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Kinetic Stabilization of Lysozyme upon Interactions with β-Cyclodextrin through Partial Unfolding

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Protein aggregation and denaturation are two major limitations in bioprocess engineering and protein processing. In the present study, we investigated the effects of β-cyclodextrin, as an artificial chaperone, on the structure and function of lysozyme using UV-Vis, fluorescence spectrophotometry, isothermal titration calorimetry (ITC) and theoretical approaches of docking. Lysozyme was entirely aggregated in a solution containing 0.1% lysozyme (w/v), 150 mM sodium phosphate buffer (pH 7.2), and 10 mM dithiotreitol (DTT). The absorption changes were monitored at 37 °C for 50 min using a UV-Vis spectrophotometer at 360 nm. The residual lysozyme activity was determined using chitosan, a polysaccharide, whose structure is similar to a bacterial cell wall, as a substrate. The effect of β-cyclodextrin on lysozyme activity was determined in the presence and absence of DTT. Our findings indicated that β-cyclodextrin increased the enzyme activity and its kinetics stabilization by binding to Trps 62 and 63, which are embedded in the core of the enzyme inducing its disaggregation. These occurred through the disruption of hydrophobic interactions as demonstrated by ANS fluorescence spectrophotometry. Furthermore, ITC analysis indicated that the binding of β-cyclodextrin to lysozyme was an endothermic reaction and reduced thermodynamic stability by partial unfolding of the enzyme. Thus, the interaction of β-cyclodextrin with lysozyme reduces themodynamic stability by inducing partial unfolding of the enzyme.

Keywords: β-Cyclodextrin, Lysozyme, Disaggregation, Chitosan, Kinetic stabilizer, Isothermal titration calorimetry (ITC)

INTRODUCTION

Protein aggregation and denaturation present major problems during bioprocess engineering or protein processing [1]. Target proteins secreted by wild type or engineered organisms can be aggregated or denatured by the heat generated through metabolism or during fermentation [2]. However, target proteins can be stabilized and made resistant to such factors by inclusion of additives, which prevent aggregation. Many substances have been developed to

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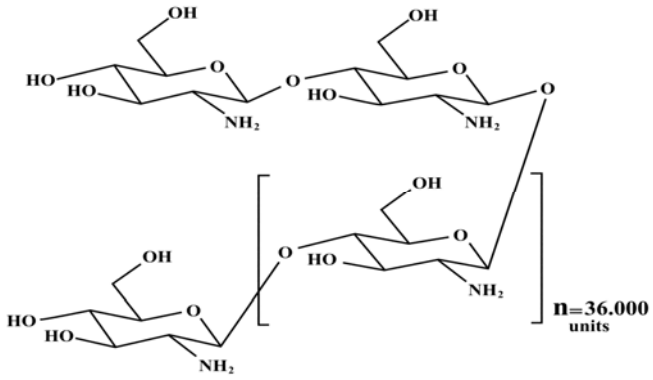
suppress aggregation or assist in refolding the protein molecules [3] reducing aggregation and denaturation *in vitro*. Guanidine and urea are good suppressors that influence intermolecular interactions on the hydrophobic sections of the proteins [4-5].

Arginine [5-9], proline [10], sugar [11], micelles [12], liposomes [13] can also prevent aggregation of partially refolded proteins. Investigators have employed surfactants together with cyclodextrins [14-18] or surfactants and cycloamylose [19] to prevent protein aggregation. Thus, chaperone-like molecules can be used to reduce protein aggregation. One of the most commonly used chaperone-like molecules is β-cyclodextrin (β-CyD) [20-22]. B-CyD is built up of 6-8 glucopyranose units, which are cyclic and water-soluble, and commonly employed as drug delivery vehicle [23-25]. Cyclodextrins are well tolerated and are not harmful to humans [14,20-21]. They have hydrophobic cavities that prevent direct interactions on the hydrophobic surfaces of proteins and in this way suppress protein aggregation [26].

