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Research Note

A Thermodynamic and Kinetic Study of Glucose Oxidase at Various pH

A.A. Moosavi-Movahedi* and S. Mansouri[1]

Thermal denaturation of glucose oxidase has been studied in the pH range of 3.2 to 8.6. The pace theoretical model was used to determine thermodynamic parameters. Hill coefficient has been determined kinetically. Thermodynamic data and decrease in enzyme activity at pH 4.4 emphasize the association of glucose oxidase. The value of ΔC_p, T_s and T_H for this enzyme indicates a high degree of hydrophobicity for glucose oxidase.

INTRODUCTION

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase-EC. 1.1.3.4) has been isolated from honey, honey-bees and several filamentous fungi (notably Penicilium species and Aspergillus niger). An enzyme catalyzes the oxidation of β-D-glucose by molecular oxygen to gluconolactone which is subsequently hydrolyzed to gluconic acid and hydrogen peroxide [1-3].

The fungal glucose oxidase is a homodimer (with two disulphide bonds and two free sulphydryl groups), each containing one molecule of flavin-adenine dinucleotide (FAD) which is tightly and non-covalently bonded to the protein. These flavin cofactors are responsible for the oxidation-reduction properties of the enzyme [3-9].

Glucose oxidase is a glycoprotein with a carbohydrate content of 10 to 24% of its molecular weight (150 to 180 kD) depending on the source. The covalently bonded carbohydrate does not appear to significantly affect catalytic activity or tertiary structure of the protein [9-13]. The isoelectric point of enzyme is reported to be 4.05 to 4.2 [14].

Primary structure for the 583 residues per subunits has been elucidated [14-15]. X-ray analysis and electron density maps have also revealed important information on the overall structure of this enzyme [16].

Dissociation of subunits is possible only under denaturation and is accompanied by the loss of the cofactor FAD [17]. The activity of enzyme was found to depend heavily on 4- and 6-OH groups of the substrate [18]. The enzyme is inhibited by halide ions, D-glucal, hydrogen peroxide and various other cations [1,2,19-22]. Thermal stability of glucose oxidase depends on its redox state [23]. The enzyme is resistant to thermal denaturation in the presence of various additives (e.g. polyols, salts or synthetic polymers) [24-26]. Dodecyl trimethylammonium bromide (DTAB) is a good denaturant for the enzyme. Microcalerimetric studies of glucose oxidase with such surfactants reveal the nature of energetics for such interactions [27-30].

Glucose oxidase has been used widely for the quantitative determination of glucose in body fluids and in food technology (for removing residual glucose or oxygen from foods and beverages) and it has also been used in the production of gluconic acid. The enzyme is reported to have antitumor, antibacterial and antivirucidal effects [31-34].

In this article, a kinetic and thermodynamic investigation of the effect of pH on glucose oxidase activity is reported, which is absent in the literature.

EXPERIMENTAL

Materials

Glucose oxidase from Aspergillus niger, sodium n-dodecyl sulphate (SDS) and O-dianisidine were obtained from Sigma. The buffers were 50 mM glycine HCl (pH range of 3.2–5) and 50 mM glycine NaOH (pH range of 8.6-10). All other materials and reagents were of analytical grade and solutions were made in double-distilled water.

*. *Corresponding author, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, I.R. Iran.*
1. *Institute of Biochemistry and Biophysics, University of Tehran, Tehran, I.R. Iran.*

Methods

The activity of the enzyme was measured using the coupled peroxidase and O-dianisidine system [35]. One unit of activity is that amount of enzyme liberating 1 μmol of H_2O_2 per min at 25°C.

In the investigation of reversibility of pH, enzyme samples were incubated in various pH for 12 hours and then the activity was measured at an optimum pH (pH = 3.2) [36].

Thermal and chemical denaturation curves were determined by measuring the optical densities at 280 nm (where Tyr, Phe and Trp contribute as chromophores to the UV spectroscopy) of the solutions containing 0.4% (W/V) glucose oxidase with a Shimadzu model 2100 Spectrophotometer and cuvette with 1 cm optical path-length.

