J. Enzyme Inhibition, 2001, Vol. 16, pp. 65–80
Reprints available directly from the publisher
Photocopying permitted by license only





# Effect of Cadmium Acetate on the Conformation of Lysozyme: Functional Implications

ROSA OLMO, M. DOLORES BLANCO, JESÚS M. SOCORRO, JUAN A. MARTÍN and JOSÉ MARÍA TEIJÓN*

*Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense de Madrid, 28040 Madrid (Spain)*

*(Received 9 February 2000)*

**Structural variations of lysozyme as a consequence of its interaction with $CdAc_2$, as well as the implications on the protein functionality have been studied. Variations in the conformation of the macromolecule are seen, however these changes are not reflected on the secondary structure. The interaction of the salt with the polypeptide chain is weak and thermodynamically unfavourable. Molecular aggregates (dimer forms) are observed at the highest salt concentrations. This interaction causes an inhibitory effect on lysozyme, the activity loss being 50% at the highest salt concentration studied. The inhibition is of mixed type with an uncompetitive component. Thus cadmium does not bind to the active site of the enzyme which is in accordance with the not very large activity loss observed. The substrate inhibition of lysozyme is favoured in the presence of the salt, so interaction with the macromolecule is at low affinity sites.**

*Keywords*: Lysozyme, Cadmium, Preferential interactions, Viscosity, Secondary structure, Enzyme inhibition, Kinetics

## INTRODUCTION

The structure of macromolecules can be modified by some salts.[1–3] When macromolecules are proteins, salts can modify their structure, and conformational changes are originated which can cause modifications in protein function. The study of these structural changes can be carried out through a knowledge of the sort of interactions that such substances establish with the protein, and the thermodynamic state of the interaction.[4,5] Lysozyme is one of the better known proteins and is a cationic protein with low molecular mass (14 000 Da). Lysozyme is formed by a single polypeptide chain of 129 amino acids,[6] and four disulphide bridges, which are important to renaturation,[7] have been reported in the protein structure.[8,9] This protein has a defensive function in the organism due to its antimicrobial nature. The function of lysozyme is essential as the first antimicrobial barrier against organisms since it breaks $\beta(1–4)$ links between N-acetylmuramic and N-acetylglucosamin, which are components of the bacterial wall. Antimetastatic action and inmunopotentiation have also been observed.[10,11] Thus, any agent that can interact with this protein so decreasing its activity would have a considerable effect on

*Corresponding author. Tel.: 91 394 14 47. Fax: 91 394 16 91. E-mail: jmt77255@eucmax.sim.ucm.es

the body's resistance to bacterial attack. Cadmium has a large influence on protein activity, being in almost all cases an inhibitor,[12–14] although some times it can be an activator.[15,16]

The aim of this work has been to study the interaction of cadmium acetate with lysozyme. The structural interaction has been characterized by studying possible conformational modification of protein tertiary structure using viscometry and densimetry, secondary structure employing circular dichroism and spectophotometry UV/V, and protein aggregation with gel filtration chromatography. The effect of the lysozyme–cadmium salt interaction on protein function was evaluated through enzyme activity measurements.

## MATERIALS AND METHODS

### Materials

The macromolecule used in this study, lysozyme of hen egg white, (E.C. 3.2.1.17), [Sigma], was dialysed against water and then lyophilized. Cadmium acetate ($CdAc_2$), $KH_2PO_4$ and $K_2HPO_4$ [Panreac] were used as received. Distilled and deionized water was employed.

### Solutions

The solvent was a binary mixture of water/metal acetate. Cadmium acetate concentrations between 2.5 mM and 15 mM were used. The protein solution was made gravimetrically; specific volumes of the binary mixtures (water/metal acetate) at 298 K were added to accurate weights of protein.

Potassium phosphate buffer (0.05 M, pH = 7.0) and *Micrococcus luteus* [Sigma] were prepared for the spectrophotometric assays. *Micrococcus luteus* was triturated with phosphate buffer in an agate mortar and the suspension was diluted such that the absorbance was 0.750±0.050 at 450 nm. (measured against air; $d = 1$ cm). Lysozyme was prepared immediately before measurement and was diluted 1:250 with repurified water (control) or water/$CdAc_2$ (1:3) mixture.

Experiments were carried out in triplicate.

### Instrumentation and Techniques

#### *Viscometry*

Viscosity measurements were carried out in a modified Ubbelohde suspended level viscometer, immersed in a thermostatically controlled bath at 298 K[17] ± 0.01 K. Flow times were reproducible within ±0.03 s.

The intrinsic viscosity, $[\eta]$, was determined as described by Fuoss and Strauss:[18]

$$C/\eta_{sp} = 1/A + (B\ C^{1/2})/A \qquad (1)$$

where A and B are constants, $\eta_{sp}$ is the specific viscosity, and C is the concentration of protein (1%–0.5% w/v). Straight lines were obtained when $(C/\eta_{sp})$ was plotted against $C^{1/2}$. Extrapolation to $C = 0$ gives $1/A$ which represents the intrinsic viscosity.

#### *Densimetry*

The density of the solvents and solutions was measured using pycnometers (4–5 ml) previously calibrated. They were syringe-filled and put in a thermostatically controlled bath at 298 K to reach temperature equilibrium (10 min). The filled pycnometers were weighed in a Sartorius balance, with 0.1 mg precision.

In these three component systems the Scatchard[19] and Stockmayer[20] notation was followed: (1) water, (2) protein and (3) salt.

The partial specific volume of lysozyme at constant concentration of metal ($\nu_2$) and constant electrochemical potential ($\phi_2$) in the solution were determined from density values of solvent ($\rho_0$) and solutions ($\rho$) as follows:[21]

$$\nu_2 = [1 - (\partial\rho/\partial g_2)_{T,P,m_3}]/\rho_0 \qquad (2)$$

$$\phi_2 = [1 - (\partial\rho/\partial g_2)_{T,P,\mu_3}]/\rho_0 \qquad (3)$$

Here $(\partial\rho/\partial g_2)_{T,P,m_3}$ is the change in the density of the solution compared to the protein concentration ($g_2$) at constant temperature (T), pressure (P), and mass of metal ($m_3$) and $(\partial\rho/\partial g_2)_{T,P,\mu_3}$ at electrochemical potential ($\mu_3$). Six different concentrations of lysozyme between 0.2% to 1% w/v were used.

### *Equilibrium Dialysis*

Dialysis was carried out using dialysis tubing to obtain the density values of the solution at constant electrochemical potential of metal ($\mu_3$), which are required to determine the preferential adsorption coefficient ($\lambda$).[22] This parameter was determined using the Kratochvill *et al.* equation:[23]

$$\lambda = [(\partial\rho/\partial g_2)_{T,P,\mu_3} - (\partial\rho/\partial g_2)_{T,P,m_3}]/(\partial\rho/\partial g_3)_{T,P,m_2} \quad (4)$$

Here, $(\partial\rho/\partial g_2)_{T,P,\mu_3}$ is the change in density with the protein concentration at constant electrochemical potential ($\mu_3$) and $(\partial\rho/\partial g_3)_{T,P,m_2}$ is the change in the density of the solutions with respect to the metal concentration at constant temperature (T), pressure (P) and mass of protein ($m_2$).

