Asian Journal of Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 1 (2): 153-161, 2006
ISSN 1815-9923

Effects of Glyoxime and Dichloroglyoxime on Lysozyme: Kinetic and Structural Studies

[1]Bijan Ranjbar, [1]Saied Afshar, [2]Ali Kakanejadifard, [1]Khosro Khajeh,
[1]Hossein Naderi-Manesh, [1]Leila Hassani and [3]Naader Alizadeh
[1]Department of Biophysics and Biochemistry, Faculty of Science,
Tarbiat, Modares University, P.O. Box 14115-175, Tehran, Iran
[2]Department of Chemistry, Faculty of Science,
University of Lorestan, Khorramabad, Iran
[3]Department of Chemistry, Faculty of Science,
Tarbiat Modares University, Tehran, Iran

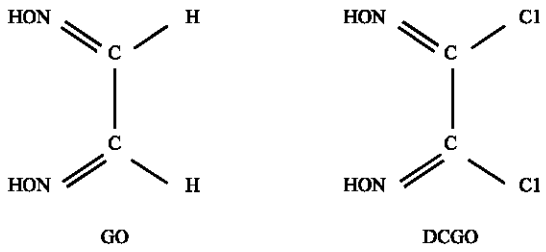
Abstract: Kinetic and structural studies have been made on the effect of glyoxime (GO) and dichloroglyoxime (DCGO) on the activity and the structure of lysozyme in 100 mM potassium phosphate buffer, pH 7.0, using UV spectrophotometry, circular dichroism (CD) and fluorescence spectroscopy techniques. GO and DCGO act as an uncompetitive inhibitors with K_i = 99 and 52 μM, respectively. Circular dichroism studies show that the secondary structure of the enzyme in the presence of different concentrations of GO and DCGO does not show considerable change. Kinetic results show that at low concentration of GO (0.12-120 μM) and DCGO (0.07-64 μM) considerable inhibition of enzyme could be seen, but the fluorescence data show that there is not noticeable change in the tertiary structure of lysozyme at low concentration of inhibitors. Also, these results indicate considerable decrease in the tertiary fold of the lysozyme at high concentrations of GO and DCGO espetially dichloroglyoxime. Results show that, lysozyme in the presence of high concentration of GO (1200 μM) and DCGO (640 μM), presents structural characteristics of a molten globule like state.

Key words: Lysozyme, α-dioxime, glyoxime, dichloroglyoxime, inhibition, molten globule state

Introduction

α-dioxime derivations have many applications in the industry and agriculture, for example, for chemical sensors that are used for special elements and for pesticides, fungicides and bactericides. Remarkable activity against gram-positive and gram-negative bacteria as well as certain yeast was observed for dichloroglyoxime. In addition, these substances effect biological systems adversely. For example, dimethyl glyoxime causes irritation of the eye, skin, digestive and respiratory systems and moreover, proven dangerous if ingested. Experimental results show that these substances are mutagens (Khalili *et al*., 1986; Bing *et al*., 1999; Ayres *et al*., 2002). Lysozyme (EC 3.2.1.17) is found in various animal and plant tissues, e.g., in tear liquid and chicken egg white. It functions as an antibacterial agent catalyzing the hydrolysis of a major cell-wall polysaccharide of

Corresponding Author: Bijan Ranjbar, Department of Biophysics and Biochemistry, Faculty of Science, Tarbiat Modares University, P.O. Box: 14115-175, Tehran, Iran Tel/Fax: (98)-21- 88009730



Scheme 1

certain bacteria (Ovchinikov, 1996). This enzyme hydrolyses β(1→4) glycosidic linkage from NAM (N–acetylmuramic acid) to NAG (N-acetylglucosamine) in the alternating NAM-NAG polysaccharide component of cell wall peptidoglycan of bacteria. Lysozyme is single domain protein and the active site is located just in the cleft (Ovchinikov, 1996; Schomburg *et al*., 1991; Smith *et al*., 1993). Hen egg white lysozyme is one of the most studied and best characterized globular proteins. It was the first enzyme to have its structure determined by X-ray diffraction and has ever since been extensively used as a system in which the underlying principles of protein structure, function, dynamics and folding can be studied through both experimental theoretical approaches (Smith *et al*., 1993; Matagne *et al*., 1998; Alizadeh *et al*., 2003; Godjaev *et al*., 1998; Fujita *et al*., 1995; Iwase *et al*., 1999; Johannesson *et al*., 1997; Schwalbe *et al*., 2001; Kristiansen *et al*., 1998).

