Journal of Sciences, Islamic Republic of Iran 21(1): 15-20 (2010) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

A Thermodynamic Study of the Interaction between Urease and Copper Ions

A.A. Saboury,[1,*] E. Poorakbar-Esfahani,[2] and G. Rezaei-Behbehani[3]

[1]*Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Islamic Republic of Iran*
[2]*Biology Department, Payam Noor University, Tehran, Islamic Republic of Iran*
[3]*Chemistry Department, Imam Khomeini International University, Qazvin, Islamic Republic of Iran*

Received: 1 November 2009 / Revised: 14 December 2009 / Accepted: 24 December 2009

Abstract

A thermodynamic study of copper ions by jack bean urease (JBU) was carried out at two temperatures of 27 and 37°C in Tris buffer (30 mM; pH=7.0) using an isothermal titration calorimetry. There is a set of twelve identical and non-interacting binding sites for copper ions. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding are 285 μM and −15.2 kJ/mol at 27°C and 346 μM and −14.6 kJ/mol at 37°C, respectively. The molar entropy of binding is 17.2 J K^{-1} mol^{-1}at 27°C and +19.1 J K^{-1} mol^{-1} at 37°C. Hence, the binding process of copper ion to JBU is not only enthalpy driven but also it is entropy driven, which the role of entropy driven should be more effective by increasing the temperature.

Keywords: Urease; Copper ion; Isothermal titration calorimetry; Enthalpy of binding; Entropy of Binding

Introduction

Jack bean urease (urea amidohydrolase; E.C. 3.5.1.5) is the first crystallized enzyme [1] and also is the first enzyme shown to contain nickel [2-3]. The crystal structure of urease has not been determined yet. The best resolution obtained at 3.5 Å only allowed to assign the octahedral crystals of this urease to a cubic spacegroup [4]. Jack bean urease has six identical subunits. Each subunit consist of a single kind of polypeptide chain containing 840 amino acid residue with relative molecular mass of 90770, excluding the two nickel ions per subunit[5]. Hence, a mass of the hexamer urease, including 12 nickel ions, being 545.340 kDa[5] (590 kDa by a sedimentation method [6]). The subunit of urease from microorganisms appear to be smaller than jack bean urease in size and number [7-8].

Urease catalyzes the hydrolysis of urea to carbonic acid and two molecules of ammonia[9]. The specificity of urease was believed to be absolute [10] until Fishbein et al. reported that N-hydroxyurea was a substrate [11-12]. N-hydroxyurea [11-13], (N,N')dihydroxyurea [14-15], semicarbazide [16], N-methylurea [17], formamid [18] and acetamide [17] are another examples of substrates for urease. The mechanisms of hydrolysis of urea by urease presently contemplated are those by Benini et al. [19] and Karplus et al. [20], which taking their origins from the mechanism proposed by Zerner's group [17]. Zerner et al. have proposed a model for jack bean urease catalysis in which one nickel coordinates the oxygen atom of urea, polarizing the carbonyl group, and a second nickel coordinates hydroxide ion, the

* Corresponding author, Tel.: +98(21)66409517, Fax: +98(21)66409517, E-mail: saboury@ut.ac.ir

catalytic nucleophile [17].

Ureases are inhibited by a number of compounds. The study of urease inhibitors may have medical or agronomic significance, as well as providing insight into the urease catalytic mechanism [21-22]. Substrate urea, product ammonium ions, and substrate analogues are weak inhibitors of urease [21, 23-24]. Thiols inhibit urease competitively in their thiolate anion form $R\text{-}S^{-}$ [22]. Amides and esters of phosphoric acid are also slowbinding inhibitors of urease, classified as the strongest inhibitors. Boric and boronic acids are rapidly binding urease inhibitors, comparatively weak. Fluoride ion was found to be a competitive inhibitor for jack bean urease.[25] There are some reports on the inhibition of urease by bismuth compounds, which are of medical importance since are widely used as bactericidal agents [26-29]. The inhibition of ureases by quinones has been mainly tested for their potential application with urea fertilizers [29-30].

