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Study the Interaction of Ni Complex of Tetradentate Schiff Base Ligand with HEN Egg White Lysozyme

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Interaction of Ni complex (Salen = N,N′-ethylene bis (salicylideneimine)) with hen egg white lysozyme (HEWL) was studied by absorption spectroscopy, competitive binding study and thermal denaturation study. The protein binding affinity of Ni complex was found to be (3.0×10^3 M^{-1}). The binding plot obtained from the absorption titration data gives a binding constant of 2.4 (± 0.3) × 10^3 M^{-1}. It was found that the charge transfer band of the metal complex was perturbed in the presence of HEWL. Thermal denaturation study of HEWL with Ni complex revealed the ΔT_m value of 5 ± 0.2 °C. The thermodynamic parameters ($\Delta H^o > 0$ and $\Delta S^o > 0$) showed that the hydrophobic interaction leads to the increasing entropy brought about by interaction with the complex. The negative ΔG^o values for the interaction of HEWL with the Ni complex indicate the spontaneity of the interaction.

Keywords: Metal-Salen complex, Ni complex, Hen egg-white HEWL, Protein-binding

INTRODUCTION

Medication tendency to plasma proteins is an important factor that must be considered in the drug design. Since the effective concentration, nature and medical potential are strongly linked to their willingness against specific binding sites on the carrier's bio-molecules, the issue of possible interactions between studied model of the drugs and carrier proteins is important. In the past decade, studies have shown that serum albumin in the blood plasma can be connected to a wide range of compounds such as phosphate, cysteine, glutathione ligands base shifts and the complexes of Cu(II), Ni(II), Mn(II), Co(II), Hg(II) and Zn(II) and thionin metal. HSA is one of the most widely studied proteins, which has provided a two-position link with a high affinity for a variety of drugs IA, and IIIA can probably be replaced in the sequence [1]

A wide range of ions and molecules are connected to this protein with a high affinity, and this protein carries them. Most drugs and metal complexes bind to serum albumin. This binding is a critical determinant of drug distribution, drug kinetics and the medicinal ability [2]. In order to design effective chemotherapeutic agents and better cancer drugs, studies on interaction of metal complexes with biomolecules are needed. Schiff base complexes, an important class of metal complexes, are in medical areas. In recent years this material as well as biological applications including anti-bacterial properties has shown excellent anti-fungal and anti-cancer properties [1,3]. Study of binding of small molecules to proteins such as HSA, BSA, and LYS provides a data model to drug design based on a label for accurate quantification of proteins [2].

Hen Egg white lysozyme [HEWL], a glycoprotein, hydrolase sugar, binds to the bacterial cell wall enzyme through the hole that the sequence of Asp 52 and Glu 35 has formed, and destroys it. This is because protein tends to bind to small molecules including metal complexes showing coordination [4]. Metal complexes binding to biomolecules are not only critical systems but also play an important role in environmental chemistry and food chemistry.

Binding of small molecules to a protein such as a biosensor generates an important signal. To design

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appropriate metal complexes with potential applications in biology.

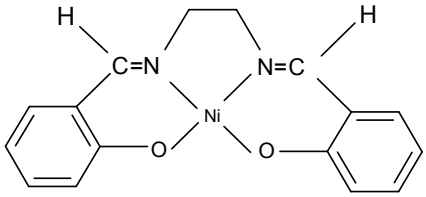
Metal complexes with Schiff Base Ligands are used for easy synthesis, low cost and a variety of side groups. Moreover, Schiff base ligands and metal complexes tend to interact with other proteins, addressing the inhibition of enzyme activity. Inhibitors are used to comply with important pathophysiological conditions. On the other hand, metal ions particularly transition metal ions include a variety of coordinations and are primarily used to design appropriate coordination complexes. Nickel, as a transition metal ion, is an essential ingredient that despite its very small amounts in bacteria, plants and animals play an important role in the structure and activity of the urease enzyme [3].

Salicylidene-ethylendiamine, often known as Salen, is one of the most suitable ligands used for the production of metal catalysts. Metal-Salen complexes are able to speed a wide range of reactions such as cyclopropanations, epoxidations and oxidations, and are valuable for kinetic resolutions [5]. These metal Schiff base complexes have been developed as nucleic acid compounds to induce damages in DNA and/or RNA. Recently, synthetic salicylic aldehyde nucleosides have been used to improve the metal-salen-base pair complexes inside the DNA, and the complexation was found to repeal the succession information [6]. To the best of our knowledge, the interaction of Ni complex with HEWL has not been reported yet.