Lysozyme binds to various small ligands which mimic the interaction of the acetamido group of its natural substrates within the active cleft of the enzyme (Johannesson *et al*., 1997; Imoto *et al*., 1972). Glyoxime (GO) and dichloroglyoxime (DCGO) were synthesized (Scheme 1) in our laboratory (Kakanejadifard *et al*., 2003, 2004) and in the present research we describe the catalytic behavior and structural changes of lysozyme in the presence of GO and DCGO.

Materials and Methods

Materials

The hen egg white lysozyme and *Micrococcus lysodeikticus* were purchased from Sigma (St. Louis, Mo, USA). The enzyme was homogenous on SDS-PAGE. All other chemical reagents were from Merck (Darmstadt, Germany) and reagent grade. The solutions were prepared in double distilled water. Glyoxime was obtained by the condensation of glyoxal and hydroxylamine in water at 0°C and dichloroglyoxime were synthesized by chlorination of glyoxime in ethanol in our laboratory (Kakanejadifard *et al*., 2003, 2004; Coburn, 1968; Willer *et al*., 1985; Kanno *et al*., 1995).

Determination of Enzymatic Activity and Protein Concentration

Enzymatic activity was determined using *Micrococcus lysodeikticus* as substrate in 100 mM potassium phosphate buffer, pH 7.0. One enzymatic unit is equal to a decrease in turbidity of 0.001 per minute at 450 nm at pH 7.0 under specified conditions (Worthington, 1993). The protein concentration was determined by Lowry *et al*. (1951) method.

Circular Dichroism (CD) Measurements

CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) using solutions with protein concentrations varying from 0.15 (far-UV) to 2 mg mL^{-1} (near-UV). The results were

expressed as molar ellipticity, [θ] (deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight (MRW) assuming its average weight for lysozyme to be equal to 111.5. The molar ellipticity was determined as [θ] = (θ×100MRW)/(cl), where c is the protein concentration in milligrams per milliliter, l is the light path length in centimeters and θ is the measured ellipticity in degrees at a wavelength λ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291}$ = 7820 deg cm^2 $dmol^{-1}$ (Schippers *et al*., 1981) and with JASCO standard nonhydroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5}$ = 7910 deg cm^2 $dmol^{-1}$ (Takakuwa *et al*., 1985). Noise in the data was smoothed using the JASCO J-715 software, including the fast Fourier-transform noise reduction routine which allows enhancement of most noisy spectra without distorting their peak shapes (Protasevich *et al*., 1997; Ataie *et al*., 2004; Khajeh *et al*., 2001).

Intrinsic Fluorescence Measurements

The fluorescence emission spectra of the enzyme were performed in a Perkin-Elmer luminescence spectrometer LS50B. The spectra were measured in 100 mM phosphate buffer, pH 7.0 and the final concentration of 10 μM for lysozyme. The fluorescence emission was scanned between 300 and 400 nm with an excitation wavelength of 280 nm.

Aggregation Measurements

Lysozyme at a concentration of 0.5 mg mL^{-1} in 100 mM phosphate buffer, pH 7.0, in the presence of different concentrations of GO and DCGO were placed in Perkin-Elmer luminescence spectrometer LS50B cuvette. The excitation and emission monochromators were set at 350 and 355 nm with the band passes of 1.5 nm and the extent of light scattering was monitored. The scattering effect, due to different concentrations of GO and DCGO, was corrected in all relevant cases.

Results presented in this paper are the mean from at least three repeated experiments in a typical run to confirm reproducibility.

Results and Discussion

Catalytic Parameters of Lysozyme in the Presence of α-dioxime Derivatives

The activity of lysozyme has been estimated in the presence of different concentrations of glyoxime and dichloroglyoxime. The enzyme activity decreases as the concentration of GO and DCGO increase (Fig. 1). Effects of the inhibitors on lysozyme is time-independent and