Inhibition of urease by heavy metal ions is important not only in view of heavy metal ion pollution, appropriate levels of urease activity in agricultural soils may be endangered, but also this inhibition may be exploited in constructing urease inhibition-based sensing systems [31-33] for in situ and real time determination of trace levels of the ions, e.g. in environmental monitoring, food control and biomedical analysis. Heavy metal ions inhibit both plant [31,34-38] and bacterial ureases[39-40] at the following order of effectiveness [36,38]:

$$Hg^{2+} \approx Ag^{+} > Cu^{2+} >> Ni^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+} > Fe^{3+} > Pb^{2+} > Mn^{2+}$$

Hg^{2+}, Ag^{+} and Cu^{2+} ions nearly always listed as the strongest inhibitors [31-36,38,41]. The inhibition has been habitually ascribed to the reaction of the metal ions with the thiol groups of the enzyme [33,41-42]. However, both copper and silver ions coordinate to nitrogen- (histidine) and possibly oxygen- (aspartic and glutamic acids) containing functional groups in urease [43-44]. Notwithstanding heavy metal ion binding to urease is important, there is not a comprehensive binding study in this case. Here, we applied isothermal titration microcalorimetry as a powerful tool for studying of copper ion binding to jack bean urease, which all thermodynamic parameters for the binding process can be found.

Materials and Methods

Materials

Jack bean urease (JBU) was obtained from Sigma Chemical Co. Copper nitrate was obtained from Merck. The buffer solution used in the experiments was 30 mM Tris using double-distilled water, pH=7.0, which was obtained from Merck. Experiments were carried out at two temperatures of 27 and 37 °C.

Methods

The experiments were performed with the 4-channel commercial microcalorimetric system, Thermal Activity Monitor 2277 (Thermometric, Sweden). Each channel is twin heat conduction calorimeter (multijuction thermocouple plates) positioned between the vessel holders and the surrounding heat sink. Both, sample and reference vessels were made from stainless steel. The limited sensitivity for the calorimeter is 0.1 μJ Copper ion solution (10 mM) was injected into the calorimetric titration vessel, which contained 1.8 ml JBU, 4 μM (2.2 mg/ml), in Tris buffer (30 mM), pH=7.0, using a Hamilton syringe. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimetric vessel. Injection of copper ion solution into the perfusion vessel was repeated 30 times and each injection included 20 μl copper ion solution. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of injection was calculated by the "Thermometric Digitam 3" software program. The heat of dilution of the copper ion solution was measured as described above except JBU was excluded. Also, the heat of dilution of the protein solution was measured as described above except that the buffer solution was injected to the protein solution in the sample cell [45]. The enthalpies of copper ion and protein solutions dilution were subtracted from the enthalpies of copper ion solutions in JBU solutions. The microcalorimeter was frequently calibrated electrically during the course of the study.

Results and Discussion

The raw data obtained from ITC at two temperatures of 27 °C and 37 °C are shown in Figure 1. Figure 1a shows the heat of each injection and Figure 1b shows the cumulative heat at each total concentration of copper ion, $[Cu^{2+}]_t$. For a set of identical and independent binding sites, we have before shown different methods of ITC data analysis[45]. For a set of identical and independent binding sites, we have previously introduced the following equation[45-46]:

$$\frac{\Delta q}{q_{max}} M_0 = \left(\frac{\Delta q}{q}\right) L_0 \frac{1}{g} - \frac{K_d}{g} \qquad (1)$$

