The Km value is an important constant in enzymes studies. It is the substrate concentration which gives half of the maximum velocity, so it is an indicator of the enzyme affinity toward its substrate. An enzyme with high affinity for its substrate has low Km value; the value of Km establishes an approximate value for the intercellular level of the substrate, while the Vmax refer to the amount of active enzyme present [32, 33].

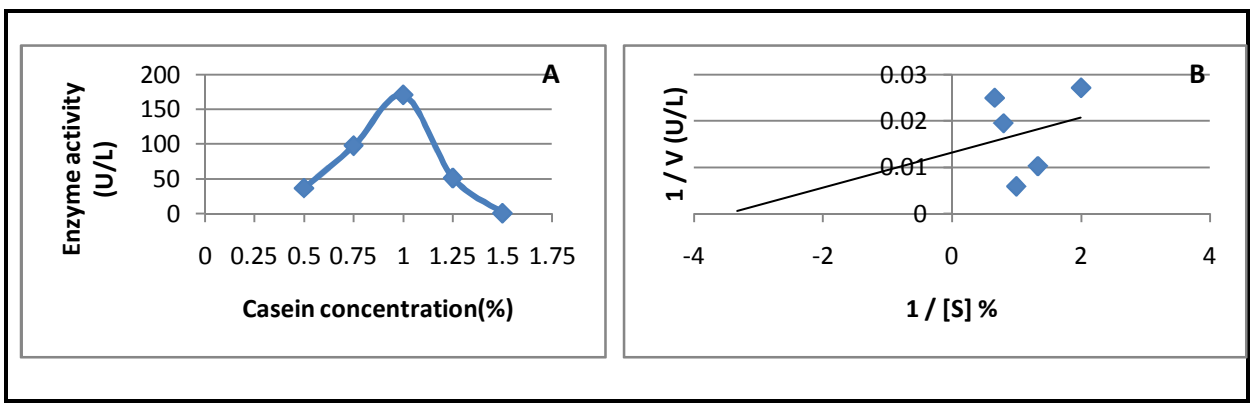

Figure 4:(a)Effect of substrate on protease activity, (b)Km and Vmax by Line Weaver–Bruk

Figure 5 shows the optimum condition of protease activity in sera of burns patients in pH, temperature, and incubation time. The results indicated a bell shaped curve when enzyme activity plotted against pH with optimum pH equal to7.5. This optimum may be due to (a) a true reversible effect on V itself, (b) an effect of pH on the affinity, the fall on either side of the optimum being due to a decreased saturation of the enzyme with substrate, due to a decreased affinity, or (c) an effect of pH on the stability of the enzyme, which may become irreversibly destroyed on one or both sides of the optimum [34].

Upon increasing the temperature, the protease activity in sera of burn patients increases, where the optimum temperatures that observed was (35ºC). But above optimum temperature, a decline in the

activity was observed .The effect of temperature on the velocity of enzyme reactions may be due to several different causes. It may be due to an effect on the stability of the enzyme; to an effect on the actual velocity of breakdown of the complex determined by the heat of activation of the reaction; to an effect on the enzyme -substrate affinity to an effect on the pH functions of any or all of the components [34]. The activity of Protease increases by increasing the incubation time allowing the enzyme to be completely saturated with the protease until reaching to the optimum time course, which in present study equal to 30 minutes.