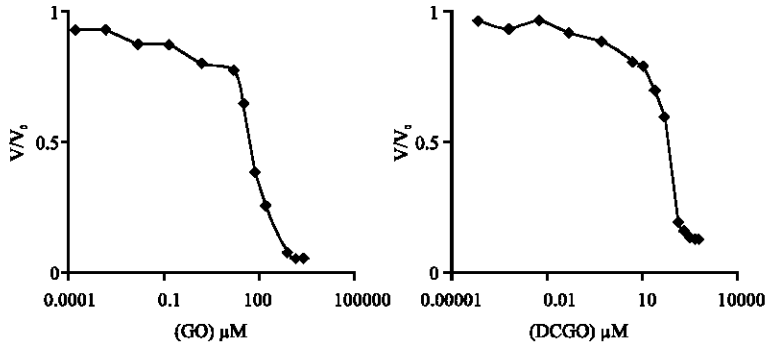


Fig. 1: Dose-response curves of lysozyme activity as a function of different concentrations of glyoxime (a) and dichloroglyoxime (b)

Table 1: Kinetic parameters for lysozyme in the absence and presence of glyoxime and dichloroglyoxime

Ligand	C (μM)	K_m (mg mL^{-1})	V_{max} (unit)
Control	-	0.69	139
	120.00	0.29	60
	12.00	0.61	124
+Glyoxime	1.20	0.68	138
	0.12	0.69	139
	64.00	0.31	62
	6.40	0.61	124
+Dichloroglyoxime	0.64	0.68	138
	0.07	0.69	139

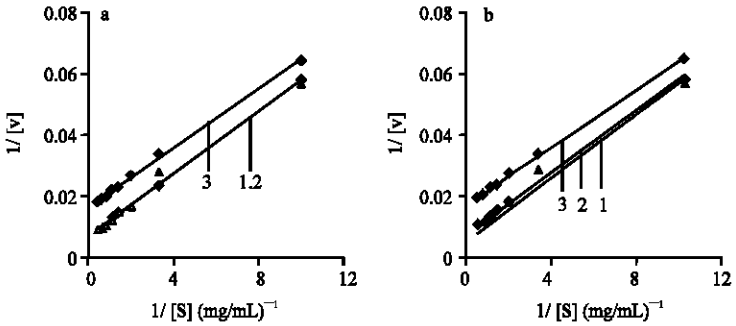


Fig. 2: Lineweaver-Burk plots of lysozyme in the absence and presence of different concentrations of glyoxime (a) (1) 0, (2) 0.12, (3) 120 μM and dichloroglyoxime (b) (1) 0, (2) 6.4 and (3) 64 μM

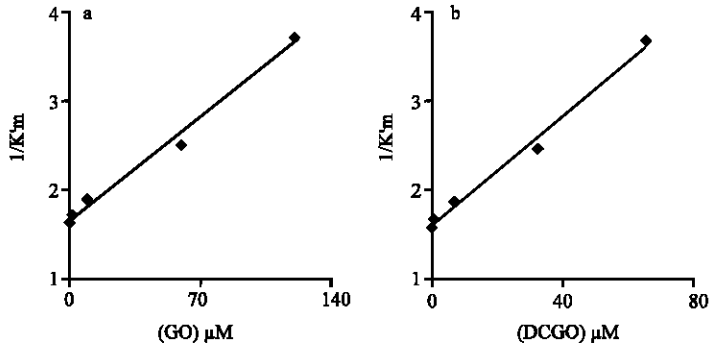


Fig. 3: Plot of $1/K'_m$ versus different concentration of inhibitors; K'_m is the apparent Michaelis constant in the presence of inhibitor glyoxime (a) and dichloroglyoxime (b)

after effects of inhibitor on lysozyme is eliminated with dialysis against buffer, activity of the enzyme is recovered (data not shown). These results show that lysozyme inhibition with GO and DCGO is reversible. To compare the inhibition effects of GO and DCGO, IC_{50} values of these compounds based on Fig. 1 were evaluated. These values are 60 and 95 μM for DCGO and GO, respectively. Our results indicate that inhibition strength of DCGO is higher compared to GO. This could be due to the presence of chloride in DCGO and its high electronegativity. Fig. 2 shows lineweaver-Burk plots of lysozyme in the absence and the presence of different concentrations of GO and DCGO. V_{max} and K_m of the enzyme, in the