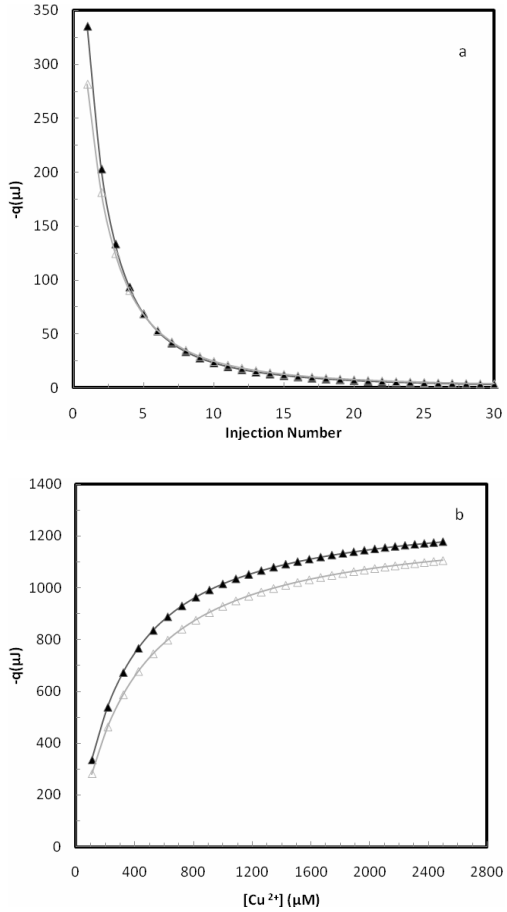

Figure 1. (a) The heat of copper ion binding to JBU for 30 automatic cumulative injections, each of 20 μl, 10 mM of the copper nitrate solution, into sample cell containing 1.8 ml 4 μM protein solution at 300 K (▲) and 310 K (Δ). (b) The total cumulative heat of binding *vs.* total concentration of copper ion calculated from Fig. 1a.

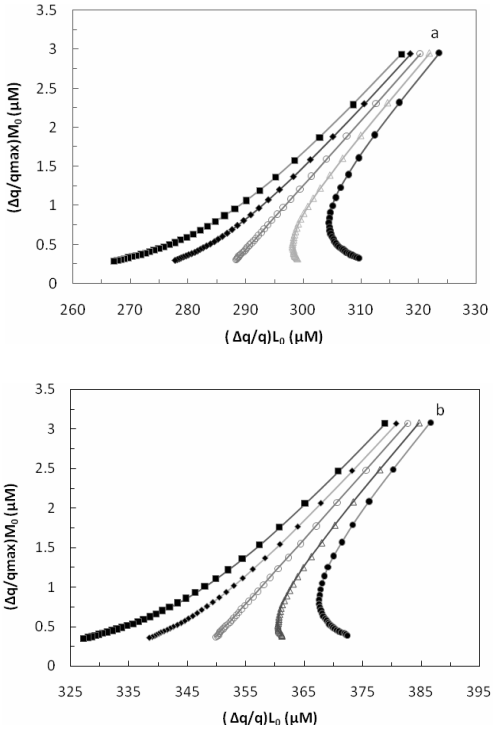

Figure 2. (a) The best linear plot of $(\frac{\Delta q}{q_{max}})M_0$ *vs.* $(\frac{\Delta q}{q})L_0$, according to Eq. (1), using values of −1303 μJ (■), −1308 (♦), −1313 μJ (○), −1318 μJ (Δ), −1323 μJ (●) at 300 K for q_{max} to obtain the best correlation coefficient value (R^2=0.999) for a linear plot. (b) The best linear plot of $(\frac{\Delta q}{q_{max}})M_0$ *vs.* $(\frac{\Delta q}{q})L_0$, according to Eq. (1), using values of −1251 μJ (■), −1256 (♦), −1261 μJ (○), −1266 μJ (Δ), −1271 μJ (●), at 310 K for q_{max} to obtain the best correlation coefficient value (R^2=0.999) for a linear plot.

where g is the number of binding sites, K_d is the dissociation equilibrium constant, M_0 and L_0 are total concentrations of biomacromolecule and metal ion, respectively, $\Delta q=q_{max}-q$, q represents the heat value at a certain L_0 and q_{max} represents the heat value upon saturation of all biomacromolecule. If *q* and q_{max} are calculated per mole of biomacromolecule then the standard molar enthalpy of binding for each binding site ($\Delta H°$) will be $\Delta H° = \frac{q_{max}}{g}$. According to the equation (1), a plot of $(\Delta q/q_{max})M_0$ *vs.* $(\Delta q/q)L_0$ should be a linear plot by a slope of 1/g and the vertical-intercept of K_d/g, which g and K_d can be obtained. The related plot for the binding of Cu^{2+} ions by JBU is shown in Figure 2. The linearity of the plot has been examined by different estimated values for q_{max} to find the best value for the correlation coefficient (near to one). The best linear plot with the correlation coefficient (R^2) value (near to one) was obtained using −1313 μJ and −1261 μJ (equal to −182.4 kJ/mol and −175.1 kJ/mol) at 27 °C and 37 °C, respectively, for q_{max} (Fig. 2). The lack of a suitable value for q_{max} to obtain a linear plot of $(\frac{\Delta q}{q_{max}})M_0$ vs. $(\frac{\Delta q}{q})L_0$ may be related to the existence of non-identical binding sites or the interaction between them. The value of g is 12, obtained from the slope and values of K_d, obtained from the vertical-intercept plot for a set of identical and independent binding sites are 285 μM and 346 μM at 27 °C and 37 °C, respectively. Dividing the q_{max} amount of −182.4 kJ/mol and −175.1 kJ/mol by