Lysozyme is a protein containing four disulfide linkages and its stability depends on the formation of these disulfide linkages. Furthermore, the 3-dimensional structure studies by X-ray crystallography [27] have revealed that the protein has a highly stable structure under harsh conditions such as extreme pH [25]. This has been mainly attributed to the hydrophobic core of the enzyme and the availability of the ionic residues on the enzyme's surface [27-28]. Lysozyme exists as a monomer at low pH but tends to oligomerize at higher pH [29-30].

Lysozyme's substrates are generally bacteria, and the enzyme attacks the polysaccharide bacterial cell walls [31-36]. Chitosan is a polysaccharide and is an appropriate non-bacterial substrate for lysozyme (Scheme 1). A chitosan with a very low fraction of acetylated units was studied as an enzyme inhibitor [37]. N-acetylated chitosan (NAC) was used to determine the enzymatic activity of the polyethyleneoxide-maleic anhydride (PEOMA) modified lysozyme with different degrees of modification [38]. The amine groups in chitosan have a pKa value of ~6.5, thus, chitosan is positively charged and soluble in acidic to neutral solutions [39]. Chitosan is furthermore hypoallergenic and has natural anti-bacterial properties further supporting its use in field bandages [39]. The kinetics, thermodynamic stabilization and biological activity of lysozyme in the presence of chitosan, as a substrate, have not been reported to date. Here, we studied the biological activity of lysozyme in the presence of chitosan, as an anti-bacterial substrate, and showed the dual effects of β-CyD on its thermodynamic and kinetic stabilization using ANS fluorescence spectrophotometry and ITC analysis.



Scheme 1. Chitosan structure

MATERIALS AND METHODS

Hen egg white lysozyme and β-cyclodextrin, chitosan, dithiotreitol (DTT), 8-anilino-1-naphthalenesulfonic acid (ANS) were from Sigma-Aldrich (St. Louis, MO, USA), and other chemicals used were of analytical grade (Merck, Germany).

Enzymatic Activity

Lysozyme activity was determined employing a solution of chitosan (0.05%; w/v) prepared in phosphate buffer (pH 7.2) at 37 ºC. The amount of the formed reducing sugar was measured by 3,5-dinitrosalysilic acid as reported by Miller [40]. One unit of enzyme activity was defined as the mg of reducing sugar formed through the hydrolytic action of lysozyme on chitosan in one minute under the above assay conditions.

Lysozyme Disaggregation

Lysozyme was aggregated in a solution containing 0.1% lysozyme in 150 mM sodium phosphate buffer (pH 7.2) and 5 mM DTT. The solution absorbancies were monitored at 37 °C for 50 min using a Jen Way UV-Vis spectrophotometer model

6505 at 360 nm. The prevention of lysozyme aggregation was quantified under the same conditions in the presence of different concentrations of β-CyD (0.3, 3 and 30 mM).

Fluorescence Spectrophotometry

The ANS (1-anilino-8-naphtalene sulfonate) fluorescence spectra of lysozyme were recorded on a Cary Eclipse Varian fluorescence spectrophotometer. The following solutions were prepared: a) lysozyme (0.177 mg ml^{-1}) in 150 mM phosphate buffer (pH 7.2); b) lysozyme (0.177 mg ml^{-1}) in the presence of 30 mM β-CyD and c) 30 mM β-CyD. All the solutions were incubated at 37 °C for 50 min and transferred to a 0.4 ml cuvette. The fluorescence intensities in the presence of 10 mM ANS (1-anilino-8-naphtalene sulfonate) were measured at the $\lambda_{excitation}$ 365 nm at 37 °C with a bandwidth of 10 nm. The final results were obtained using Sigma plot software.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) measurements were made on a VP-ITC ultra-sensitive micro-calorimeter (MicroCal, Northampton, MA). All the solutions were degassed before titrations were performed. The following procedure was adopted: β-CyD (30 mM) and lysozyme (1.26 μM) solution were prepared in phosphate buffer pH 7.2 at 37 °C. During the titration, 8 μl of β-CyD solution was injected at 5 min intervals into the calorimetric titration vessel, which contained lysozyme (1.26 μM). The cell was stirred at 307 rpm. The titration was conducted at 37 °C. The injection of β-CyD solution into the vessel was repeated 30 times, with 8 μl per injection. The calorimetric signal was measured by a digital voltmeter that was part of a computerized recording system. The heat of each injection was calculated by the ‘‘Thermometric Digitam 3’’ software program. The micro-calorimeter was frequently calibrated electrically during the course of the study. The enthalpies of β-CyD-lysozyme interactions were calculated in kJ mol^{-1} and analyzed by GRB solvation model using a nonlinear least square method.