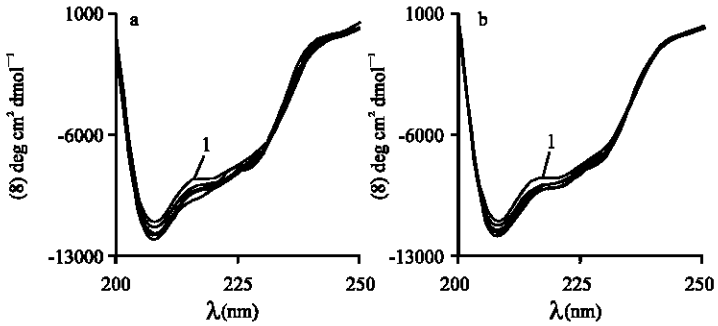


Fig. 4: Far-UV CD spectra of lysozyme in the absence and presence of the different concentrations of glyoxime (a) and dichloroglyoxime (b). (1) in the absence and others in the presence of glyoxime and dichloroglyoxime

presence and absence of inhibitors, were obtained and the type of inhibition was discussed. V_{max} and K_m decreased as the concentration of these substances increased (Table 1). These results indicate that inhibition mechanism is uncompetitive (Copeland, 2000). The secondary plot was prepared to calculate K_i (Fig. 3) (Copeland, 2000; Saboury *et al*., 2002) and subsequently, the inhibition constants for GO and DCGO were shown to be 99 and 52 μM, respectively. These results confirm the finding that inhibition strength of DCGO is higher than GO.

Comprehensive structural studies have been reported for GlcNAc oligosaccharides, urea and DMSO all of which bind to the active site of the enzyme both in crystal (Blake *et al*., 1994) and in solution (Cohen and Jardetzky, 1968; Lumb and Dobson, 1992). DMSO, at low concentration, binds to the active cleft and the protein surface and at high concentration, disrupts the tertiary fold of lysozyme (Johannesson *et al*., 1997). GO and DCGO can form as many hydrogen bonds with the enzyme as N-acetyl glucose amine or urea, the similarity of their structures with acetamido group suggests that they can bind to the active cleft and the enzyme surface. Our results show that GO and DCGO is able bind to enzyme-substrate [ES] complex.

Circular Dichroism and Fluorescence Measurements of GO- and DCGO- Induced Equilibrium Denaturation

The far-UV-CD spectra of the lysozyme obtained in phosphate buffer, pH 7.0 at different GO and DCGO concentrations are shown in Fig. 4. Percentage of secondary structures in lysozyme, in the absence and presence of glyoxime and dichloroglyoxime, are displayed in Table 2.

Upon the addition of the mentioned inhibitors (0.00064-640 μM of DCGO and 0.0012-1200 μM of GO) CD spectra of lysozyme show a very slight increase in the negative ellipticity at 208 and 222 nm with respect to that of the proteins in the buffer only. In fact, these results show that the enzyme ellipticity at 222 nm, characteristic of the α-helical conformation, does not change in the presence of GO and DCGO, indicating that a native-like secondary structure persists even at 640 μM of DCGO and 1200 μM of GO. The fluorescence emission spectra of lysozyme in phosphate buffer, pH 7.0 at different concentrations of GO and DCGO are shown in Fig. 5. The observed effect of initial increase of lysozyme fluorescence could be interpreted as the consequence of inhibitor binding in the cleft region, in the proximity of Trp62, Trp63 or Trp108, which decreases the accessibility of indole rings for water molecules. Upon addition of these substances, the tryptophan fluorescence of the lysozyme gradually decreases. The decrease is observed in the fluorescence intensity, suggesting that

Table 2: Secondary structure percentage of lysozyme in the absence and presence of glyoxime and dichloroglyoxime

Ligand	C (μM)	α-helix (%)	α-structure (%)	Random coil (%)
Control	-	29.3	32.5	38.3
	1.2×10^{-3}	30.1	31.6	38.2
	1.2×10^{-1}	30	32.4	37.6
	12	30.1	31.6	38.3
+Glyoxime	120	29.4	31.9	38.8
	580	29.7	32.7	37.6
	870	30.2	32.4	37.4
	1200	31.3	31.5	37.2
	6.4×10^{-4}	30.7	30.9	38.6
	6.4×10^{-2}	30.6	31.4	38.0
	6.4	30.9	30.9	38.2
+Dichloroglyoxime	64	30.1	32.1	37.8
	320	30.7	32.2	37.1
	480	30.3	31.5	38.1
	640	30.8	30.8	38.4