g=12, therefore, gives $\Delta H° = -15.2$ kJ/mol and −14.6 kJ/mol at 27 °C and 37 °C, respectively.

The molar enthalpy of each binding site (ΔH°) and its dissociation equilibrium constant (K_d) in a set of biomacromolecule binding sites can also be obtained via a simple graphical nonlinear fitting method using the following equation [45, 47-48].

$$\Delta H° = 1/A\left\{(B+K) - \left[(B+K_d)^2 - C\right]^{1/2}\right\} \quad (2)$$

A, B and C are constants in each injection, which have been defined as follows:

$$A = V/2q \quad B = gM_0 + L_0 \quad C = 4gM_0L_0 \quad (3)$$

where V is the volume of the reaction solution in the calorimetric sample cell in each injection step. Equation (2) contains two unknown parameters, K_d and ΔH°. A series of reasonable values for K_d is inserted into equation (2) and corresponding amounts for ΔH° are calculated and the graph ΔH° versus K_d is constructed. Curves of all titration steps will intersect in one point, which represents true amounts for ΔH° and K_d. The plots of ΔH° versus K_d, according to Eq. (2) for all injections are shown in Figure 3. The intrinsic dissociation equilibrium constant and the molar enthalpy of binding were obtained 285 μM and −15.2 kJ/mol at 27 °C and 346 μM and −14.6 kJ/mol at 37 °C, respectively (see Figure 3). These results are identical with results obtained by previous method described above.

To compare all thermodynamic parameters in metal binding process for JBU, the change in standard Gibbs free energy (ΔG^o) should be calculated according to the equation (4), which its value can use in equation (5) for calculating the change in standard entropy (ΔS^o) of binding process.

$$\Delta G^o = -RT \ln K_a \quad (4)$$

$$\Delta G^o = \Delta H^o - T\Delta S^o \quad (5)$$

where K_a is the association binding constant (the inverse of the dissociation binding constant, K_d). The K_a values are obtained 3509 and 2891 M^{-1} at 27 °C and 37 °C, respectively. Hence:

$\Delta G^o = -20.4$ kJ/mol $\Delta S^o = +17.2$ J/ K mol (at 27 °C)

$\Delta G^o = -20.6$ kJ/mol $\Delta S^o = +19.1$ J/ K mol (at 37 °C)

It means that the binding process is spontaneous resulted by not only enthalpic but also entropic driven.

All thermodynamic parameters for the interaction between JBU and copper ion at two temperatures of 27 and 37 °C have been summarized in Table 1. There is a set of twelve identical and non-interacting binding sites for copper ions to JBU. The binding process is exothermic at both temperatures. The binding process of is not only enthalpy driven but also it is entropy driven. The role of entropy driven in the binding process should be more effective by increasing the temperature. The molar entropy of binding means that the difference between the entropy of Cu^{2+}–JBU complex (S_{Cu-JBU})