The velocity of the enzyme was determined by measuring the activity, the maximal velocity ($V_{\max}$) was obtained from Lineweaver-Burk plot.

RESULTS AND DISCUSSIONS

The enzyme activity at pH range of 3 to 10 with a minimum at pH 4.4 is shown in Figure 1. The loss of activity is probably related to the formation of inactive enzyme species.

Kinetic studies have been carried out in the pH range of 3.2 to 8.6, in order to investigate pH effect on glucose oxidase denaturation. Table 1 shows Hill coefficient (n_H) at various pH corresponding to Hill equation. This can be written as [36]:

$$\log V/(V_{\max} - V) = n_H \log[S] - \log K_m \qquad (1)$$

where $[S]$ is substrate concentration and K_m is Michaelis constant (measured as $[S]$ is required for $\frac{1}{2}V_{\max}$). The high pH value in the alkaline region appears to affect n_H which indicates some conformational changes and a possible alteration in the active site position; substrate would then be expected to bind to the active site, as well as other sites since n_H is less than one. Based on crystallography data [16], low activity in alkaline solutions can be explained by deprotonation of histidine residues and a possible rearrangment of the nearby side chains which may partially block the entrance to the active site.

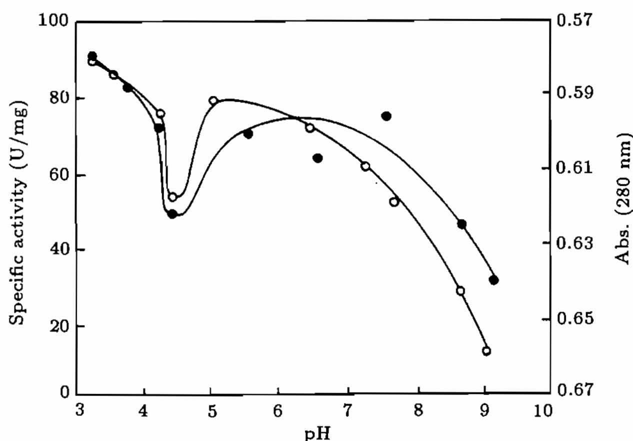

Figure 1. UV-absorption at 280 nm (•) and enzyme activity for glucose oxidase as a function of pH (o).

Figure 1 also shows absorbance change at 280 nm as a function of pH (3 to 10). The change in absorbance in the alkaline region greatly reflects effects of pH on conformational changes of the enzyme. Spectral changes largely reflect effects of the pH on the environment of the Trp, Phe and Tyr in the enzyme. On the basis of Figure 1 and Table 1, it can be concluded that pH effect in the alkaline region not only leads to changes in active site geometry but also to conformational changes in the three dimensional structure of the enzyme, resulting in loss of activity. pH changes in the acidic region, however, does not appear to affect the value of n_H indicating that the conformational changes probably do not occur at pH 3-5 (Table 1). Therefore, activity loss at pH 4.4 may be due to changes in the active site. This was investigated further using thermodynamic techniques. Effect of temperature on the absorbance change of glucose oxidase is shown as a sigmoidal curve in Figure 2. Assuming two state models to calculate the thermodynamic parameters based on the Pace method [37]:

$$F_D = (Y_N - Y_{\text{obs}})/(Y_N - Y_D) , \qquad (2)$$

where F_D is the fraction of denaturation, Y_{obs} is the observed variable parameter e.g., absorbance intensity, Y_N and Y_D are the values of Y characteristic of the native and denatured conformation.