Theoretical Approaches

One of the predetermined lysozyme structures that is registered in protein structure data bank was taken with 2VB1 code. The docking of β-cyclodextrin was performed for lysozyme by HEX 6.0 software. The best docking result selected was based on the best HEX docking score. SPDBV was the software used to draw the accessible surface area.

RESULTS AND DISCUSSION

Natural polymers are commonly used for drug delivery in humans [41]. Chitosan is a good polymer among the enzymatic degradable polymers developed for drug delivery [42]. It has low toxicity, good biocompatibility and can be hydrolyzed by lysozyme [43]. Hydrolyzed chitosan is used as a preservative in food and exhibits antimicrobial activity [44]. We therefore, utilized chitosan as a non-bacterial substrate to study lysozyme’s biological activity. Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit; Scheme 1).

Figure1 shows the changes in enzyme specific activity in the presence or absence of β-CyD *vs*. the duration of reaction (ranging from 0-3600 s at an interval of 600 s) using chitosan as a substrate at 37 °C. The specific activity of lysozyme in the absence of β-CyD was increased up to 1,800 s, and then decreased. However, in the presence of β-CyD the enzyme specific activity increased up to 1,800 s, and then remained constant. Figure 2 shows the absorption of lysozyme at 360 nm vs. time. The DTT was used in this experiment to induce aggregation of lysozyme in the presence of different concentrations of β-CyD. The aggregation of lysozyme was reduced and delayed in the presence of β-CyD. Figure 3 shows the changes of enzyme specific activity in the presence or absence of β-CyD *vs*. time in reactions containing DTT (the aggregation condition: 0.1% Lysozyme (W/V) and 10 mM DTT for 30 min) using chitosan as a substrate at 37 °C. These results indicated the kinetic stabilization of lysozyme in the presence of 30 mM β-CyD, whereas in the absence of β-CyD the specific activity of lysozyme sharply decreased due to the aggregation of the enzyme. Inclusion of DTT as a chemical aggregator agent in the solution resulted in the aggregation of the protein at room temperature [45-46]. The specific activity measurements of lysozyme were started at 300 s, in the presence or absence of DTT, associated with or without β-CyD (Figs. 1 and 3). The discrepancy of curves in the presence of DTT (Fig. 3) indicated that the deactivation of lysozyme occurred during 1,800 s. In contrast, in the presence

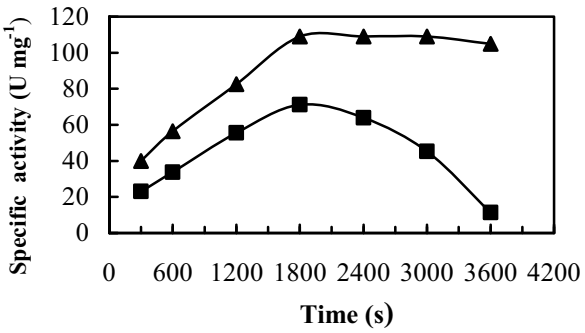


Fig. 1. Enzyme Specific activity in the absence (■) or presence (▲) of β-CyD (30 mM), pH 7.2 at 37 °C.