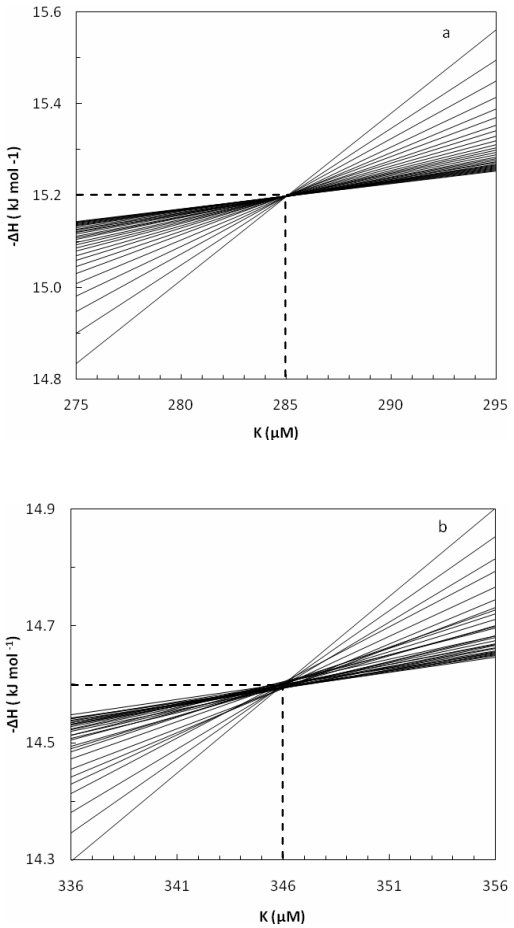

Figure 3. ΔH versus K_d for all 30 injections at 300 K (a) and 310 K (b) using data in Fig.1b. The coordinates of the intersection point of the curves give the true values for ΔH and K_d.

Table1. Thermodynamic parameters of binding for copper ions to JBU obtained by ITC

T (K)	g	K (μM)	K_a (M^{-1})	ΔH° (kJ/mol)	ΔG° (kJ/mol)	ΔS° (J/mol K)
300	12	285	3509	−15.2	−20.4	17.2
310	12	346	2891	−14.6	−20.6	19.1

and the entropy of native JBU (S_{JBU}): $\Delta S = S_{Cu-JBU} - S_{JBU}$. Hence, the disorder of the protein structure has been increased due to the binding of copper ions.

Acknowledgement

Financial support from the University of Tehran is gratefully acknowledged.