Table 1. Thermodynamic parameters characterizing the thermal denaturation and Hill coefficient of glucose oxidase at various pH.

pH	n_H	ΔH_m(kJ mol^{-1})	ΔS_m(kJ mol^{-1}K^{-1})	ΔC_p(kJ mol^{-1}K^{-1})	T_s(K)	T_H(K)
3.2	1.01	351.2	1.6	30.7	319.65	319.06
4.4	1.04	181.2	0.5	7.0	309.65	308.62
4.9	1.02	242.3	0.7	13.7	316.25	315.66
8.6	0.82	298.6	0.9	41.2	317.75	317.64

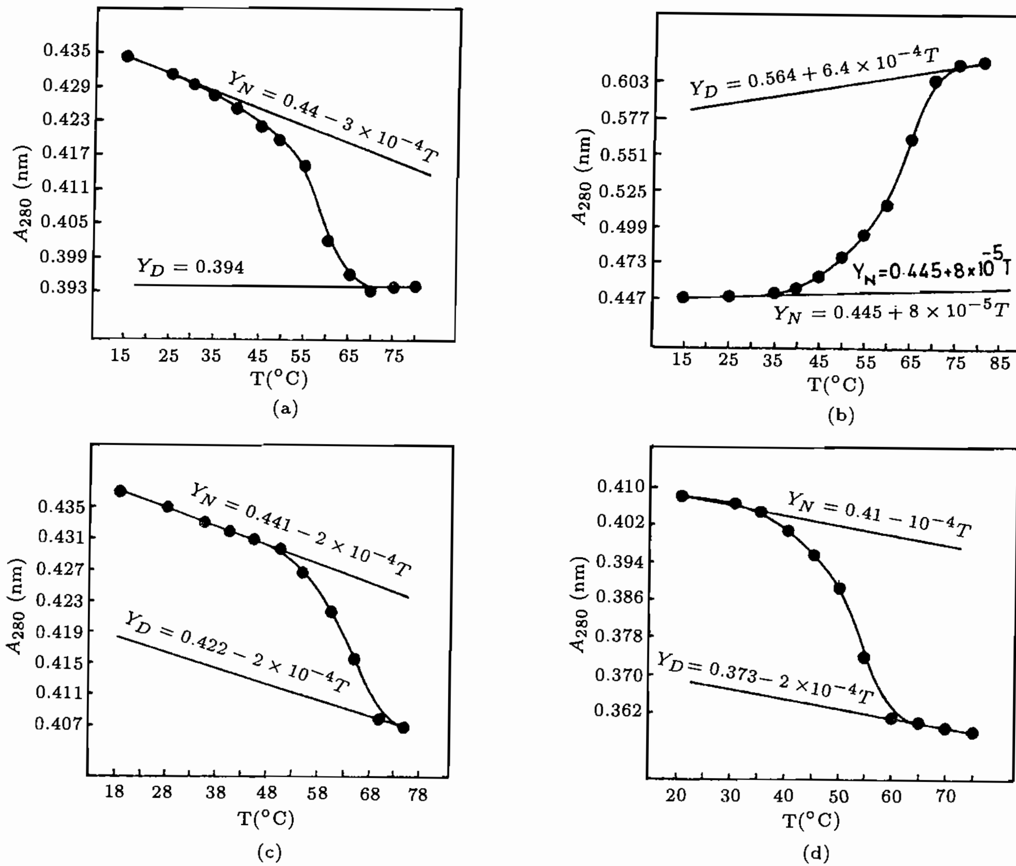

Figure 2. Thermal denaturation curve for glucose oxidase at various pH, a: pH 3.2 , b: pH 4.4 , c: pH 4.9, d: pH 8.6.

The difference in free energy between the native and denatured conformation, ΔG, can then be calculated using the equation:

$$\Delta G = -\text{RT } \ln[F_D/(1 - F_D)]$$
$$= -\text{RT } \ln[(Y_n - Y_{\text{obs}})/(Y_{\text{obs}} - Y_d)] \ , \quad (3)$$

where R is the gas constant and T is the absolute temperature. ΔC_p can be calculated by Gibbs-Helmoltz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m)$$
$$- \Delta C_p[T_m - T + T \ln T/Tm] \ , \quad (4)$$