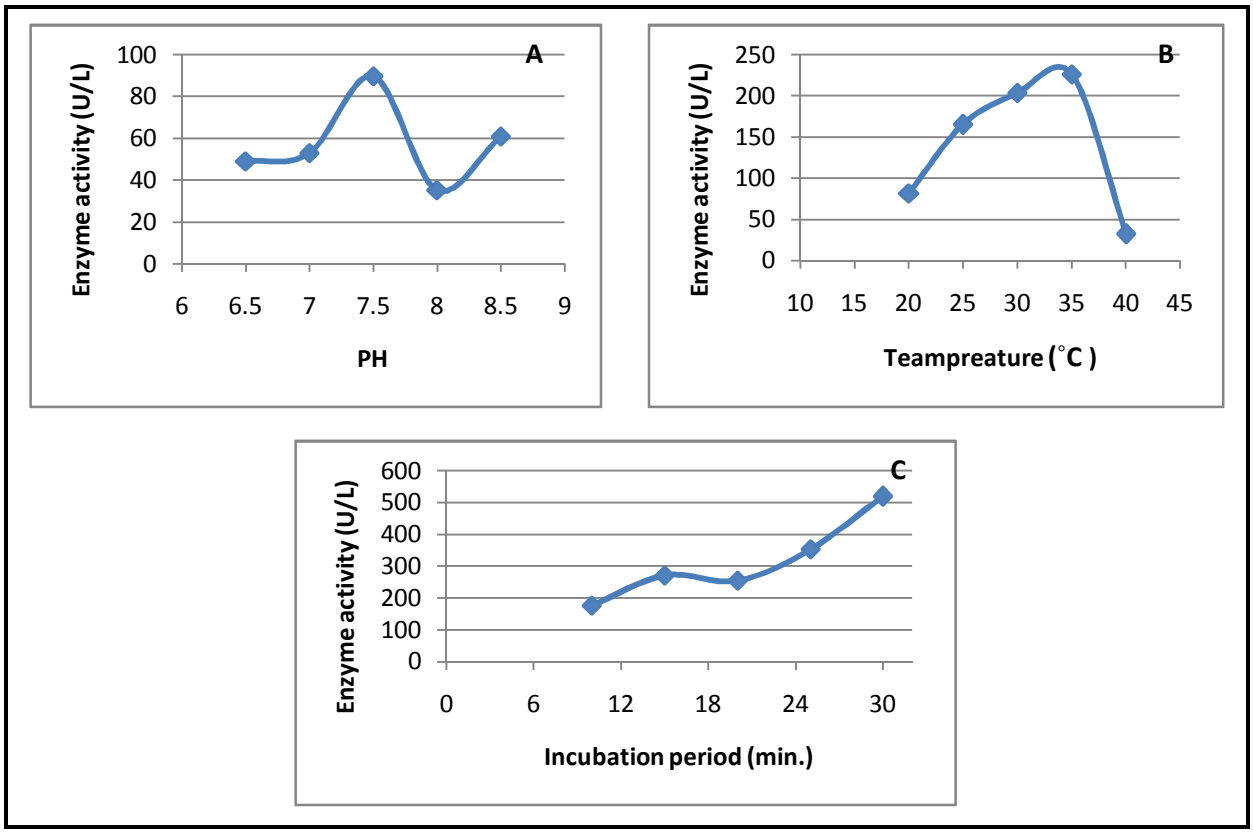

Figure 5:Effect of PH (a),Teampreature (b),Incubation time, (c) on protease activity

According to our knowledge, the present study is the first to carry out the effect of gold and nickel nanoparticles colloids on the protease activity.Results presented in Figure 6 showed that both nanoparticles have an inhibitor effect on the enzyme activity in patients and control groups. Gold nanoparticle have been more inhibitor effect (78.29%) on protease activity than NNPs (76.02%), while in control group, NNPs have been more inhibitor effect(12.69%) on protease activity than GNPs (7.47%).