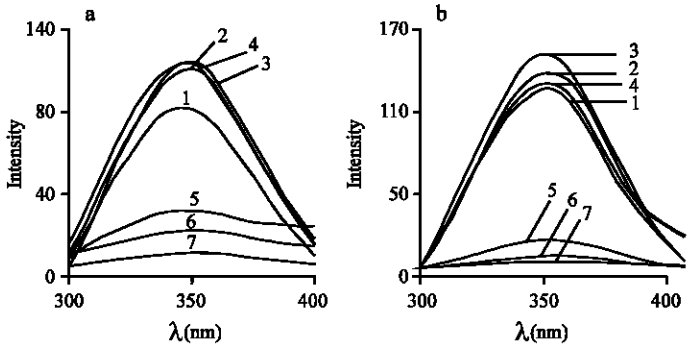


Fig. 5: Fluorescence emission spectra of lysozyme in the absence and presence of different concentrations of glyoxime (a) (1) 0, (2) 1.2×10^{-3}, (3) 1.2×10^{-1}, (4) 12, (5) 580, (6) 870 and (7) 1200 μM and dichloroglyoxime (b) (1) 0, (2) 6.4×10^{-4}, (3) 6.4×10^{-2}, (4) 6.4, (5) 320, (6) 480 and (7) 640 μM

at high concentrations of GO (580-1200 μM) and DCGO (320-640 μM), there is a change in the tertiary structure of the protein, resulting in the exposure of the buried tryptophans to the polar solvent. In other words, the intrinsic fluorescence reduction along with the red shift shows that the enzyme has denatured at high concentrations of glyoxime and dichloroglyoxime. The tertiary fold reduction of lysozyme at high concentrations of these reagents (especially DCGO) could probably be the consequence of water structure breaking down by the formation of strong hydrogen bonds between these reagents and the water molecules. The expansion of the protein structure, occurs at the 1200 μM and 640 μM of GO and DCGO, respectively. Previously, the neutron diffraction and the computer simulation studies of DMSO-water mixtures revealed similar results (Soper *et al*., 1992; Luzar *et al*., 1993; Iwase *et al*., 1999). Present results indicate that at high concentrations of both reagents, lysozyme adopts the features of the molten globule state, with substantial secondary structure and at the same time, the tertiary structure is less organized than that of the native state (Hosseinkhani *et al*., 2004; Asghari *et al*., 2004; Boren *et al*., 1999).

Aggregation Measurements

Light scattering methods provide a sensitive measure of aggregate formation in protein solutions (Rajaraman *et al*., 1996). Figure 6 shows the light scattering profile of the lysozyme (0.2 mg mL^{-1}) as

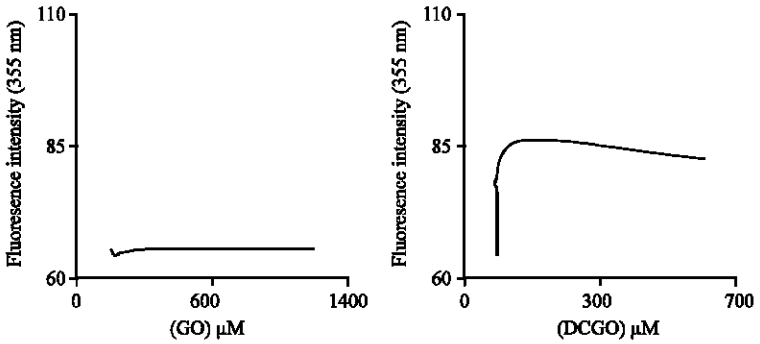


Fig. 6: Light scattering profiles of lysozyme as a function of different concentrations of glyoxime (a) and dichloroglyoxime (b)

a function of different concentrations of GO and DCGO. Results indicate that in the presence of DCGO, significant aggregation takes place since the folding or unfolding intermediates of proteins, including the molten globule intermediates, expose hydrophobic surfaces and create a tendency to aggregate (Fig. 6).

Thus, lysozyme activity, at low concentrations of GO and DCGO is inhibited by a mechanism that is uncompetitive and in the presence of high concentrations of these reagents, the enzyme displays structural characteristics of a molten globule like state. Several proteins in this state have been shown to be sticky and prone to aggregation (Goto, 1991; Sivaraman *et al*., 1997). However, in the light of the aggregation data obtained, the probability of molten globule state induction for lysozyme is higher in the presence of DCGO

Acknowledgement

The financial support by Tarbiat Modares University is gratefully acknowledged.