References

1. Sumner, J.B., The isolation and crystallization of the enzyme urease ,J. Biol. Chem., **69**: 435–441, (1926).
2. Dixon, N.E.; Gazzole, C.; Blakeley, R. P.; Zerner, B., Jack bean urease (EC 3.5.1.5), A metalloenzyme.A simple biological role for nickel. J. Am. Chem. Soc., **97**: 14, 4131–4133 (1975).
3. Mamiya, G.; Takishima, K.; Masakuni, M.; Kayumi, T.; Ogawa, K., Complete amino acid sequence of jack bean urease J. Protein Chem., **6**:1, 55–59(1987).
4. Jabri, E.; Lee, M. H.; Hausinger, R. P.; Karplus, P.A. , Preliminary Crystallographic Studies of Urease from Jack Bean and from Klebsiella_Aerogenes. J. Mol. Biol., **227**:3, 934–937(1992).
5. Takishima, K.; Suga, T.; Mamia, G., The structure of jack bean urease. The complete amino acid sequence, limited proteolysis and reactive cysteine residues. Eur. J. Biochem., **175**:1, 151–165(1988).
6. Dixon, N. E.; Hinds, J. A.; Fihelly, A. K. ; Gazzola, C.; Winzor, D. J.; Blakeley, R. L.; Zerner, B., Jack bean urease (EC 3.5.1.5). IV. The molecular size and the mechanism of inhibition by hydroxamic acids. Spectrophotometric titration of enzymes with reversible nhibitors. Can. J. Biochem., **58**:1-2, 1323–1334(1980).
7. Hausinger, R. P., Purification of a nickel-containing urease from the rumen anaerobe Selenomonas ruminantium. J. Biol. Chem., **261**: 17, 7866–7870(1986).
8. Christians, S.; Hausinger, R. P., Nickel-content of urease from Bacillus pasteurii. Arch. Microbiol., **145**: 1, 51–55 (1986).
9. Mobley, H. L. T.; Hausinger, R. P., Microbial ureases: significance, regulation, and molecular characterization. Microbiol. Rev., **53**:1, 85–108 (1989).
10. Varner, J. E. The Enzymes, Vol **4**, 2nd Edition, Academic Press, New York, 247-256(1960).
11. Fishbein, W. N.; Winter, T. S.; Davidson, J. D., Ureae catalysis. I. Stoichiometry, specificity and kinetics of a second substrate: Hydroxyurea. J. Biol. Chem., **240**, 2402–2406 (1965).
12. Fishbein, W. N.; Carbone, P. P., Urease Catalysis. J. Biol. Chem., **240**, 2407–2414 (1966).
13. Blakeley, R. L.; Hinds, J. A.; Kunze, H. E.; Webb, E.C.; Zerner, B. , Jack bean urease (EC 3.5.1.5). Demonstration of a carbamoyl-transfer reaction and inhibition by hydroxamic acids. Biochemistry, **8**:5, 1991–2000 (1969).
14. Fishbein, W. N., A simple, sensitive, and specific colorimetric assay for dihydroxyurea. Anal. Chim. Acta, **40**:2, 269 –275 (1968).
15. Fishbein, W. N., Urease catalysis. 3. Stoichiometry, kinetics, and inhibitory properties of a third substrate: dihydroxyurea. J. Biol. Chem., **244**: 5, 1188–1193(1969).
16. Gazzole, C.; Blakeley, R. L.; Zerner, B., On the substrate specificity of jack bean urease (urea amidohydrolase, EC 3.5.1.5). Can. J. Biochem., **51**:9, 1325–1330 (1973).
17. Dixon, N. E.; Riddles, P. W. C. Gazzole, R.L. Blakeley and B. Zerner, Jack bean urease (EC 3.5.1.5). V. On the mechanism of action of urease on urea, formamide, acetamide, N-methylurea, and related compounds.,Can. J. Biochem., **58** :12 ,1335–1344(1980).
18. Fishbein, W. N., Formamide- minimum-structure substrate for urease. Biochem. Biophys. Acta., **484**:2, 433–442 (1977).
19. Benini, S.; Rypniewski, W. R.; Wilson, K. S.; Miletti, S.; Ciurli, S.; Mangani, S., A new proposal for urease mechanism based on the crystal structures of the native and inhibited enzyme from Bacillus pasteurii: why urea hydrolysis casts two nickels. Structure, **7**:2, 205–216 (1999).
20. Karplus, P. A.; Pearson, M. A.; Hausinger, R. P., 70 years of crystalline urease: What have we learned?. Acc. Chem. Res., **30**:8, 330–337(1997).
21. Krajewska, B., Ureases I. Functional, catalytic and kinetic properties: A review .J. Mol. Catalysis B: Enzymatic, **59**:1-3, 9–21 (2009).
22. Todd, M. J.; Hausinger, R. P., Competitive inhibitors of Klebsiella aerogenes urease. Mechanisms of interaction with the nickel active site. J. Biol. Chem., **264**:27, 15835–15842(1989).
23. Saboury, A. A.; Moosavi-Movahedi, A. A. , A Simple Novel Method For Studying The Combined Inhibitory Effects of Ethylurea and N, N-Dimethylurea on Jack Bean Urease . J. Enzyme Inhib. , **11**:3, 217–22(1997).
24. Saboury, A. A., Isothermal titration microcalorimetric method for studying the combined ligand binding with application to the binding of ethylurea and (N,N)dimethylurea on urease Thermochim. Acta, , **320**:1-2, 97-100(1998).
25. Saboury, A. A.; Moosavi-Movahedi, A. A., A simple novel method for determination of an inhibition constant by isothermal titration microcalorimetry. The effect of fluoride ion on urease J. Enzyme Inhib., **12**:4, 273–279(1997).
26. Zhang, L.; Mulrooney, S. B.; Leung, A. F. K.; Zeng, Y.; Ko, B. B. C.; Hausinger, R. P.; Sun, H., Inhibition of urease by bismuth(III): Implications for the mechanism of action of bismuth drugs. Biometals, **19** :5,503 – 511(2006).
27. Asato, E.; Kamamuta, K.; Akamine, Y.; Fukami, Y.; Nukada, R.; Mikuriya, M.; Deguchi, S.; Yokota, Y. , Bismuth(III) complexes of 2-mercaptoethanol: Preparation, structural and spectroscopic characterization, antibactericidal activity toward Helicobacter pylori, and inhibitory effect toward H-pylory-produced urease Bull. Chem. Soc. Jpn., **70**:3, 639–648(1997).
28. Murafuji, T.; Azuma, T.; Miyoshi, Y.; Ishibashi, M.; Rahman, A. F. M. M.; Migita, K.; Sugihara, Y.; Mikata, Y., Inhibition of jack bean urease by organobismuth compounds Bioorg. Med. Chem. Lett., **16**:6 ,1510–1513(2006).