It is possible to consider that $\lambda$ can be given as,

$$\lambda = (\partial g_3/\partial g_2)_{T,\mu_1,\mu_3},$$

where $g_i$ is the weight of component $i$ in grams; for diluted solutions the error is not significant.[24]

The preferential hydration, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ can be obtained from the equation:[25,26]

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -(1/g_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (5)$$

The preferential interaction $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, shows the relationship between the mutual perturbation of the electrochemical potentials of components 2 and 3[27,28] as follows:

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,P,m_2} \quad (6)$$

The term $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ can be determined from the following expression:[29]

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (7)$$

where $M_i$ and $m_i$ are the molecular weight and molality of component $i$ respectively and the self-interaction term $(\partial\mu_3/\partial m_3)_{T,P,m_2}$ can be calculated from:

$$(\partial\mu_3/\partial m_3)_{T,P,m_2} = nRT/m_3 + nRT(\partial \ln\gamma\pm/\partial m_3)_{T,P,m_2} \quad (8)$$

where $n = 3$ for $CdAc_2$, R is the gas constant and $\gamma\pm$ is the ionic activity coefficient of the salt.

Starting from the preferential interaction parameter, it is possible to determine the transfer free energy, $\Delta\mu_2$, of the protein according to the equation:[30,31]

$$\Delta\mu_2 = \mu_{2,m_3} - \mu_{2,w} = \int (\partial\mu_2/\partial m_3)_{T,P,m_2}\, dm_3 \quad (9)$$

where $\mu$, $[(\mu_{2,m_3}); (\mu_{2,w})]$, reflects the relative affinities of salt and water for the protein at the given solvent composition, and the subscript w refers to water.

### *Solubility Measurements*

Protein solubility was determined by dissolving the protein in the desired solvent and adding protein until the solution turned turbid or viscous. The mixture was then dialyzed against several changes of the same solvent for ca. 24 h at 298 K. Afterwards the protein (solution plus precipitate) was removed from dialysis and centrifuged for 10 min in a Biofuge B (Heraeus) at 40,000 × g. The concentration of protein in the supernatant defined as the solubility was measured spectrophotometrically by diluting it with the dialyzing solvent. Protein concentration was determined spectrophotometrically on a Unicam Model 8700 instrument using Bradford's method.[32]

### *Ion Selective Electrode*

Binding experiments were done with an ion selective electrode for cadmium [Metrohm

6.0726.100] connected to a pHmeter/voltameter (Metrohm 654) which registers variation in potential.

The electrode was calibrated by placing it in a vessel containing 70 ml of a solution of $CdAc_2$ 0.1 M at a constant stirring rate and 298 K. At intervals of 2–3 min, aliquots of cadmium salt were added to obtain a final concentration of 10 mM. The electromotive force was measured after each metal addition, the accuracy being ±0.01 mV. The potentiometric study of the protein–metal interaction was carried out in a similar manner except that the starting solution was a 70 μM lysozyme solution. The concentration of free ligand and bound ligand and binding constants were calculated using Nerst's equation.

### *UV, CD Spectral Measurements*

UV spectra of lysozyme in different solutions were recorded with a Unicam 8700 spectrophotometer from 240 to 320 nm (lcm) using a protein concentration of about 1% (W/V).

CD spectra were obtained in a Jobin Yvon Mark III dichrograph fitted with a 250 W xenon lamp. The spectra were recorded at 0.5 nm/s scanning speed. The protein solutions were analyzed in 1 cm optical path cells in the far-ultraviolet (below 250 nm) region. CD results were expressed as mean residue weight ellipticities, in units of degree $\cdot$ cm$^2$ $\cdot$ dmol$^{-1}$. Calculation of the secondary structure of the protein solution was performed by the method of Convex Constraints Analysis (CCA).[33]

### *Aggregation Studies*

Size exclusion chromatography was carried out to determine the formation of protein aggregates in the solvent medium. A column (Pharmacia, 40 × 1.8 cm I.D) with Sephadex G-75 (Pharmacia) as stationary phase was employed at 4 °C. The column was calibrated using a standard protein Kit (Sigma MW-GF-70). Fractions (3 ml) were collected and their absorbance 280 nm determined in a UV/Vis spectrophotometer (Unicam 8700).

### *Activity Study*

A 2.95 ml volume of *Micrococcus luteus* was pipetted into a cuvette and the reaction was started with 0.05 ml of lysozyme solution. Changes in absorbance were measured with at 298 K and the decrease in turbidity of the suspension was recorded at 450 nm. The parameter ΔA/min was calculated from the linear part of the curve. The activity was calculated using the equation:[34] Activity = (1000/0.05) $\cdot$ (ΔA/min) (units/ml lysozyme solution).

### *Enzyme Kinetics*

Vmax and the Michaelis constant (Km) were determined using concentrations of *Micrococcus luteus* between 1–6 $\cdot$ 10$^{-6}$ M in the activity assays and a Lineweaver-Burk plot.[35] The type of inhibition and the inhibitor constant were determined in the presence of $CdAc_2$.

## RESULTS AND DISCUSSION

A ternary system constituted by a solvent, a macromolecule and a substance, which could cause a conformational change, needs to be studied in order to determine the relationships that are established between them for the purpose of obtaining information about the possible structural variations of the protein. Moreover, those conformational changes of the protein must be related to possible changes in its activity, which would affect the protein function.

The preferential adsorption or solvation coefficient ($\lambda$) indicates which of the two components of the solvent mixture (water (1) or salt (3)) preferentially interacts with the protein structure (2), but does not imply that the other component of the mixture is not interacting with the protein. Thus, if the preferential solvation coefficient

exhibits a negative value, the component of the solvent mixture preferentially bound to the protein will be the salt (Figure 1). On the other hand, the preferential hydration parameter is a measurement of the balance between the total hydratation and the total salt bound to protein.[36]

The preferential adsorption coefficient ($\lambda$) of lysozyme in the presence of different concentrations of cadmium acetate is shown in Figure 2. The parameter shows negative values, thus the salt is preferentially adsorbed in the inside part of the macromolecule.[37–39] Preferential hydration parameter values (Figure 2) corroborate this behaviour because of the positive values of this parameter indicating that the water is found in the immediate domain of the macromolecule. The same behaviour is exhibited for lysozyme and bovine serum albumin (BSA) with the acetates of sodium, barium, magnesium and calcium, that indicate a salt deficiency in the protein immediate domain with regard to its concentration in the solution, thus the hydration shows positive values.[26]

Conformational variations due to fluctuations in the molecule's degree of solvation have been described for lysozyme. These fluctuations are accompanied by changes in the vibrational freedom of the molecular structure which can cause structural modifications.[40,41,33] The large values for the preferential hydration are similar to those observed for albumin in different saline systems[42,43] and for lysozyme in $AlCl_3$,[1] showing salt exclusion from the protein immediate domain.