References

Ayres, G.H. and Martin J.B., 2002. Spectrophotometric determination of palladium with glyoxime and chloroform extraction. Anal. Chim. Acta, 35: 300-309.

Alizadeh, N., B. Ranjbar and M. Mahmodian, 2003. Electrochemical study of thermodynamics of interaction of lysozyme with sodium dodecyl sulfate in binary ethanol-water mixtures. Colloids and Surfaces A: Physicochem. Eng. Aspects, 212: 211-218.

Ataie Jafari, G., S. Safarian, A. Divsalar, A.A. Saboury, A.A. Moosavi-Movahedi, B. Ranjbar, G. Cristalli and S. Mardanian, 2004. Kinetic and structural analysis of the inhibition of adenosine deaminase by acetaminophen. J. Enzyme Inhibition Med. Chem., 19: 71-78.

Asghari, S.M., K. Khajeh, F. Moradian, B. Ranjbar and H. Naderi-Manesh, 2004. Acid-induced conformational changes in *Bacillus amyloliquesfaciens* α-amylase: Appearance of a molten globule like state. Enzyme Microbial Technol., 39: 51-57.

Bing, C., R. Deen, G.N. Khang, C.L. Sai and L. Kryger, 1999. Chemical accumulation and voltammetric determination of traces of nickel (II) at glassy carbon electrodes modified with dimethyl glyoxime containing polymer coatings. Talanta, 49: 651-659.

Boren, K., P. Andersson, M. Larsson and U. Carlsson, 1999. Characterization of a molten globule state of bovine carbonic anhydrase III: Loss of asymmetrical environment of the aromatic residues has a profound effect on both the near- and far-UV CD spectrum. Biochim Biophys. Acta, 1430: 111-118.

Blake, D.A., P. Chakrabarti, F.M. Hatcher and R.C. Blake, 1994. Enzyme immunoassay to determine heavy metals. in Proceedings of the 17th Annual EPA Conference on Pollutants in the Environment, US EPA Office of Water, EPA Publication 821-R-95-008, pp: 293-316.

Coburn, M.D., 1968. Phenylamine substituted heteroeveles II-furazanes. J. Heterocyclic. Chem., 5: 83.

Copeland, R.A., 2000. Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis. 2nd Edn., A John Willy and Sons, INC.

Cohen, J.S. and O. Jardetzky, 1968. Nuclear magnetic resonance studies of the structure and binding sites of enzymes. II. Spectral assignments and inhibitor binding in hen egg-white lysozyme. Proc. Natl. Acad. Sci. USA., 60: 92-99.

Fujita, Y., Y. Hidaka and Y. Noda, 1995. Thermal stability of alkylated and hydroxyalkylated lysozymes. Thermichim. Acta, 253: 117-125.

Godjayev, N.M., S. Akyuz and L. Ismailova, 1998. The conformational properties of Glu 35 and Asp 52 of lysozyme active center. ARI, 51: 56-60.

Goto, Y., 1991. Role of electrostatic repulsion in the acidic molten globule of cytochrome. C. J. Mol. Biol., 222: 679-686.

Hosseinkhani, S., B. Ranjbar, H. Naderi-Manesh and M. Nemat-Gorgani, 2004. Chemical modification of glucose oxidase: Possible formation of molten globule-like intermediate structure. FEBS Letters, 561: 213-216.

Iwase, H., M. Hirai, S. Arai, S. Mitsuya, S. Shimizu, T. Otomo and M. Furusaka, 1999. Comparison of DMSO-induced denaturation of hen egg-white lysozyme and bovine α-lactalbumine. J. Phys. Chem. Solids, 60: 1379-1381.

Imoto, T., L. Johnson, A. North, D. Phillips and J. Rupley, 1972. Vertebrate Lysozymes, The Enzymes 7, P. Boyer, Academic Press, NY.

Johannesson, H., V. Denisov and B. Halle, 1997. Dimethyl sulfoxide binding to globular proteins: A nuclear magnetic relaxation dispersion study. Protein Sci., 6: 1756-1763.

Khalili, F. and A. Mahasneh, 1986. Preparation and antimicrobial activity of glyoximes. Dirasat Sci., 13: 185-188.

Kristiansen, A., K.J. Varum and H. Grasdalen, 1998. Quantitative studies of the non-productive binding of lysozyme to partially N-acetylated chitosans. Biochim. Biophys. Acta, 1425: 137-150.