So,in the present work, we describe the synthesis and characterization of Ni complex (Scheme 1) and its interaction with HEWL. This interaction has been investigated in view of thermodynamic using UV-Vis differential absorption. The aim of this study is to determine the binding constant, thermodynamic parameters and the mechanism and the kind of binding by analyzing absorption data using a simple binding model. Also, to investigate protein stability, the thermal denaturation of HEWL is studied and the melting temperature point (T_m) of HEWL in the presences and absences of Ni complex is determined.

SYNTHESIS OF NI COMPLEX

Salen ligand was synthesized by the following procedures established in Ref. [7]. At first, 0.1 mol aliquot of ethylene diamine was dissolved in 25 ml of ethanol, and then this mixture was added to the solution of 0.2 mol of salicyl aldehyde in 150 ml of ethanol under stirring conditions. The obtained solution was refluxed for 1 h, and finally cooled down and kept at room temperature for 3 h. The formed yellow solid was filtered and recrystallized from ethanol. Ni complex was prepared using the following method: 0.01 mol of salen ligand was dissolved in 50 ml of ethanol, and then the mixture was heated to boiling temperature. Then, a solution of 0.01 mol of metal salt ($Ni(CH_3COOH)_2.4H_2O$) was added to 125 ml of ethanol. The resultant solution was stirred and refluxed for 1 h. After the solution was cooled to room temperature, the product was separated by filtration and recrystallized from CH_3OH.



Scheme 1. Ni complex structure

Yield: 95%. Anal. Calcd. for $C_{16}H_{14}NiN_2O_2$: C, 68.79; H, 6.87; N, 11.46. Found: C, 68.70; H, 6.81; N, 11.39. FT-IR (KBr) ν_{max} (cm^{-1}): 2931, 2795, 1626, 1456, 1288, 1154, 1014.

MATERIALS AND METHODS

Chemical and Preparations

Ni complex was prepared and purified by the methods described in the literature [7]. HEWL 99% was obtained from Cinajen Chemical Co and was used without more purification. The fresh protein solution was prepared before any measurement, and double distilled water was used throughout the experiments. The molar coefficient of HEWL is 68000 M^{-1} cm^{-1} and its concentration was determined using UV-Vis method.

Optical Absorption

The absorbance monitoring was performed on a Perkin-Elmer UV-Vis Lambda 2 equipped with thermostat cell compartment. The UV-Vis titration experiments were made by addition of Ni(II)salen solution into a 1.4 ml curette containing the HEWL solution of appropriate concentration at various temperatures with precision of ±1 °C.

The melting curves of both free HEWL and HEWL-Ni complex in phosphate buffer were obtained by measuring the HEWL absorbance at 281 nm as a function of temperature. Melting temperature was measured in phosphate buffer pH 7.0 containing 9.7 μM HEWL. The temperature was scanned from 25 until 93 °C.

Binding Analysis of Interaction of Ni Complex with HEWL

The total absorbance of Ni complex-HEWL complex at the wavelength of 392 nm is Eq. (1):

$$A_t^{392} = \varepsilon_b^{392}\, b[Sa]_b + \varepsilon_f^{392} b[Sa]_f \quad (1)$$

And the total concentration of salen complex is:

$$[Sa]_t = [Sa]_b + [Sa]_f \quad (2)$$

Where $[Sa]_t$, $[Sa]_f$ and $[Sa]_b$ are total, free and binding complex concentrations and ε_b, ε_f are molar absorption coefficients for the free and bound forms of the HEWL, respectively. Then, by dividing Eq. (1) to total concentration of $[HEWL]_t$, we obtain:

$$\frac{A^{392}}{[HEWL]_t} = \varepsilon_b r + (\varepsilon_b - \varepsilon_f)\frac{[Sa]_b}{[HEWL]_t} \quad (3)$$

where r is the $\frac{[sa]_t}{[HEWL]_t}$ ratio.

By plotting $\frac{A^{392}}{[HEWL]_t}$ *vs.* r, ε_b is calculated at saturated point. ε_f is calculated in Beer law plot for free salen and is applied in other calculations.