The existence of conformational changes in macromolecules can be determined through the intrinsic viscosity parameter, since if a conformational change takes place a variation of this parameter will be observed, whereas if the change does not occur the intrinsic viscosity of the protein will increase in a linear way with metal concentration.[44,2,3]

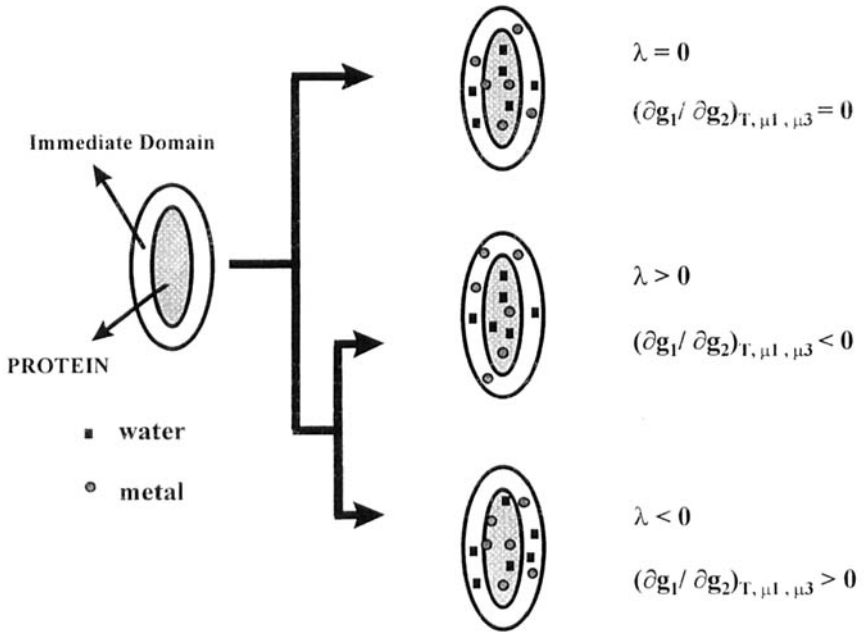


FIGURE 1 Preferential interactions in a three component system [water(1)/protein(2)/salt(3)]. The sign of the preferential adsorption ($\lambda$) and preferential hydration [$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$] parameters is shown. Thus, $\lambda = 0$ means there are the same number of water molecules in the inside part of the protein as in the immediate domain. When $\lambda > 0$ water is mainly in the inside part of the protein, and if $\lambda < 0$ it is mainly in its immediate domain.

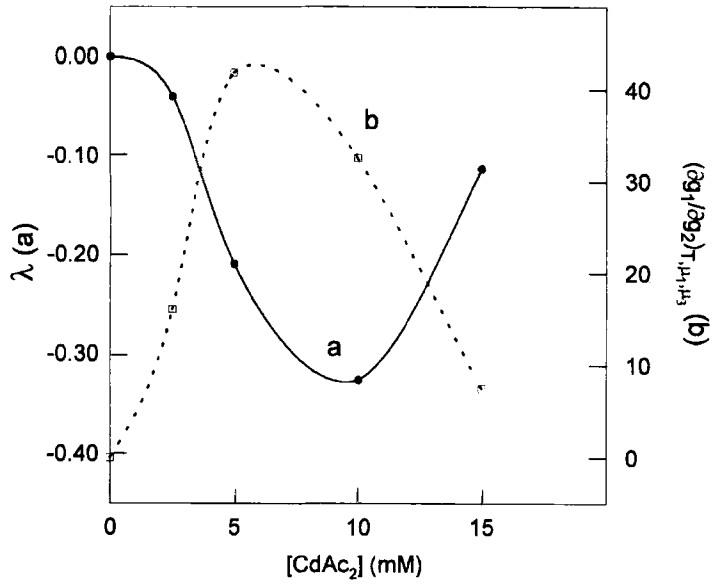


FIGURE 2 Dependence of preferential adsorption coefficient ($\lambda$) (a) and preferential hydration $[(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}]$ (b) of lysozyme with the concentration of cadmium acetate ($CdAc_2$).

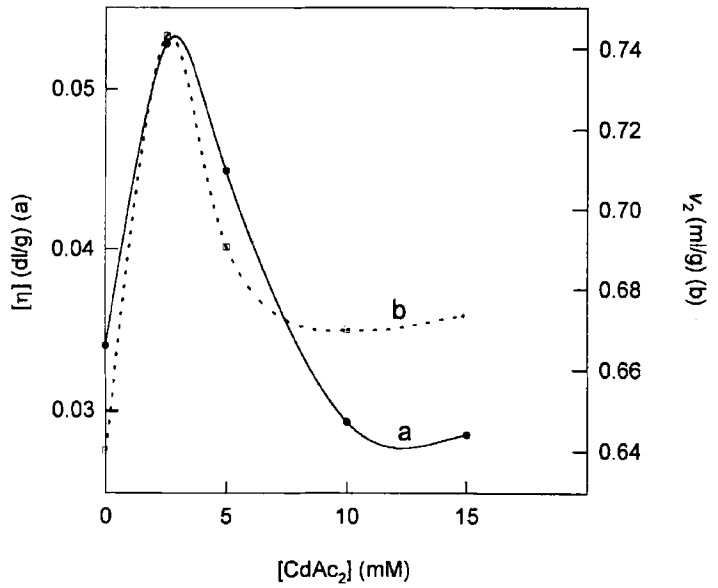


FIGURE 3 Variation of intrinsic viscosity, $[\eta]$, (a) and partial specific volume, $v_2$, (b) of lysozyme as a function of the concentration of cadmium acetate ($CdAc_2$).

The intrinsic viscosity of lysozyme as a function of $CdAc_2$ concentration is shown in Figure 3. Variation of the parameter is observed with increased salt addition, suggesting that conformational transitions in the protein take place. The changes in intrinsic viscosity can be explained on the basis that the increase in cadmium concentration induces an expansion of the poly-

peptide chain due to electrostatic repulsions between the salt and the protein, which lead an initial increase of viscosity. Further increases in salt concentration neutralize those electrostatic repulsions, which leads to a decrease of the intrinsic viscosity. The same behaviour has been observed for the complex formed between SDS and RCAM-BSA (reduced-carboxyamidomethylated bovine serum albumin).[45] At 2.5 mM salt there was an expansion of the protein structure and the maximum value of the intrinsic viscosity was obtained, probably due to interactions between the salt and the protein charged groups. A conformational change could originate at this salt concentration which allows more ions to interact with the protein, as the more negative values of the preferential adsorption parameter indicate (Figure 2). This interaction leads to an expansion of the protein that is reflected in the maximum of both intrinsic viscosity and partial specific volume at constant metal concentration, $v_2$ (Figure 3) at that salt concentration. The last parameter is correlated with $\lambda$ because it depends on the establishment of preferential interactions in the solvent system. This is not the case of $\phi_2$, calculated at equilibrium (at constant electrochemical potential). So there is a clear relationship between conformational changes (observed at 2.5 mM) and preferential interactions. The same correlation has been described for lysozyme with copper (II),[46] cobalt (II),[47] lead (II),[41] zinc (II),[48] mercury (II),[49] aluminium (III)[1] and lithium (I).[50]

In order to determine whether the interaction of lysozyme with cadmium acetate is thermodynamically favourable or unfavourable, the preferential interaction parameter and the transfer free energy were determined.