where $\Delta G(T)$ is ΔG at temperature T, T_m is the midpoint of thermal denaturation curve and ΔH_m is the enthalpy change at T_m, $\Delta H_m = (T_m \text{ in } K)(-\partial \Delta G/\partial T)_{T=T_m}$. The temperature of maximum stability (T_s) occurs at the temperature where $\Delta S = 0$ and is given by:

$$T_s = T_m \exp(-\Delta H_m)/(T_m \cdot \Delta C_p) \ , \quad (5)$$

also T_H defining minimum solubility. where enthalpy change of unfolding becomes zero, can be written as [38]:

$$T_H = T_m - \Delta H(T_m)/\Delta C_p \ . \quad (6)$$

Thermodynamic results for glucose oxidase at various pH is shown in Table 1. ΔCp depends on order of water molecules around the non-polar groups that are brought into contact with water when a protein unfolds and its value is attributed to amount of hydrophobic interaction in the enzyme [39]. The high value of ΔC_p and T_s observed for glucose oxidase in comparison with other enzymes e.g., adenosine deaminase [40] suggests a high degree of hydrophobicity (Table 1). Furthermore, the temperature shift of the enthalpy and entropy functions ($T_S - T_H$) is very important for stabilization of the native protein [41]. The large value of observed T_H also indicates high solubility of glucose oxidase due to high percentage of carbohydrate and charged residues in this enzyme.

The observed similarity in T_H and T_S values further suggests the existence of some charged residues and carbohydrates in the hydro-interacting region of the enzyme during the unfolding process leading to exothermic enthalpy. Decrease in the ΔC_p and $DeltaS$ at pH 4.4, however, suggests that an association state may occur in enzyme subunits. Ye and Combs [5] have reported that the dimer and trimer forms of glucose oxidase possess enzymatic activity, whereas the corresponding monomer and tetramer do not appear to be active. The finding that activity decreases at

pI (pH = 4.2) probably corresponds to the tetramer contribution because of the association state.

Figure 3 shows that absorption changes versus temperature at different [SDS] is binary (combined of two sigmoids). This may indicate more than one oligomeric form of glucose oxidase; a decrease in electrostatic repulsion at this pH (4.4) results in an association state as it is near the isoelectric point (4.2).