RESULTS AND DISCUSSION

Effect of Concentration

In order to identify the solution properties of Ni complex, we employed UV-Vis spectroscopy. The optical absorption Spectrum of Ni complex shows two bands in 321, 392 nm, and a weak band at 443 nm. We choose 392 nm due to the wavelength overlap with HEWL spectrum near 321 nm.

In 25 °C, the maximum band of Ni complex obeys Beers law over concentration range of 3.8×10^{-6}-5.34×10^{-5} M in 5 mM phosphate buffer, pH 7.0. From this observation, we can conclude that Ni complex does not show concentration dependent aggregation.

Effect of Temperature

The effect of temperature on the UV-Vis spectrum of Ni complex 43.1 μM in phosphate buffer is shown in Fig. 1. As shown in this figure, the wavelength of maximum absorption λ_{max} does not show considerable changes and no new band appears. The binding isotherm, binding capacity and scatchard plots Eqs. (4) and (5) were plotted. The results show that the HEWL has one binding site set and presents positive cooperativity in Fig. 2. The results show that scatchard plots [8] are nonlinear, therefore, by using Hill plot due to Eq. (5) [9] binding constant at each temperature was determined .The results are shown in Figs. 3 and 4 and Table 1.

$$\frac{\nu}{[L]} = K_a(n - \nu) \quad (4)$$

$$\log(\tfrac{\nu}{n-\nu}) = \log k_a + n_H \log[L] \quad (5)$$

where n is the number of sites and nH is Hill coefficient related to cooperativity. By increasing temperature, K_a is increased because of an endothermic interaction between HEWL and Ni complex, and n_H is decreased because of protein saturation.

Investigation of Thermodynamic Interaction of Ni Complex with HEWL

The thermodynamics of the interaction of Ni complex with HEWL was investigated in terms of the difference in

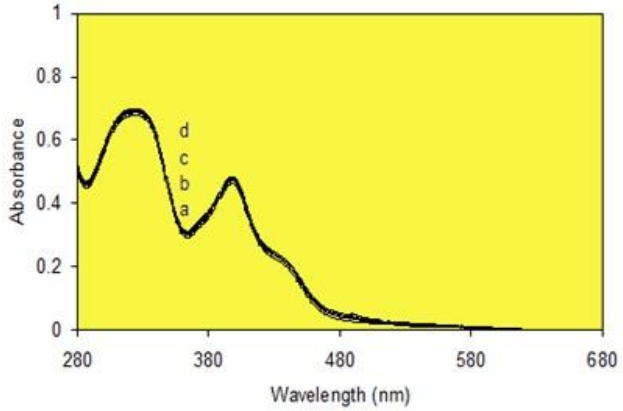


Fig. 1. The effect of temperature change on the UV-Vis spectrum of Ni complex solution 43.1 μM in phosphate buffer in temperatures 45 (d), 40 (c), 35 (b) and 30 (a).

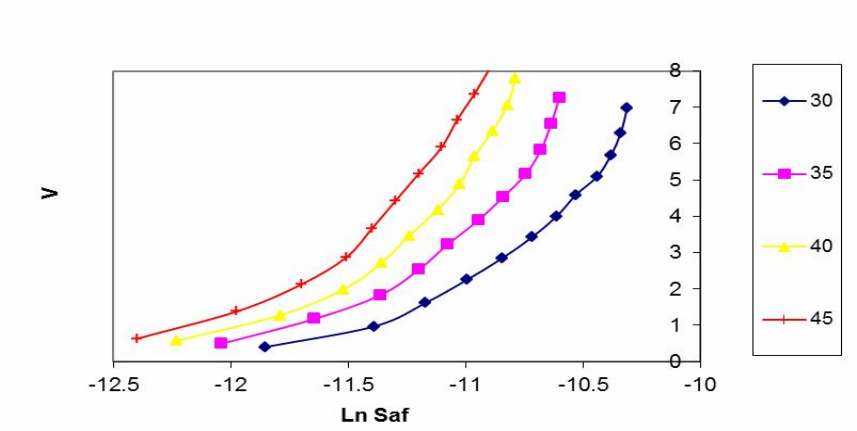


Fig. 2. The binding Isotherms of interaction HEWL with Ni complex in 5 mM phosphate buffer solution, pH = 7 at λ_{max} 392 and temperatures 45 (●), 40 (▲), 35 (■) and 30 (♦).