Starting with the preferential adsorption parameter, the preferential interaction parameter can be obtained. This parameter is a measurement of the change in thermodynamic stability change of the systems which is induced by a variation in the solvent composition at a determined composition; thus it reflects the relative affinities of salt and water for the protein at a determined solvent composition.[51]

The transfer free energy indicates the affinity difference of the protein for a determined solvent system and for pure water. This parameter is totally independent of both interaction mechanisms with solvent components and the protein state whether natural or denaturalized. The parameter can be obtained by integration of the polynomial function at which the preferential interaction parameter variation with the salt concentration is fitted. Thus:

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -3{,}69\,10^3 + 2{,}79\,10^6\,m_3 - 3{,}19\,10^8\,m_3^2 + 1{,}1\,10^{10}\,m_3^3 \quad (10)$$

The preferential interaction parameter and the transfer free energy are shown in Figure 4. In this case, the integration limits have been taken as the smaller salt concentration studied ($m_3 =$ 2.5 mM) for the lower one and each one of the next salt concentration used for the higher limit. Due to no linear dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ with respect to $m_3$ being observed, the function behaviour between 0 and 2.5 mM cannot be known, so the smaller salt concentration studied is used as the lower integration limit. The preferential interaction parameter exhibits positive values at all salt concentrations studied indicating that there is a preferential interaction with cadmium. At 5 mM salt a maximum in the parameter is observed, thus at this salt concentration the protein–salt interaction is thermodynamically more unfavourable. The transfer free energy parameter shows the protein solution is thermodynamically favoured in water and it is unfavourable in the presence of the salt. This agrees with the hydration parameter; hydration of the immediate domain of the protein (Figure 2) is maximum at this salt concentration.

The solubility, determined according to Arakawa *et al.*,[51] can be related with the transfer free energy, which allows a knowledge of the value of this parameter in the precipitated state as well

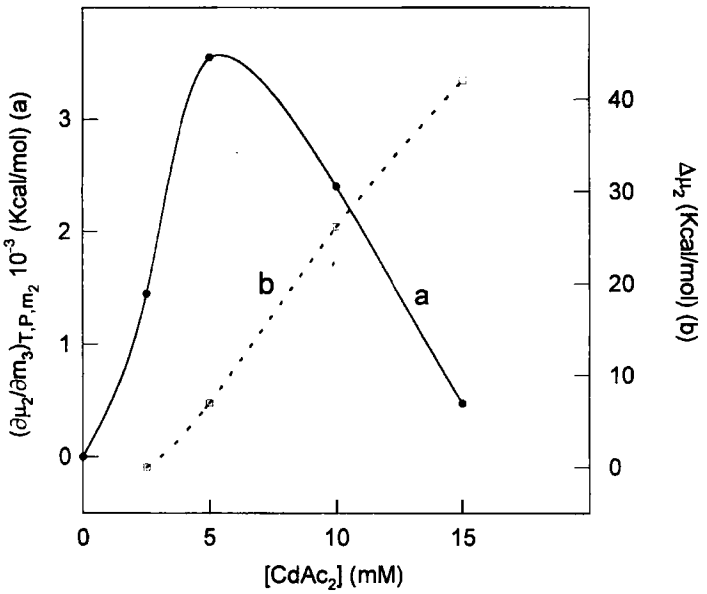


FIGURE 4 Preferential interaction parameter $[(\partial\mu_2/\partial m_3)_{T,P,m_2}]$ (a) and tranfer free energy from water to salt solution ($\Delta\mu_2$) (b) of lysozyme as a function of cadmium acetate concentration ($CdAc_2$).

as in the solution state. The transfer free energy from water to saline solution can be related to the protein solubility in such a medium according to the equation:

$$-\ln(S_{2,m_3}/S_{2,water}) = \Delta\mu_2/RT \qquad (11)$$

where $S_2$ is the protein solubility in concentration units. From this equation it is possible to obtain:

$$-RT[\ln S_{2,m_3} - \ln S_{2,water}] = \Delta\mu_2^1 - \Delta\mu_2^s \qquad (12)$$

where 1 and s refer to the liquid and solid phases, respectively. The transfer free energy of the macromolecule in the precipitated state from an aqueous medium to a saline one ($\Delta\mu_2^s$) can be calculated using the latter equation starting from both experimental solubility and preferential interaction in solution data. If the salt addition decreases the protein solubility, the salt will be a salting-out agent, the opposite behaviour leads to a salting-in agent.

The experimental solubility data are shown in Table I. The lysozyme solubility is not substantially modified in the presence of different concentrations of cadmium salt.

Another factor used to classify a salt as a salting-out or salting-in agent is the value of the transfer free energy of the protein in the precipitated state ($\Delta\mu_2^s$) and in the solution state ($\Delta\mu_2^1$). In salting-out systems the interaction of the protein with the solvent has to be less unfavourable in the precipitated state than in solution. This means that $\Delta\mu_2^s < \Delta\mu_2^1$ for the salting-out agents. The opposite behaviour is characteristic of the salting-in agents. The salting-in or salting-out behaviour of some agents for a protein depends on the contribution of both the anion and the cation, because each of them can behave as salting-out or salting-in agent, the general behaviour of the salt being the balance of these effects. For example, it has been observed that $MgCl_2$ has no effect on the solubility of the ATGEE (acetyltetraglycine ethyl ester) model peptide, whereas NaCl produces a salting-out effect on it.[52,53] This different behaviour is due to the differences between $Mg^{2+}$ and $Na^+$. One possibility is that the salting-out effect of both $Mg^{2+}$ and $Cl^-$ was zero, thus the salting-out effect of the salt is due to $Na^+$. Another

TABLE I Partial specific volume at constant electrochemical potential ($\phi_2$), preferential binding parameter $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, solubility, transfer free energy in the precipitate state ($\Delta\mu_2^s$) and in solution ($\Delta\mu_2^l$), molecular weight of lysozyme (M), inhibiton constant for substrate, Michaelis constant (Km) and maximum rate (Vmax) of lysozyme in the presence of cadmium acetate ($CdAc_2$)