Kakanejadifard, A., S.M.F. Farnia and G.R. Najafi, 2004. A modified one step synthesis of diamino glyoxime. Iran. J. Chem. Chem. Eng., Vol. 13.

Kakanejadifard, A., F. Delfani and B. Ranjbar, 2003. Synthesis of N, N' 3, 4-Dialkylamino-1, 2, 5-Oxadiazoles. Iran. J. Chem. Chem. Eng., 22: 13-15.

Kanno, H. and H. Yamamoto, 1995. Dichloroglyoxime. European patent application, 0636605A1.

Khajeh, K., B. Ranjbar, H. Naderi-Manesh, Ebrahim A. Habibi and M. Nemat-Gorgani, 2001. Chemical modification of bacterial α-amylases: Changes in tertiary structures and the effect of additional calcium. Biochim. Biophys. Acta, 1548: 229-237.

Lowry, O.H, N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Lumb, K.J. and C.M. Dobson, 1992. 1H nuclear magnetic resonance studies of the interaction of urea with hen lysozyme. Origins of the conformational change induced in hen lysozyme by N-acetylglucosamine oligosaccharides. J. Mol. Biol., 227: 9-14.

Luzar, A. and J. Chandler, 1993. Structure and hydrogen bond dynamics of water-dimethyl sulfoxide mixtures by computer simulations. J. Chem. Phys., 98: 8160.

Material Safety Data Sheet, Dimethyl Glyoxime, ACC # 07225.

Matagne, A. and C.M. Dobson, 1998. The folding process of hen lysozyme: A perspective from the 'new view'. CMLS, Cell. Mol. Life Sci., 54: 363-371.

Ovchinnikov, Y.A., 1996. Bioorganic Chemistry; Proteins and Peptids. Moscow, Nauka, pp: 158-163.

Protasevich, I., B. Ranjbar, V. Lobachov, A. Makarov, R. Gilli, C. Briand, D. Lafitte and J. Haiech, 1997. Conformation and thermal denaturation of apocalmodulin: Role of electrostatic mutations. Biochemistry, 36: 2017-2024.

Rajaraman, K., B. Raman and C.M. Rao, 1996. Molten-globule state of carbonic anhydrase binds to the chaperone-like α-crytallin. J. Biol. Chem., 271: 27595-27600.

Schomburg, D. and M. Salzmann, 1991. Lysozyme. Enzyme Handbook, 4: 1-11.

Smith, L.J., M.J. Sutcliffe, C. Redfield and C.M. Dobson, 1993. Structure of hen lysozyme in solution. J. Mol. Biol., 229: 930-944.

Schwalbe, H., S.B. Grimshaw, A. Spencer, M. Buck, J. Boyd, C.M. Dobson, C. Redfield and L.J. Smith, 2001. A refined solution structure of hen lysozyme determined using residual dipolar coupling data. Protein science, 10: 677-688.

Saboury, A.A., A. Divsalar, G. Ataie Jafari, A.A. Moosavi-Movahedi, M.R. Housaindokht and G.H. Hakimelahi, 2002. A product inhibition study on adenosine deaminase by spectroscopy and calorimetry. J. Biochem. Mol. Biol., 35: 302-305.

Schippers, P.H. and H.P.J.M. Dekkers, 1981. Direct determination of absolute circular dichroism data and calibration of commertial instrument. Anal. Chem., 53: 778-788.

Sivaraman, T., T.K.S. Kumar, G. Jayaraman and C. Yu, 1997. The mechanism of 2,2,2-trichloroacetic acid-induced protein precipitation. J. Protein Chem., 16: 291-297.

Soper, A.K. and A. Luzar, 1992. A neutron diffraction study of dimethyl sulfoxide-water mixtures. J. chem. Phys., 97: 1320.

Takakuwa, T., T. Konno and H. Meguro, 1985. A new standard substance for calibration of circular dichroism: Ammonium d-10-camphorsulfonate. Anal. Sci., 1: 215-225.

Willer, R.L. and D.W. Moore, 1985. Synthesis and chemistry of some furazans. J. Org. Chem., 50, 5123.

Worthington, V., 1993. Lysozyme. Worthington enzyme manual, Worthington biochemical corporation, 250-255.

Zaks, A. and A.J. Russell, 1988. Enzymes in organic-solvents: Properties and applications. J. Biotechnol., 8: 259-270.