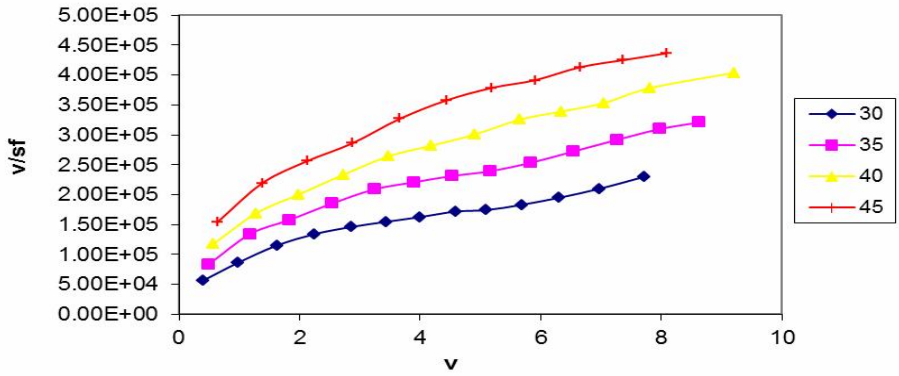


Fig. 3. The Scatchard plots of interaction HEWL with Ni complex in 5 mM phosphate buffer solution, pH = 7 at λ_{max} 392 and temperatures 45 (●), 40 (▲), 35 (■) and 30 (♦).

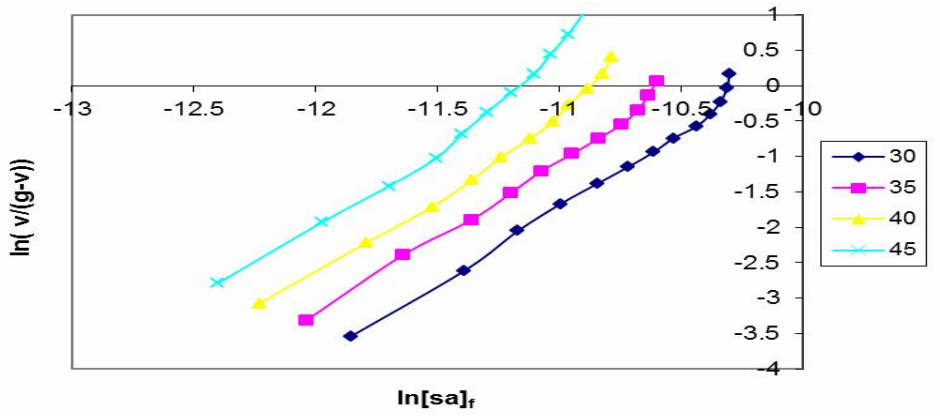


Fig. 4. The Hill equation of interaction HEWL with Ni complex in 5 mM phosphate buffer solution, pH = 7 at λ_{max} 392 and temperatures 45 (●), 40 (▲) 35 (■) and 30 (♦).

Table 1. Binding Constants K_a, n_H of Interaction Ni Complex to HEWL at 5 mM Phosphate Buffer, pH 7.0 and Various Temperatures

t (°C)	n_H	$\ln K_a$
30	4.110	10.029
35	3.810	10.458
40	3.430	10.680
45	3.150	11.130

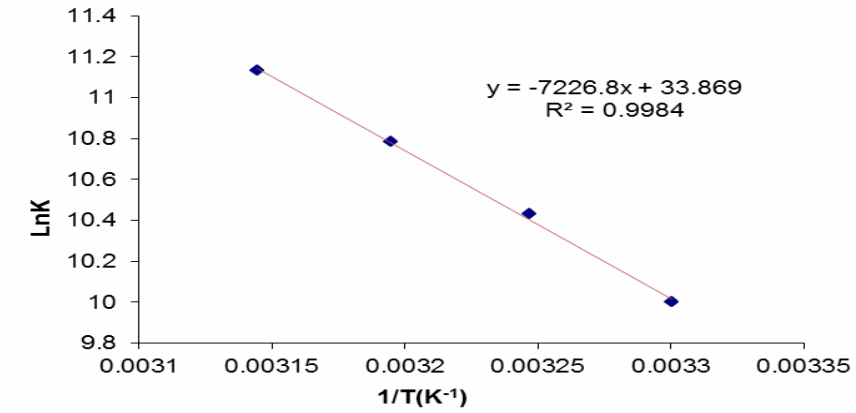


Fig. 5. Van't Hoff diagram for HEWL with Ni complex interaction in 5 mM phosphate buffer solution, pH = 7 at different temperatures.