| [$CdAc_2$] mM | $\phi_2\ 10^2$ (ml/g) | $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ | Solubility $10^4$ (g prot/g $H_2O$) | $\Delta\mu_2^l$ (Kcal/mol) | $\Delta\mu_2^s$ (Kcal/mol) | $\Delta\mu_2^l - \Delta\mu_2^s$ (Kcal/mol) | M | $K_i$ (A) substrate | $K_M$ (A) | Vmax (A/min) |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | – | – | $1.1 \pm 0.3$ | – | – | – | 14000 | $0.97 \pm 0.04$ | $1.09 \pm 0.09$ | $0.096 \pm 0.007$ |
| 2.5 | $76.18 \pm 2.01$ | $-2.1720 \pm 0.03$ | $0.9 \pm 0.5$ | – | – | – | 14000 | $0.84 \pm 0.10$ | $0.69 \pm 0.06$ | $0.073 \pm 0.002$ |
| 5 | $78.77 \pm 0.19$ | $-11.3193 \pm 1.02$ | $1.3 \pm 0.2$ | $6.948 \pm 0.025$ | $7.046 \pm 0.037$ | $-0.098 \pm 0.012$ | 14000 | $0.83 \pm 0.05$ | $0.62 \pm 0.04$ | $0.066 \pm 0.006$ |
| 10 | $82.07 \pm 1.03$ | $-17.6246 \pm 3.41$ | $1.1 \pm 0.1$ | $26.147 \pm 0.044$ | $26.147 \pm 0.035$ | $0.000 \pm 0.009$ | 20106 | $0.88 \pm 0.09$ | $0.56 \pm 0.12$ | $0.040 \pm 0.008$ |
| 15 | $81.54 \pm 3.30$ | $-6.0784 \pm 0.59$ | $1.4 \pm 0.5$ | $42.040 \pm 0.030$ | $42.183 \pm 0.055$ | $-0.143 \pm 0.025$ | 20106 | $0.90 \pm 0.12$ | $0.47 \pm 0.06$ | $0.042 \pm 0.001$ |

possibility is that both $Na^+$ and $Cl^-$ have a salting-out effect; in this case the salting-in effect has to be due to the $Mg^{2+}$ cation, and this effect is balanced by the opposite one due to $Cl^-$ anion:[29]

Acetate anion has been described as a salting-out agent when it forms magnesium or calcium salts.[29] When $Mg^{2+}$ is a part of $MgCl_2$ salt, this cation behaves as a salting-in agent, thus in the magnesium acetate salt the acetate anion contribution can be considered to be salting-out.

The cadmium acetate salt shows two different behaviours, at 5 and 15 mM salt it behaves as a salting-in agent ($\Delta\mu_2^s < \Delta\mu_2^1$), however at 10 mM, $\Delta\mu_2^s = \Delta\mu_2^1$. Besides, the solubility value does not give clear information about the salt behaviour at 10 mM salt, because it is the same as in water. Therefore cadmium acetate would behave as a salting-in agent for lysozyme, this behaviour being due to the cadmium cation, and at 10 mM salt a balance of the cation and anion effects would take place.

Effects on different parameters caused by cadmium salt can be due either to the interaction of the salt with some amino acid residues or to conformational changes induced by the salt. In order to discriminate between both possibilities, the effect of the solvent system used (water/$CdAc_2$) on the lysozyme conformation using CD has been studied.

Circular dichroism spectra, determined in the far UV region, of lysozyme in water and in different concentrations of cadmium salt are shown in Figure 5. The lysozyme spectrum in water was fitted using the CCA method,[33] which

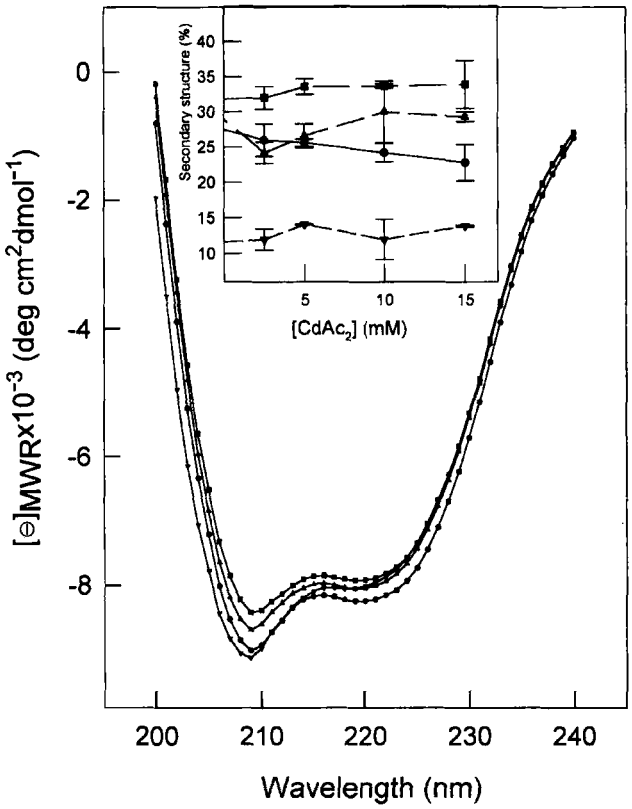


FIGURE 5 Circular dichroism spectrum in far UV region for lysozyme in the presence (●) 0 mM, (■) 5 mM, (▲) 10 mM and (▼) 15 mM of cadmium acetate. Inset: Secondary structure of lysozyme from circular dichroism spectra in far UV as a function of cadmium acetate concentration: (●) α-helix (a), (■) β-sheet (b), (▲) β-turn (c) and (▼) random coil (d). Lysozyme spectra have been fitted using CCA method.

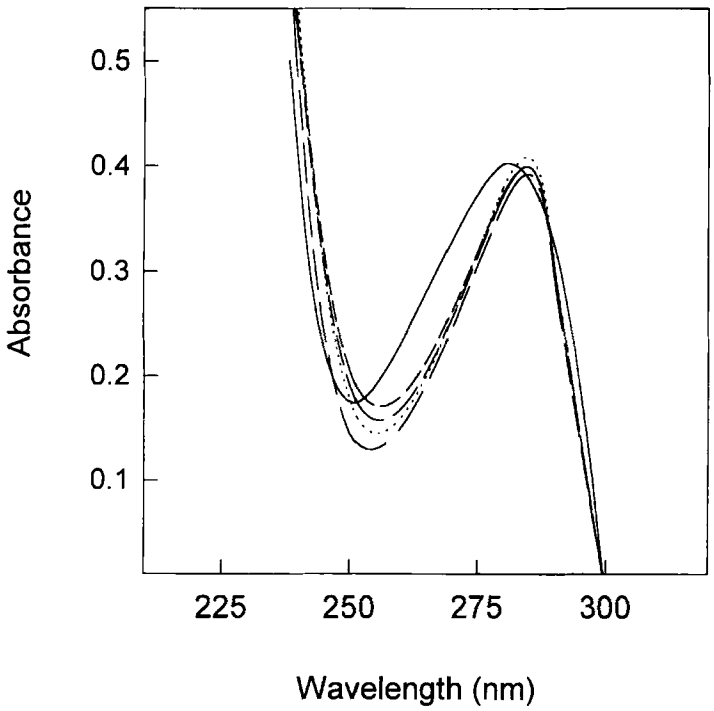


FIGURE 6 UV/Vis spectrum of lysozyme in the presence of (—) 0 mM, (—–) 2.5 mM, (––) 5 mM, (– – –) 10 mM and (. . .) 15 mM of cadmium acetate.

allows determination of the secondary structure composition: 27% $\alpha$-helix, 32% $\beta$-sheet, 12% $\beta$-turn and 29% random coil. These results are in accordance with those obtained by Kuwajima, *et al.* using different fitting systems.[54] Different percentages of secondary structure at each of the salt concentration studied are shown in the inset of Figure 5. The effect of the cadmium salt on the lysozyme secondary structure is not very significant. At smaller salt concentrations the effect of cadmium is almost inappreciable; at the highest salt concentration a very small conformational change is observed, which originates from a small increase in $\beta$ structure content and a decrease of the $\alpha$-helix content, thus the secondary structure obtained is: 21% $\alpha$-helix, 36% $\beta$-sheet, 13% $\beta$-turn and 30% random coil.