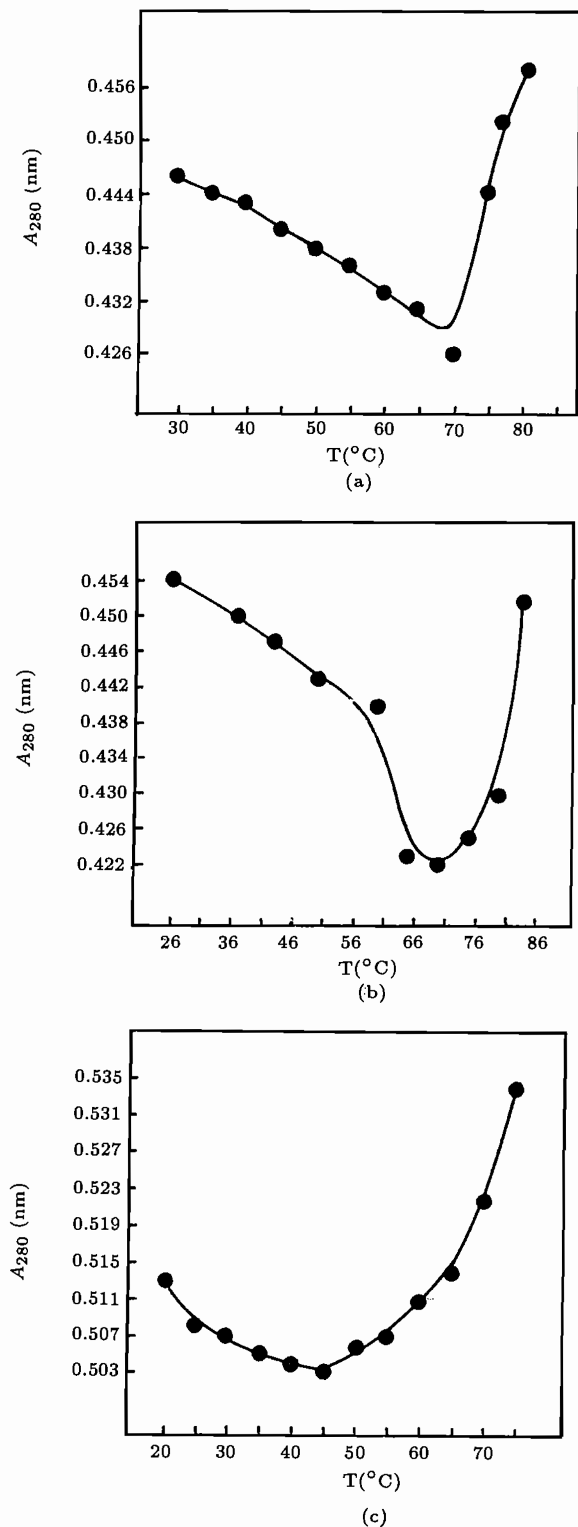

Figure 3. Thermal denaturation curve for glucose oxidase at pH 4.4 in the presence of SDS; a: 0.5 mM; b: 1.4 mM; c: 10 mM.

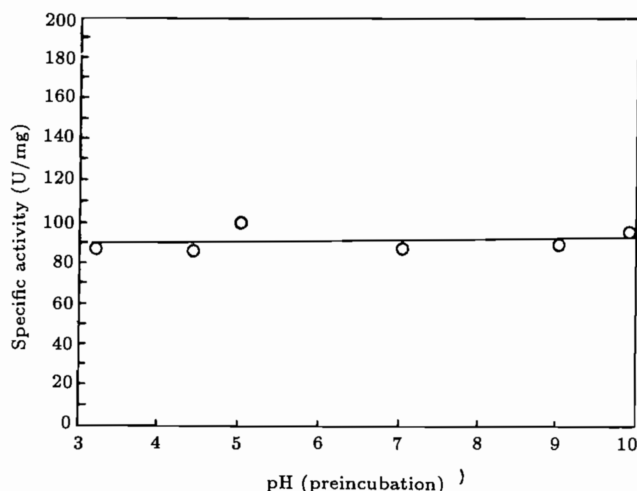

Figure 4. Specific activity of glucose oxidase at optimum pH = 3.2 as a function of incubation pH.

Figure 4 shows specific activity of samples of enzyme at optimum pH of 3.2 which demonstrates the effect of pH on enzyme activity and stability. pH stability depends on many factors including temperature, ionic strength and chemical nature of the buffer, therefore the enzyme should be preincubated for a long period of time. The horizontal linearity suggests that the effect of pH on the active site, conformational structure and the oligomeric state is reversible.

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REFERENCES

1. Schepartz, A.I. "The glucose oxidase of honey", *Biochem. Biophys. Acta*, **118**, pp 637-40 (1966).
2. Schepartz, A.I. and Subers, M.H. "The glucose oxidase of honey", *Biochem. Biophys. Acta*, **85**, pp 228-37 (1964).
3. Swoboda, B.E.P. and Massey, V. "Purification and properties of the glucose oxidase from Aspergillus niger", *J. of Biol. Chem.* **240**(5), pp 2209-15 (1965).
4. Swoboda, B.E.P. "The relationship between molecular conformation and the binding of flavin adenine dinucleotide in glucose oxidase", *Biochem. Biophys. Acta*, **175**(2), pp 365-79 (1969).
5. Ye, W.N. and Combes, D. "The relationship between the glucose oxidase subunit structure and its thermostability", *Biochem. Biophys. Acta*, **999**(1), pp 86-93 (1989).

6. Okuda, J., Negamine, J. and Yagi, K. "Exchange of free and bound conenzyme of flavin enzymes studied with [C] FAD", *Biochem. Biophys. Acta*, **566**, pp 245-52 (1979).