K_a values determined at various temperatures, and the results are listed in Table 1.

Thermodynamic parameters include standard bonding Gibbs free energy change ΔG_b°, standard binding enthalpy change, ΔH_b°, and binding entropy change, ΔS_b°. The standard energy, enthalpy change, is usually calculated by Eqs. (6)-(8) that follow the relationship:

$$\frac{\partial \ln K_a}{\partial(1/T)} = -\frac{\Delta H_b^\circ}{R} \tag{6}$$

$$\Delta G_b^\circ = -RT \ln K_a \tag{7}$$

$$\Delta S_b^\circ = \frac{\Delta H_b^\circ - \Delta G_b^\circ}{T} \tag{8}$$

The van't Hoff (6) plot for interaction of Ni complex with HEWL is shown in Fig. 5. The calculated thermodynamic parameters for binding of Ni complex-HEWL are listed in Table 2.

The binding and stage Gibbs free energy change of Ni complex-HEWLis usually calculated by the following relationship (9) [8,9] as shown in Fig. 6, [L] in Eq. (9) is free ligand concentration.

$$\Delta G^\circ_{b,v} = -RTn_H \ln K_a + RT(1-n_H)\ln[L] \tag{9}$$

By analyzing the results of UV-Vis spectroscopy, the detrmined thermodynamic parameters ($\Delta H^o > 0$ and $\Delta S^o > 0$) showed that the interaction between HEWL and Ni complex leads to the increasing enthalpy and entropy. The negative ΔG^o values for interaction of HEWL with the Ni complex indicate the spontaneity of the complexation. Therefore, the dominant force is entropy and the mode of this interaction is hydrophobic [10].

Thermal Denaturation of HEWL

Melting experiments were made in phosphate buffer solutions pH 7.0 containing 97 μM HEWL. The temperature was scanned from 25 to 93 ºC. The melting temperature (Tm) was taken for the free HEWL in the absence of ligand, and for the different molar ratios of Ni complex to HEWL. Results of such studies for Ni complex-HEWL complex are shown in Fig. 7. By using Igor software and determining $\delta\Delta\varepsilon/\delta\Delta T$, T_m was determined graphically and was listed in Table 3. The results represent the increasing of T_m upon addition of Ni complex concentration.

From the thermodynamic point of view, the denaturation process can be described as a transition between two macroscopic states; that is, from the native state (*N*) to a denaturated state (D).

$$N \Leftrightarrow D \tag{10}$$

The stability of a globular protein is usually quantified in the Gibbs free energy values since ΔG_D is the work required for disruption of the native protein structure. For that reason, the difference in Gibbs energy at a given temperature can be expressed by the Gibbs-Helmholtz Eq. (11):

$$\Delta G_D(T) = \Delta H_m(1-\frac{T}{T_m}) - \Delta Cp\left[(T_m - T) + T\ln\frac{T}{T_m}\right] \tag{11}$$

The evaluation of thermodynamic parameters, obtained from spectroscopic techniques, is based on the equilibrium constant K for a transition between the native state and the denaturated state [11]. The equilibrium constant was deduced from Eq. (12):

$$K = \frac{[D]}{[N]} \tag{12}$$

or as a function of spectroscopic parameters:

$$K = \frac{(A_N - A_0)}{(A_0 - A_D)} \tag{13}$$

where A_N is the absorbance of the pure native state, A_D is the corresponding absorbance of the pure denatured state, and A_0 is the observed absorbance at any temperature in the transition zone.