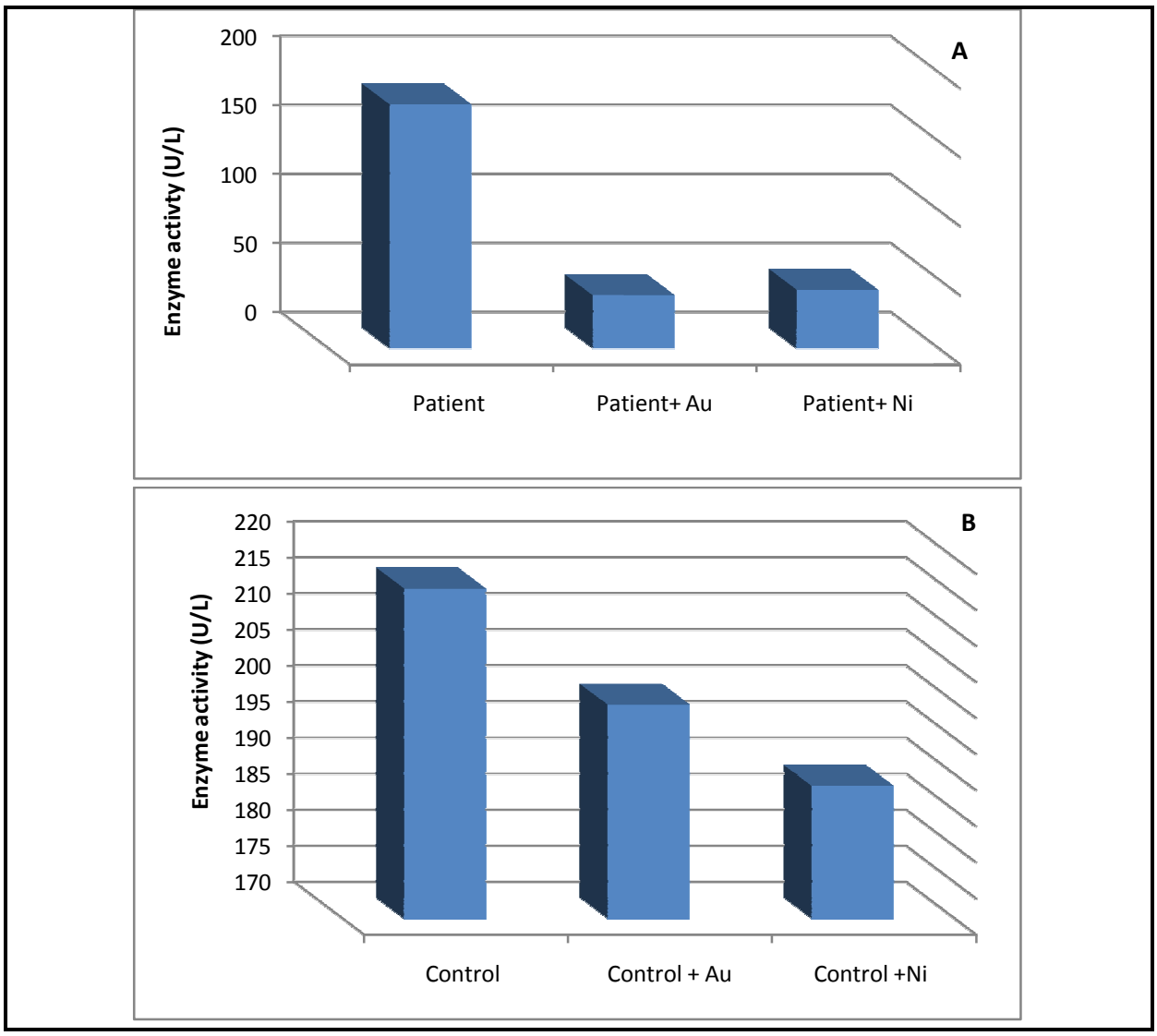

Figure 6:Nanoparticles effect on protease activity in sera of burns patients and controls