29. Bundy, L. G.; Bremner, J. M., Effects of substituted p-benzoquinones on urease activity in soils. Soil. Biol. Biochem., **5**, 847–853 (1973).
30. Mulvaney, R. L.; Bremner, J. M., Control of urea transformations in soils. *Soil Biochem.*, **5**: 153–196 (1981).
31. Preininger, C.; Wolfbeis, O. S., Disposable cuvette test with integrated sensor layer for enzymatic determination of heavy metals Biosens. Bioelectron., **11**:10, 981–990 (1996).
32. Krawczyk, T. K. V.; Moszczynska, M.; Trojanowicz, M., Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor Biosens. Bioelectron., **15**: 11-12, 681–691 (2000).
33. Kuswandi, B., Simple optical fibre biosensor based on immobilised enzyme for monitoring of trace heavy metal ions . Anal. Bioanal. Chem., **376**: 7, 1104–1110 (2003).
34. Shaw, W. H. R., The Inhibition of Urease by Various Metal Ions. J. Am. Chem. Soc., **76**, 2160–2163(1954).
35. Shaw, W. H. R.; Raval, D. N., The Inhibition of Urease by Metal Ions at pH 8.9. J. Am. Chem. Soc., **83**, 3184–3187 (1961).
36. Krajewska, B., Urease immobilized on chitosan membrane- inactivation by Heavy_metal Ions. J. Chem. Technol. Biotechnol., **52**: 2, 157–162 (1991).
37. Krajewska, B.; Zaborska, W.; Chudy, M., Multi-step analysis of Hg2+ ion inhibition of jack bean urease J. Inorg. Biochem., **98**: 6, 1160–1168 (2004).
38. Zaborska, W.; Krajewska, B.; Olech, Z., Heavy metal ions inhibition of jack bean urease: Potential for rapid contaminant probing J. Enzyme Inhib. Med. Chem., **19**, 65–69 (2004).
39. Nakano, H.; Takenishi, S.; Watanabe, Y., Purification and Properties of Urease from Brevibacterium ammoniagenes. Agric. Biol. Chem., **48**, 1495–1502 (1984).
40. Kenny, G. E. Inhibition of the Growth of ureaplasma-urealyticum by a new ureases inhibitor, Flurofamide Yale. J. Biol. Med., **56**: 5-6, 717–722 (1983).
41. Toren Jr., E. C.; Burger, F., Trace determination of metal ion inhibitors of the glucose-glucose oxidase system. J. Mikrochim. Acta, 56, 1049–1058 (1968).
42. Hellerman, L.; Chinard, F. P.; Deitz, V. R., Ascorbic Acid and Urease. J. Biol.Chem., **147**,443–462 (1943).
43. Krajewska, B., Mono- (Ag, Hg) and di- (Cu, Hg) valent metal ions affects on the activity of jack bean urease.Probing the modes of metal binding to the enzyme. J.Enzyme Inhib. Med. Chem., **23**, 535–542 (2008).
44. Follmer, C.; Carlini, C. R., Effect of chemical modification of histidines on the copper-induced oligomerization of jack bean urease (EC 3.5.1.5) Arch. Biochem. Biophys., **435**, 15–20 (2005).
45. Saboury, A. A., A review on the ligand binding studies by isothermal titration calorimetry J. Iran. Chem. Soc., **3**:1, 1–21 (2006).
46. Saboury, A. A.; Atri, M. S.; Sanati, M. H.; Moosavi-Movahedi, A. A.; Hakimelahi, G. H.; Sadeghi, M. , A thermodynamic study on the interaction between magnesium ion and human growth hormone Biopolymers, **81**:2, 120–126 (2006).
47. Ghadermarzi, M. Saboury, A. A.; Moosavi-Movahedi, A. A., A microcalorimetry and spectroscopy study on the interaction of catalase with cyanide ion. Polish J. Chem., **72**, 2024–2029 (1998).
48. Saboury, A. A., New methods for data analysis of isothermal titration calorimetry J. Therm. Anal. Cal., **72**:1, 93–103 (2003).