To avoid large errors in the estimation of the thermodynamic parameters we have used all the experimental data points obtained, and have fitted them in the equations in the following manner:

$$A_0 = \frac{(A_N + KA_D)}{(1+K)} \tag{14}$$

Table 2. Thermodynamic Parameters for Binding of Ni Complex to HEWL at 5 mM Phosphate Buffer, pH 7.0 and Various Temperatures

T (K)	ΔH°_{b} (kJ mol^{-1})	ΔG°_{b} (kJ mol^{-1})	ΔS°_{b} (J K^{-1} mol^{-1})
303	60.08	-25.19	281.44
308	60.08	-26.71	281.79
313	60.08	-28.07	281.63
318	60.08	-29.43	281.48

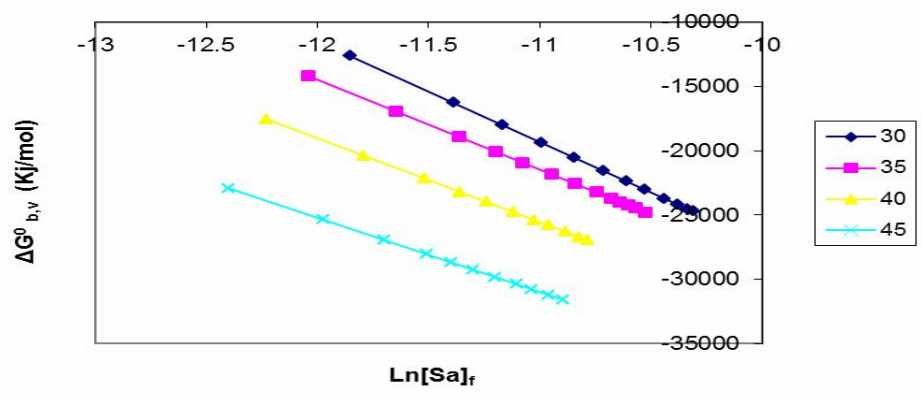


Fig. 6. Gibbs free energy change HEWL with Ni comolex in 5 mM phosphate buffer solution, pH = 7 and at temperatures 45 (●), 40 (▲), 35 (■) and 30 (♦).

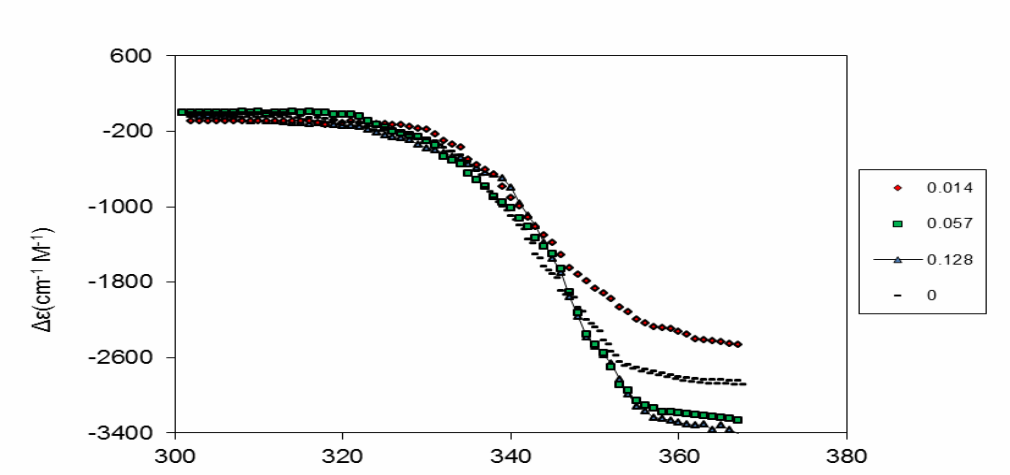


Fig. 7. Molar Absorbance coefficient change in (λ_{max} = 281 nm) with temperature for different mole ratios of Ni complex to HEWL 0.0 (▲), 0.014 (♦), 0.056 (■) and 0.127 (—).