7. Sanner, C., Macheroux, P., Ruterjans, H., Muller, F. and Bacher, A. "15N - and 13C - NMR investigations of glucose oxidase from Aspergillus niger", *Eur. J. of Biochem.*, **196**(3), pp 663-72 (1991).

8. Malley, J.J. and Weaver, J.L. "Subunit structure of glucose oxidase from Aspergillus niger", *Biochemistry*, **11**(19), pp 3527-32 (1972).

9. Tsuge, H., Natsuaki, O. and Ohashi, K. "Purification, properties and molecular features of glucose oxidase from Aspergillus niger", *J. of Biochem.*, **78**(4), pp 835-43 (1975).

10. Lee, Y.C., Scocca, J.R. and Muir, L. "Rapid automatic analysis of sugar components in glycoproteins", *Physical Biochemistry*, **27**, pp 559-66 (1969).

11. Pazur, J.H., Kleppe, K. and Cepure, A. "A glycoprotein structure for glucose oxidase from Aspergillus niger", *Arch. Biochem. Biophys.*, **111**(2), pp 351-7 (1965).

12. Nakamura, S. and Hayahi, S. "A role of the carbohydrate moiety of glucose oxidase", *FEBS letters*, **41**(2), pp 327-30 (1974).

13. Nakamura, S., Hayashi, S. and Koga, K. "Effect of perodate oxidation on the structure and properties of glucose oxidase", *Biochem. Biophys. Acta*, **445**(2), pp 294-308 (1976).

14. Fredrick, K.R., Tung, J., Emerick, R.S., Masiarz, F.R., Chamberlian, S.H., Vasavada, A., Rosenberg, S., Chakaraborty, S., Schoper, L.M. and Massey, V. "Glucose oxidase from Aspergillus niger", *J. of Biol. Chem.* **265**(7), pp 3793-3802 (1990).

15. Kriechbaum, M., Helimann, H.J., Wientijes, F.J., Hahn, M., Jany, K.D., Gassen, H.G., Sharif, F. and Alaeddinoglv, G. "Cloning and DNA sequence analysis of the glucose oxidase gene from Aspergillus niger NRRL-3", *FEBS letters*, **255**(1), pp 63–66 (1989).

16. Hecht, H.J., Kalisz, H.M., Hendle, J., Schmid, R.D. and Schomburg, D. "Crystal structure of glucose oxidase from Aspergillus niger refined at 2.3A° resolution", *J. of Mol. Biol.*, **229**(1), pp 153-72 (1993).

17. Jones, M.N., Manely, P. and Wilkinson, A. "The dissociation of glucose oxidase by sodium n-dodecyl sulphate", *Biochem. J.*, **203**(1), pp 285-292 (1982).

18. Sierks, M.R., Bock, K., Refn, S. and Svensson, B. "Active site similarities of glucose dehydrogenase glucose oxidase and glucoamylase probed by deoxygenated substrates", *Biochemistry*, **31**(37), pp 8972-7 (1992).

19. Kleppe, K. "The effect of hydrogen peroxide on glucose oxidase from Aspergillus niger", *Biochemistry*, **5**(1), pp 139-43 (1966).

20. Rogers, M.J. and Brandt, K.G. "Interaction of halide ions with Aspergillus niger glucose oxidase", *Biochemistry*, **10**(25), pp 4630-5 (1971).

21. Rogers, M.J. and Brandt, K.G. "Interaction of D-glucal with Aspergillus niger glucose oxidase", *Biochemistry*, **10**(25), pp 4624-30 (1971).

22. Rogers, M.J. and Brandt, K.G. "Multiple inhibition analysis of Aspergillus niger glucose oxidase by D-glucal and halide ions", *Biochemistry*, **10**(25), pp 4636-41 (1971).

23. Nakamura, S. and Koga, K. "Alteration of thermal stability on glucose oxidase associated with the redox states", *Biochem. Biophys. Res. Commun.*, **78**(2), pp 806-10 (1977).

24. Ye, W.N., Combes, D. and Monsan, P. "Influence of additives on the thermostability of glucose oxidase", *Enzyme Microb. Technol.*, **10**, pp 498-502 (1988).