In order to determine whether the observed changes are due to the modification of the microenvironment of the aromatic residues, UV absorption spectra of lysozyme in both the presence and the absence of cadmium salt were examined (Figure 6). In the presence of salt, the spectrum does not show essential modifications with regard to the control which means that the aromatic residues microenvironment is not modified due to the presence of salt and, besides, direct interaction seems not exist between cadmium and these aminoacids.

The light decrease observed in the ellipticity values at larger salt concentrations (Figure 5) could be explained if protein aggregation takes place, since the effective concentration of the chromophores in solution decreases. To determine the formation of molecular aggregates due to the interaction with the salt, size exclusion chromatography was carried out (Figure 7). In view of the molecular masses obtained (Table I), lysozyme molecular aggregates (dimers) could be formed at the largest salt concentrations studied. This agrees with the increase in the partial specific volume at constant electrochemical

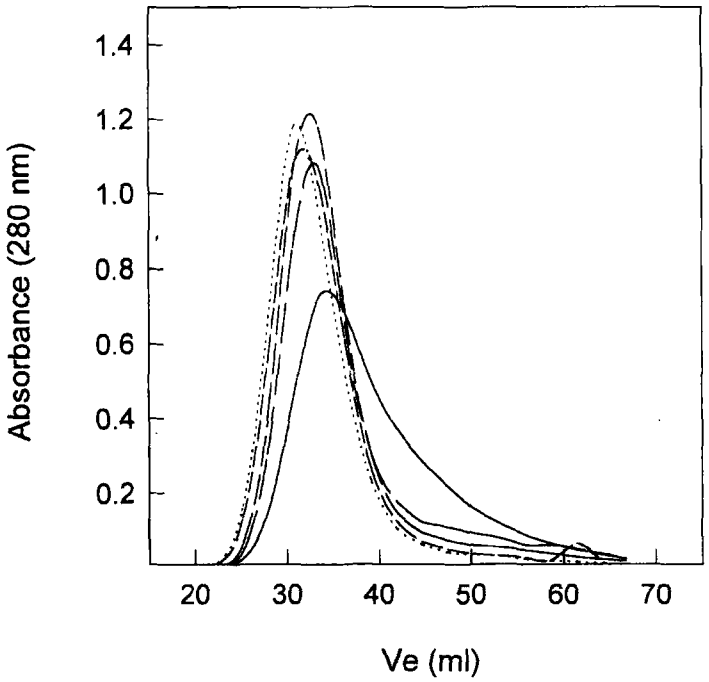


FIGURE 7 Elution profile from Sephadex G-75 of lysozyme in the presence of (—) 0 mM, (——) 2.5 mM, (– –) 5 mM, (- - -) 10 mM and (. . .) 15 mM of cadmium acetate.

potential, $\phi_2$ (Table I), which reflects an increase in the size of macromolecule, independent of preferential interactions (Table I). The lysozyme molecular mass in the presence of 10 and 15 mM cadmium acetate does not become a dimer; this can be due to the monomer and the dimer being in rapid equilibrium so that only one wide peak is obtained.[55]

Potentiometric studies show that the interaction between lysozyme and cadmium acetate is of a weak electrostatic type, since the electromotive force induced by cadmium is almost the same in the absence and in the presence of lysozyme. Thus the concentration of free ligand as well as of bound ligand cannot be determined and binding parameters cannot be calculated. However, when the interaction of a cation with a protein is stronger this method allows determination of the concentrations of free ligand and bound ligand; the interaction studies of lysozyme with mercury using potentiometry lead to a knowledge of the binding type of such cation and protein.[56]

With the purpose of analyzing the influence of these structural modifications on protein functionality, lysozyme activity in the presence of salt was determined (Figure 8). The cadmium acetate causes an inhibitory effect on lysozyme at all salt concentrations studied. This activity loss is almost 50% that of a control and the largest activity decrease takes place at the initial salt concentration used and activity is relatively constant with increasing salt concentrations.

The decrease in the enzymatic activity of lysozyme in the presence of $ZnAc_2$ and $HgAc_2$[49] is greater than in the presence of $CdAc_2$. Thus, at 15 mM of $ZnAc_2$ lysozyme activity is 35% that of a control, and at 5 mM of $HgAc_2$ no activity of the enzyme is detected. Peseck and Schneider[48] have shown that $Hg^{2+}$ binds to Asp-52 of the lysozyme active site, which would explain the dramatic decrease in activity. However, the

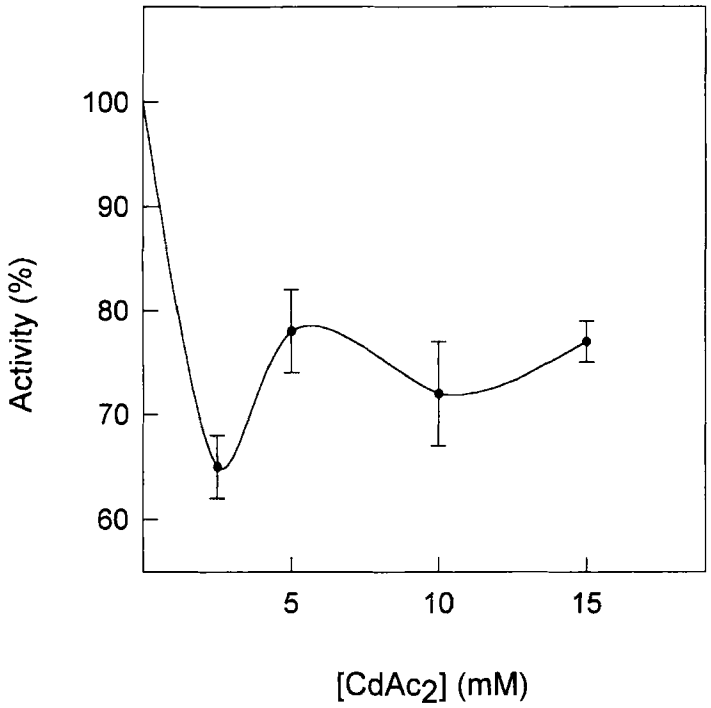


FIGURE 8 Effect of cadmium acetate concentration on lysozyme activity.



smaller decrease in activity caused by $Cd^{2+}$ would indicate that this cation does not interact with the residues of the active site of the enzyme, although it can originate distortion in that site but to a lesser extent than $Zn^{2+}$.