Table 3. Melting Point (λ_{max} = 281 nm) for HEWL for Different Mole Ratios of Ni Complex to HEWL

$\frac{[\text{Nicomplex}]}{[\text{HEWL}]}$	0.0	0.014	0.056	0.127
T_m (K)	342.45	344.05	346.55	347.45

Table 4. Six Parameters in Equation 20 that Were Determined by Fitting Using Sigmaplot Software for Different Mole Ratios of Ni Complex to HEWL

[Ni complex]/[HWEL]	0.0	0.014	0.056	0.127
a_N $(M\ cm)^{-1}$	129.5 ± 322.0	18.4 ± 122.0	104.1 ± 203.2	6.7 311.6 ± 8
m_N $(M\ cm\ K)^{-1}$	-0.3 ± 1.3	0.1 ± 0.4	-1.2 ± 0.5	-3.7 ± 0.3
a_D $(M\ cm)^{-1}$	1931.3 ± 91.5	-994.6 ± 1044.0	-3438.1 ± 792.0	-1208.0 ± 377.6
m_D $(M\ cm\ K)^{-1}$	1.3 ± 2.1	-3.92 ± 0.6	0.64 ± 0.3	0.83 ± 1.0-
T_m (K)	341.8 ± 0.3	343.2 ± 0.7	345.7 ± 0.3	348.4 ± 0.8
ΔH_m $(kJ\ mol^{-1})$	0. 127	172.6 ± 4.1	175.3 ± 4.4	175.9 ± 3.2
ΔCp $(J\ mol^{-1})$	1035 ± 43.1	568.3 ± 29 4	474.1 ± 21.2	242.0 ± 51.0

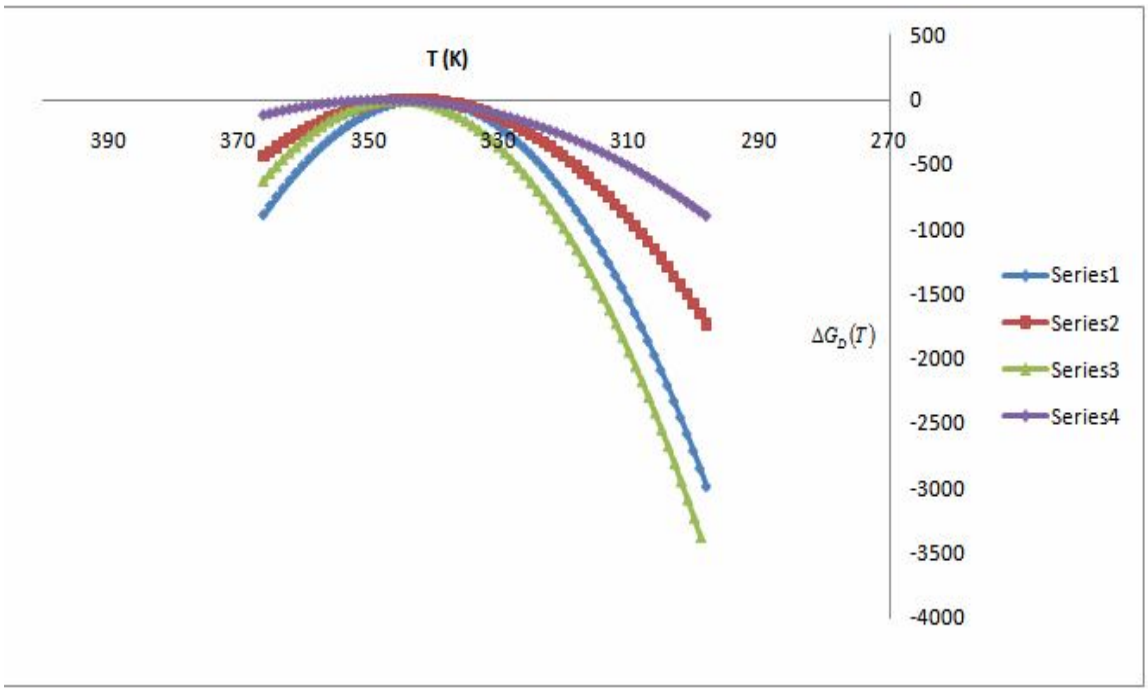


Fig. 8. Denaturation Gibbs Free Energy changes with temperature for different mole ratios of Ni complex to HEWL 0.0 (▲), 0.014 (♦), 0.056 (■) and0.127 (—).