25. Gerlsma, S.Y. "Reversible denaturation of ribonuclease in aqueous solutions as influenced by polyhydric alcohols and some other additives", *J. of Biol. Chem.*, **243**(5), pp 957-61 (1968).

26. Gekto, K. "Calorimetric study on thermal denaturation of lysozyme in polyol-water mixtures", *J. of Biochem.*, **91**(4), pp 1197-1204 (1982).

27. Moosavi-Movahedi, A.A., Housaindokht, M.R. and Moghaddasi, J. "Thermodynamic studies of the interaction of glucose oxidase with anionic and cationic surfactants", *Thermochemica. Acta*, **219**, pp 143-150 (1993).

28. Housaindokht, M.R. and Moosavi-Movahedi, A.A. "Determination of binding affinities of glucose oxidase for sodium n-dodecyl sulphate", *Int. J. of Biol. Macromol.*, **16**(2), pp 77-80 (1994).

29. Housaindokht, M.R. and Moosavi-Movahedi, A.A. "Thermodynamic denaturation of glucose oxidase in aqueous dodecyl trimethyammonium bromide solution between 25°and 65°C", *Thermochemica. Acta*, **235**, pp 189-196 (1994).

30. Housaindokht, M.R., Moosavi-Movahedi, A.A., Moghaddasi, J. and Jones, M.N. "Interaction of glucose oxidase with ionic surfactants: microcalorimetry study", *Int. J. of Biol. Macromol.*, **15**(6), pp 337-41 (1993).

31. Combes, S.H., Morgan, C.D. and Enerse, T. "Antitumor activity of an immobilized peroxidase system against murine ehrlich ascites mediated by the immune system", *Proc. of Soc. Exp. Biol. Med.*, **204**(3), pp 306-311 (1993).

32. Yamagaguchi., Y., Semmel, M., Stanislawshi, L., Stroberg, A.O. and Stanislawski, M. "Virucidal effects of glucose oxidase and peroxidase or their protein conjugates on human immunodeficiency virus type 1", *Antimicrob. Agents. Chemother.*, **37**(1), pp 26-31 (1993).

33. Kiang, SW., Kuan, J.W., Kuan, S.S. and Guilbault, G. "Measurment of glucose in plasma with use of immobilized glucose oxidase and peroxidose", *Clin. Chem.*, **22**(8), pp 1378-82 (1976).

34. Tiina, M. and Sandholm, M. "Antibacterial effect of the glucose oxidase-glucose system on food-poisoning organisms", *Int. Food. Microbiol*, **8**(2), pp 165-74 (1989).

35. *Worthington Enzyme Manual*, Freehold, New Jersey, USA, pp 19-21 (1972).
36. Segal, I.H. "Biochemical calculation" 2nd Ed., John Wiley and Sons Inc., New York, USA, Chapter **4**, pp 208-323 (1976).
37. Pace, N.C. "Measuring and increasing protein stability", *TIBS*, **15**(1) pp 14-19 (1990).
38. Moosavi-Movahedi, A.A., Moghadamnia, H. and Hakimelahi, G.H. "Thermodynamic and kinetic studies of competitive inhibition of adenosine deaminase by ring opened analogues of adenine nucleoside", *Int. J. of Biol. Macromol.*, **15**, pp 125-129 (1993).
39. Baldwin, R. "Temperature dependence of the hydrophobic interaction in protein folding", *Proc. Natl. Acad. Sci.*, **83**, USA, pp 8069–72 (1986).
40. Moosavi-Movahedi, A.A., Samiee, B. and Hakimellahi, G.H. "Thermal analysis of adenosine deaminase in the presence of dodecyl trimethylammonium bromide", *J. of Colloid and Interface Science*, **161**, pp 53-6 (1993).
41. Privalov, P.L. "Thermodynamic problems of protein structure", *Ann. Rev. Biophys. Chem.*, **18**, pp 47-69 (1994).