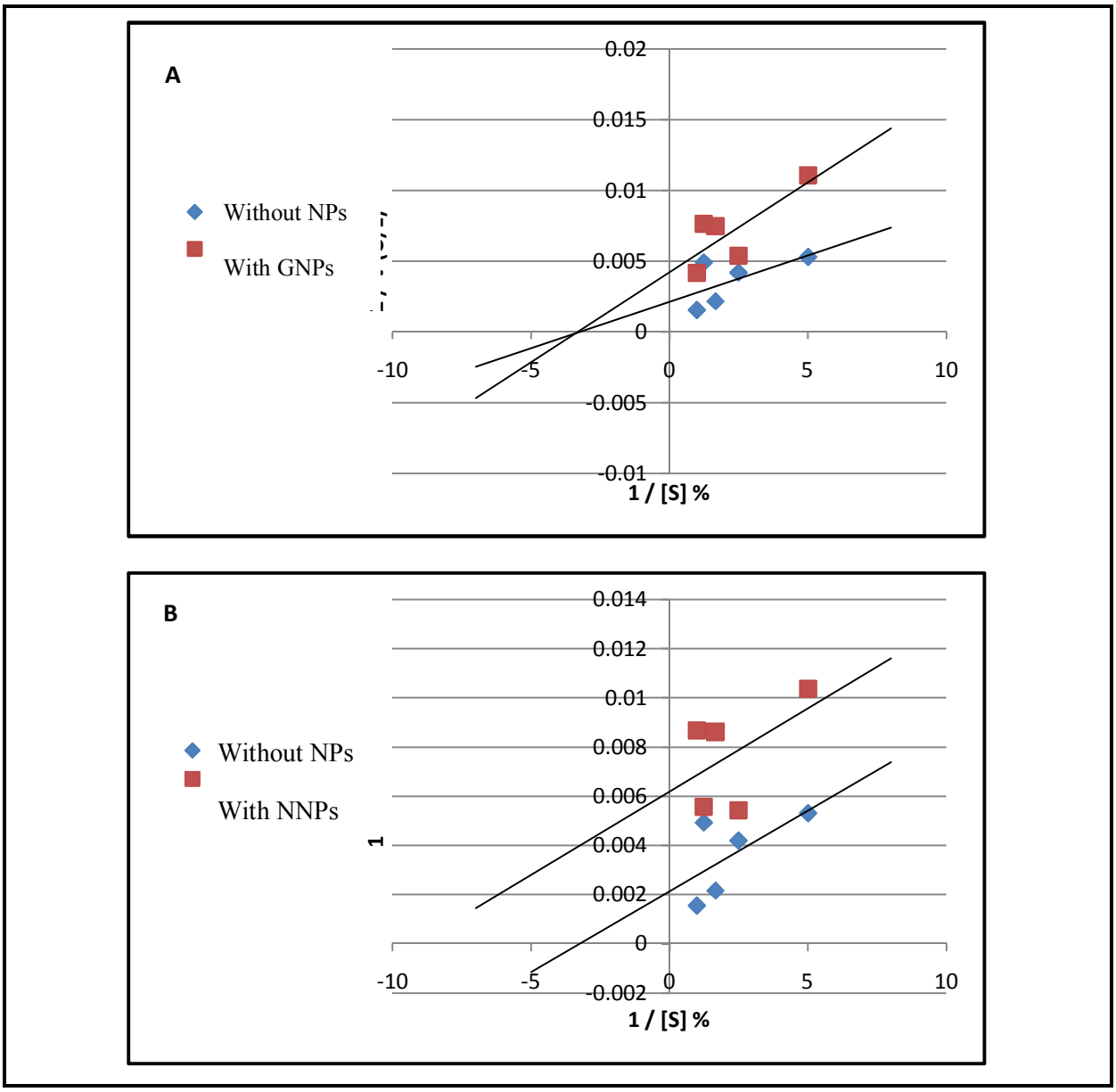

Figure 7:Inhibition type of GNPs and NNPs on protease activity in sera of burn patients

The inhibition types of GNPs and NNPs were determined using Line weaver-Burk plot. A non-competitive inhibitor for GNPs and uncompetitive inhibitor for NNPs were indicated as shown in figure 7. Noncompetitive inhibitor is binds reversibly connects to the free enzyme or to enzyme-substrate complex yielding an inactive ESI complex [35].While in NNPs inhibition type was uncompetitive; a classical uncompetitive inhibitor is a compound that binds reversibly to the enzyme-substrate complex yielding an inactive ESI complex. The inhibitor does not bind to the free enzyme. [36]. The Km and V max of protease in presence of Gold and Nickel nanoparticles were presented in Table 2.

Table 2:Kinetic parameters of protease with GNPs and NNPs in sera of burn patients

Nano particles	Kmi (%)	V maxi (U/L)	Inhibition Type
Gold	0.312	238.095	non-competitive inhibition
Nickel	0.111	163.934	Uncompetitive inhibition

Thermodynamic parameters of the transitional state were calculated by using Arrhenius equation, which connects between speed constant K or reaction maximum speed Vmax (in the biochemical reactions) and temperature inverse in Kelvin using the following equation:

Log Vmax = log A- Ea /2.303 R × 1/T

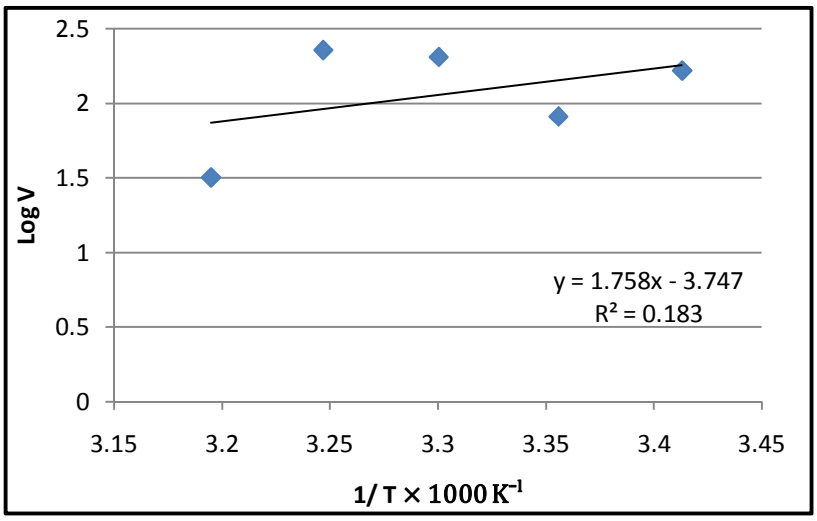

Figure 7:Arrhenius Plot of protease in sera of burn patients

Table 3:Thermodynamic parameters of protease in sera of burns patients

(t) ^{o}C	T(K)	Ea(kJ/mol.)	ΔH^{*}(kJ/mol.)	ΔG^{*}(kJ/mol.)	ΔS^{*}(J/mol.k)
20	293	0.0336664	-2.4023356	61.1371351	-216.858261
25	298	0.0336664	-2.4439056	62.22235339	-217.000869
30	303	0.0336664	-2.4854756	63.30826962	-217.141073
35	308	0.0336664	-2.5270456	64.39487171	-217.278952
40	313	0.0336664	-2.5686156	65.48214848	-217.414581

It is noticed from Table 3 that (ΔH^{*}) value is a negative, which indicates that the formation of enzyme complex (protease-casein) is exothermic reaction. The positive value of (ΔG^{*}) indicates that the reaction thermodynamically unfavorable . In other meaning the reaction needs energy to reach the transitional state.

The negative value of (ΔS^{*}) indicates that the active complex structure is more order than the reactant. ΔH^{*} and ΔS^{*} are both negative this mean that favorability of the reaction thermodynamic can depend on the temperature.

Conclusion

We conclude that nanoparticles such as gold and nickel can be used as treatment of burns through their role in body homeostasis due to their inhibition impact on protease activity .

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