Table 5. Maximum Stability Tempratures for HEWL for Different Mole Ratios of Ni Complex to HEWL

$\frac{[\text{Ni complex}]}{[\text{HEWL}]}$	0.0	0.014	0.056	0.127
Ts (K)	341.15	343.15	345.15	348.55

Table 6. UV Spectra Peaks in nm for Lysozyme-Ni(Salen) Complex

Lysozyme-Ni(Salen) interaction	Maximum wavelength absorbance (nm)
Pure Lysozyme	281, 200
Lysozyme-Ni(Salen) complex	281, 202, 196

Table 7. IR Specta Peaks in cm^{-1} for Lysozyme-Ni(Salen) Complex

Lysozyme	Lysozyme-Ni(Salen) complex
3436	3436
2083	2093
1637	1735
1365	1637
1217	1365
1057	1229
714	1217
-	1051
-	710

the other hand, the equilibrium constant can be expressed by the Gibbs energy function:

$$K = \exp\left(\frac{-\Delta G}{RT}\right) \tag{15}$$

where R is the gas constant and T is the absolute temperature. Substituting the value of K in Eq. (14) we get:

$$A_0 = \left(\frac{A_N + A_D e^{\left(\frac{-\Delta G}{RT}\right)}}{1 + e^{\left(\frac{-\Delta G}{RT}\right)}}\right) \tag{16}$$

Finally, substituting the $\Delta G_D(T)$ expression in Eq. (16), we get:

$$A = \frac{A_N + A_D \exp\left[\frac{-\Delta H_m}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) - \Delta Cp\left[\frac{T_m}{T} - 1 + \ln\left(\frac{T}{T_m}\right)\right]\right]}{1 + \exp\left[\frac{\Delta H_m}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)\right] - \Delta Cp\left[\frac{T_m}{T} - 1 + \ln\left(\frac{T}{T_m}\right)\right]} \tag{17}$$

Since A_N and A_D have been found to be linear functions of temperature, they can be written as:

$$A_N = a_N + m_N T \tag{18}$$

$$A_D = a_D + m_D T \tag{19}$$

$\Delta\varepsilon_{281}$ is an Absorbance coefficient change in any temperature with temperature 25 °C in λ_{max} = 281 NM for HEWL in the absence and presence of Ni complex.

$$\Delta\varepsilon_{281} = \frac{\Delta\varepsilon_N + \Delta\varepsilon_D \exp\left[\frac{-\Delta H_m}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) - \Delta Cp\left[\frac{T_m}{T} - 1 + \ln\left(\frac{T}{T_m}\right)\right]\right]}{1 + \exp\left[\frac{\Delta H_m}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)\right] - \Delta Cp\left[\frac{T_m}{T} - 1 + \ln\left(\frac{T}{T_m}\right)\right]} \tag{20}$$

There are six parameters in Eq. (20) that were determined by fitting, using sigmaplot software. These parameters are summarized in Table 4. Using these parameters, we can calculate the $\Delta G_D(T)$ at 25 to 93 ºC by Eq. (11). The result is represented in Fig.8. The result shows more stable state about T_s = 341.15 to 348.15 K at different mole ratios of Ni complex to HEWL.The accurate values are brought in Table 5. The $\Delta G_D(T)$ values for HEWL, in the absence and presence of Ni complex of different concentration, are near in maximum stability temperatures.

CONCLUSIONS

In summary, we investigated the binding of HEWL with a Ni complex. The thermodynamic parameters such as binding constant K_a, the binding gibbs free energy, ΔG°_b *binding enthalpy changes,* ΔH°_b, *and binding entropy changes,* ΔS°_b, were calculated by analyzing the UV-Vis

data with a simple binding model. With the obtained results of UV-Vis spectroscopy, the thermodynamic parameters ($\Delta H^o > 0$ and $\Delta S^o > 0$) were determined. These results show that the interaction between HEWL and Ni complex leads to an increasing enthalpy and entropy. The negative ΔG^o values for the interaction of HEWL with the Ni complex indicate the spontaneity of the complexation. Therefore, the dominant force is entropy and the mode of this interaction is hydrophobic [10].

The binding isotherm, binding capacity and scatchard plots were ploted .The positive values of n_H show that HEWL has one binding site set and presents positive cooperativity. Increase in T_m upon addition of Ni complex concentration shows an increase in protein stability with complex formation.

There are six parameters in Eq. (20) that were determined by fitting experimental $\Delta\varepsilon_{281}$ against T using sigmaplot software. These parameters are summarized in Table 4.

The $\Delta G_D(T)$ values for HEWL, in the absence and presence of Ni complex of different concentration , are close to the maximum stability temperatures.

ACKNOWLEDGEMENTS

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