With the aim of determining the mechanism by which the salt produces a decrease in lysozyme activity, enzyme kinetic studies were carried out, and the initial rate of the reaction at different concentrations of substrate was determined. An example of the variation of the initial rate with the substrate concentration in aqueous medium as well as in the presence of $CdAc_2$ is shown in Figure 9. In this plot a maximum of rate can be observed, which indicates that lysozyme exhibits substrate inhibition at high concentrations of *Micrococcus luteus*.[57,58] This kind of inhibition has been observed with another enzymes, i.e. fructose bisphosphatase.[59] Starting from the plot of the inverse of the initial rate (1/V) versus *Micrococcus luteus* concentration ([A]) a curve with a minimum is obtained, from which the points corresponding to high substrate concentrations can be fitted to a straight line, whose intercept on the x-axis is the substrate inhibition constant. Values of this constant (Table I) indicate that the cadmium salt favours the inhibitory effect that the substrate causes. The effect is very minor at the highest concentrations. The cadmium acetate causes a distortion on the protein affecting its low affinity sites, which facilitates substrate binding to these sites so that the inhibitory effect of the substrate increases.

At low substrate concentrations, lysozyme behaves according to Michaelis-Menten kinetics, thus Km and Vmax can be determined. In Table I these parameters, obtained from Lineweaver-Burk plot (Figure 10), are given. Cadmium acetate gives an inhibition of mixed type on lysozyme which means that a non-competitive component exists. The other component of the

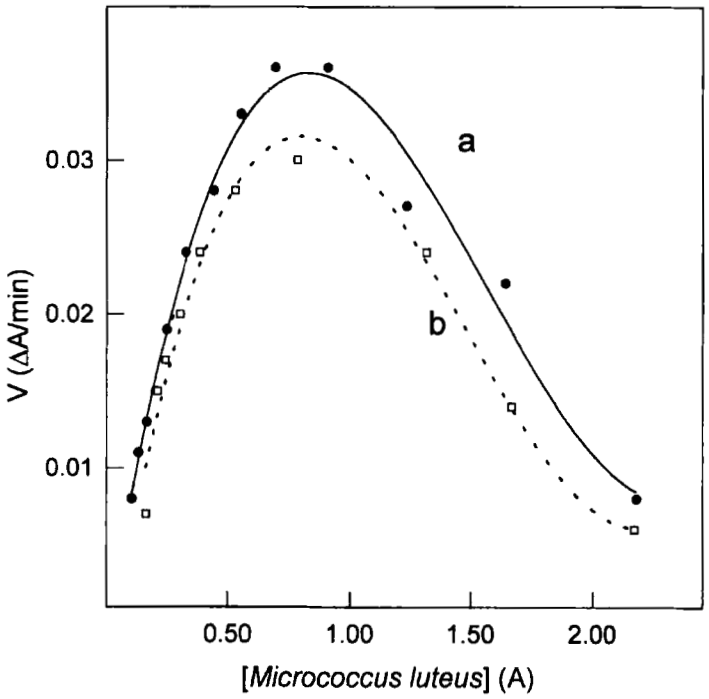


FIGURE 9 Dependence of velocity (v) on substrate concentration (*Micrococcus luteus*) of the reaction catalysed by lysozyme in aqueous medium (a) and in the presence of 2.5 mM cadmium acetate (b).

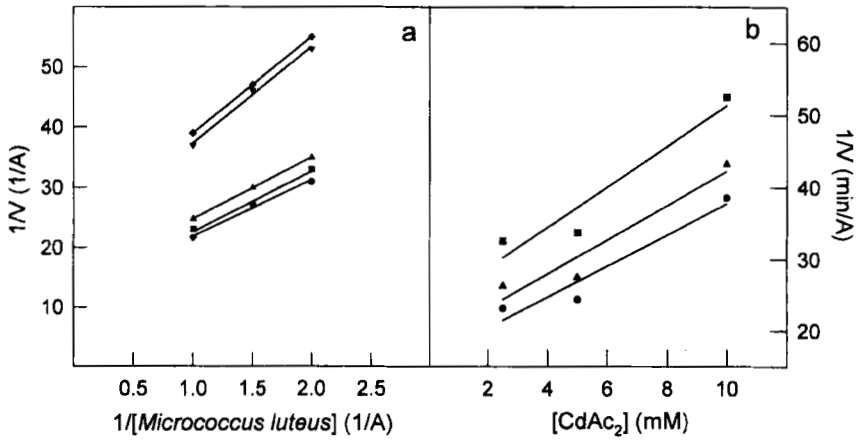


FIGURE 10 (a) Lineweaver-Burk plot for the reaction catalysed by lysozyme in the presence of (●) 0 mM, (■) 2.5 mM, (▲) 5 mM, (▼) 10 mM and (◆) 15 mM of cadmium acetate. (b) Dixon plot for lysozyme at (●) 1 absorbance, (▲) 0.5 absorbance and (■) 0.3 absorbance of substrate concentration (*Micrococcus luteus*).

inhibition was found by a Dixon plot[60] (Figure 10) to be uncompetitive. Thus the cation does not interact with the active site of the enzyme, which is in accordance with the variation of the lysozyme activity (Figure 8), since from 2.5 mM salt, concentration at which a conformational change in the protein is observed, the enzyme activity becomes stable, its value being 70% of a control.

The inhibition constant of the inhibitor, $Ki = 1.2 \pm 0.3$ A, was determined from the plot of the inverse of Vmax (1/Vmax) versus salt concentration ([$CdAc_2$]).

Results show that the interaction of cadmium acetate with lysozyme originates fluctuations on the lysozyme tertiary structure, which are not reflected in large modifications of the secondary structure. The distortion of the structure is not general. However, the salt causes an inhibitory effect on enzymatic activity. The inhibition that cadmium salt causes on lysozyme is longer than that caused by aluminium;[1] this may be due to the smaller size of aluminium compared to cadmium so that it causes a small distortion on the protein structure. The inhibition is mixed type with an uncompetitive component. Structurally cadmium should not interact directly with residues in the active site because of its uncompetitive inhibition component but its action could be due to modifications in the tertiary structure or to weak interactions with residues in the substrate binding subsites. A, B and C subsites have the largest affinity for the substrate, so cadmium atoms could interact with other subsites (D, E, F) with lower affinity. This could explain the moderate decrease in activity.

Cadmium has been traditionally considered an element very toxic for both the environment and organisms. According to our results it may be concluded that there are not drastic effects on the lysozyme protein, component of the first defensive barrier in many organism. Results seem to confirm that the structure of lysozyme in the presence of cadmium acetate is slightly different to the one in the native state but that loss of activity at reasonably high $Cd^{++}$ concentrations shows only a moderate effect.

## *Acknowledgements*

This work was funded by a grant (08.7/0002/2/98) from Comunidad Autónoma de Madrid, C.A.M. (Spain).

## *References*

[1] J.M. Socorro, R. Olmo, M.D. Blanco and J.M. Teijón (1995) *J. Inorg. Biochem.*, **57**, 293.
[2] C. Gómez, M.D. Blanco, R. Olmo, R. Lozano and J.M. Teijón (1995) *Polym. International*, **36**, 345.
[3] R. Olmo, M.D. Blanco, C. Teijón, J.M. Socorro and J.M. Teijón (1998) *Polym. International*, **47**, 179.
[4] S.W. Cho and G.J. Joshi (1989) *J. Neurochem.*, **53**, 616.
[5] N.C. Kaarsholm, H.C. Ko and M.F. Dunn (1989) *Biochemistry*, **28**, 4427.
[6] C. Redfield and C.M. Dobson (1988) *Biochemistry*, **28**, 122.
[7] C.M. Dobson, P.A. Evans and S.E. Radford (1994) *TIBS.*, **19**, 31.
[8] B. Raman, T. Ramakrishna and Ch. Mohan Rao (1996) *J. Biol. Chem.*, **271**(29), 17067.
[9] L. Stevens (1991) *Comp. Biochem. Physiol.*, **100B**, 1.
[10] S. Reitans, K. Kaykho and E.F. Osserman (1982) *Fed. Proc.*, **41**, 608.
[11] S. Das, S. Banerjee and J.D. Gupta (1992) *Expt. Chemotherapy*, **38**, 350.
[12] A. Sobieszek (1999) *Biochim. Biophys. Acta (Mol. Cell Res.)*, **1450**, 77.
[13] D. Markovich and K.M. James (1999) *Toxicol. Appl. Pharmacol.*, **154**, 181.
[14] A.M. Vitale, J.M. Montserrat, P. Castilho and E.M. Rodríguez (1999) *Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol.*, **122**, 121.
[15] H.C. Krasny and D.J. Jr. Holbrook (1988) *Biochem. Pharmacol.*, **27**, 122.
[16] K.A. Saltsman, H.L. Prentice and R.E. Kingston (1999) *Molec. Gen. Genetics*, **261**, 161.
[17] I. Katime, J.R. Ochoa and J.M. Teijón (1985) *J. Chem. Soc. Faraday Trans.*, **81**, 783.
[18] R.M. Fuoss and U.J. Strauss (1948) *Polym. Sci.*, **3**, 602.
[19] G.J. Scatchard (1946) *J. Amer. Chem. Soc.*, **68**, 2315.
[20] W.J. Stockmeyer (1950) *J. Chem. Phys.*, **18**, 58.
[21] E. Reisler and H. Eisenberg (1969) *Biopolymers*, **6**, 1077.
[22] I. Katime and C. Strazielle (1977) *Makromol. Chem.*, **178**, 2295.
[23] P. Kratochvil, J. Poychly and B. Sedlacek (1972) *J. Polym. Sci.*, **10**, 2057.
[24] J.C. Lee, K. Gakko and S.N. Timasheff (1979) *Meth. Enzymol.*, **6**, 26.
[25] E. Reisler, Y. Haik and H. Eisenberg (1977) *Biochemistry*, **16**, 197.
[26] S.N. Timasheff and H.J. Krommam (1959) *Arch. Biochem. Biophys.*, **83**, 60.
[27] E.F. Cassasa and H. Eisenberg (1961) *J. Phys. Chem.*, **65**, 427.
[28] E.F. Cassasa and H. Eisenberg (1964) *Advan. Protein Chem.*, **19**, 287.
[29] T. Arakawa and S.N. Timasheff (1984) *Biochemistry*, **23**, 5912.
[30] V. Vlachy and S. Lapanje (1978) *Biopolymers*, **17**, 2041.
[31] T. Arakawa and S.N. Timasheff (1985) *Meth. Enzymol.*, **114**, 49.
[32] M.M. Bradford (1976) *Anal. Biochem.*, **72**, 248.
[33] A. Perczel, G. Tusnády, M. Hollósi and G.D. Fasman (1991) *Prot. Eng.*, **4**, 669.
[34] O. Shugar (1952) *Biochim. Biophys. Acta*, **3**, 303.
[35] H. Lineweaber and D. Burk (1934) *J. Amer. Chem. Soc.*, **56**, 658.

[36] S.N. Timasheff (1993) *Ann. Rev. Biophys. Biomol. Struct.*, **22**, 67.
[37] M.D. Blanco, J.M. Teijón, J.A. Onrubia and I. Katime (1988) *Polymer Bull.*, **20**, 563.
[38] I.D. Katime, M.D. Blanco and J.M. Teijón (1989) *Polymer Bull.*, **21**, 89.
[39] M.D. Blanco, J.M. Teijón and I. Katime (1990) *Eur. Polym. J.*, **26**, 249.
[40] H.C. Freeman (1967) *Adv. Protein Chem.*, **22**, 234.
[41] J.M. Teijón, M.D. Blanco, A. Rodriguez, J.A. Onrubia and I. Katime (1987) *Rev. Esp. Fisiol.*, **43**, 81.
[42] H.B. Bull and K. Breese (1968) *Arch. Biochem. Biophys.*, **128**, 488.
[43] I.D. Kuntz and W. Kauzmann (1974) *Adv. Protein Chem.*, **28**, 239.
[44] T. Koseki, K.B. Kitabatake and E. Doi (1988) *J. Biochem.*, **103**, 425.
[45] S. Shinagawa, K. Kameyama and T. Takagi (1993) *Biochim. Biophys. Acta*, **1161**, 79.
[46] I. Katime, C. Abad and A. Campos (1983) *Afinidad*, XL, 257.
[47] I.M. Klotz and B.J. Campbell (1962) *Arch. Biochem. Biophys.*, **80**, 96.
[48] J.J. Pesek and J.F. Schneider (1988) *J. Inorg. Biochem.*, **32**, 233.
[49] R. Olmo, P. Huerta, M.D. Blanco and J.M. Teijón (1992) *J. Inorg. Biochem.*, **47**, 89.
[50] J.A. Martín, R. Olmo, J.M. Socorro, M.D. Blanco and J.M. Teijón (1997) *Polym. International*, **42**, 218.
[51] T. Arakawa, R. Bhat and S.N. Timasheff (1990) *Biochemistry*, **29**, 1914.
[52] S.N. Timasheff (1992) *Biochemistry*, **31**, 9857.
[53] D.R. Robinson and W.P. Jenks (1965) *J. Am. Chem. Soc.*, **87**, 2470.
[54] K. Kuwajima, Y. Hiraoka, M. Ikeguchi and S. Isugai (1985) *Biochemistry*, **21**, 874.
[55] L.W. Nichol and D.J. Winzor (1972) In *"Migration of Interacting Systems"*, pp. 53–54. Clarendon Press; Oxford.
[56] R. Olmo (1995) Implicaciones estructurales y funcionales de la interacción de las sales de zinc, cadmio y mercurio con lisozima y hexoquinasa. PhD. Thesis, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, Spain.
[57] J.L. Webb (1963) *Enzyme and metabolic inhibitors*, pp. 111–139. Academic Press; New York and London.
[58] I.M.A. Verhamme, G.W.K. Van Dedem and A.R. Lauwers (1988) *Eur. J. Biochem.*, **172**, 615.
[59] N.C. Price and L. Stevens (1989) *Fundamentals of Enzymology (2nd Edition)*, p. 148. Oxford University Press; Oxford.
[60] M. Dixon (1953) *Biochem. J.*, **55**, 170.