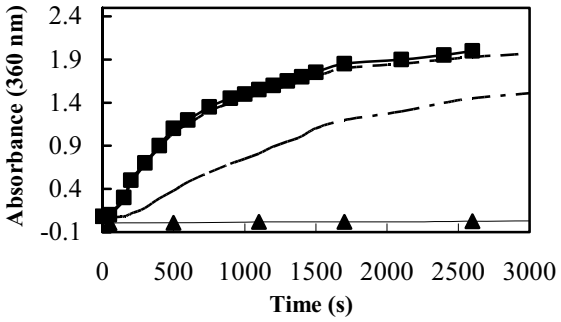


Fig. 2. Chemical aggregation of lysozyme (0.1%) by DTT in the absence (■) and presence of different concentration of β-CyD,0.3 mM (dash), 3 mM (dash dot), and 30 mM (▲) at 37 °C.

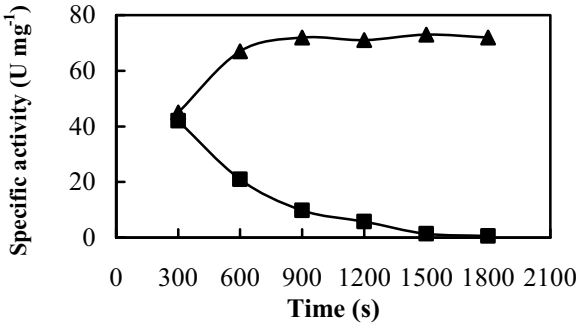


Fig. 3. Specific activity of lysozyme in the absence (■) and presence of β-CyD (▲), in the presence of DTT.

of β-CyD, the activation of lysozyme occurred at the initial time which led to saturation in later times. The specific activity curves without DTT (Fig. 1) for lysozyme activation occurred up to 1,800 s, then deactivated and remained constant in the absence or presence of β-CyD at later times, respectively. Figure 4 shows the absorption of lysozyme at 360 nm, which increased in the absence of β-CyD with time, indicating that lysozyme molecules were aggregated at 0-3,000 s. When β-CyD was added after 1,000 s of aggregation, the aggregation of the lysozyme was immediately suppressed. Molecular chaperones bind only to aggregation-prone proteins [47-48]. To prove this phenomenon for β-CyD, β-CyD was added to the reaction mixture when the aggregation reached to about half maximum. This treatment immediately stopped lysozyme aggregation (Fig. 4). Our results confirmed that the reduction and prevention of lysozyme aggregation could be reasonably accounted for by the formation of soluble enzyme-β-CyD complexes. Thus, β-CyD interactions with the accessible hydrophobicity of lysozyme caused an increase in its solubility and a reduction in its aggregation, and ultimately led to kinetic stabilization of the enzyme.

Molecular chaperones should have the following characteristics: i) the ability to suppress aggregation during protein refolding from a denatured state, ii) the ability to protect from aggregation during protein unfolding under stress conditions, and iii) recover lost biological activity [48]. We showed here that β-CyD could efficiently suppress the induced lysozyme aggregation (Figs. 2, 4) and enhance the recovery of enzyme activity (Figs. 1, 3). The interaction of β-CyD with proteins may occur through hydrophobic moiety, hydrogen bonding, or van der Waals interactions. Here the hydrophobic interactions appeared to be the major cause in lysozyme aggregation.

To determine changes in the hydrophobic parts of lysozyme, we employed ANS for monitoring the effects of β-CyD on lysozyme. The ANS fluorescence analysis indicated changes in the lysozyme conformation (Fig. 5). The ANS fluorescence intensity of the spectra increased in the presence of β-CyD, and the spectral maxima shifted towards shorter wavelength relative to the enzyme in the presence or absence of β-CyD or β-CyD alone. In the presence of DTT, the enzyme's disulfide bounds were broken, and therefore, the hydrophobic patches of lysozyme were exposed. Interactions

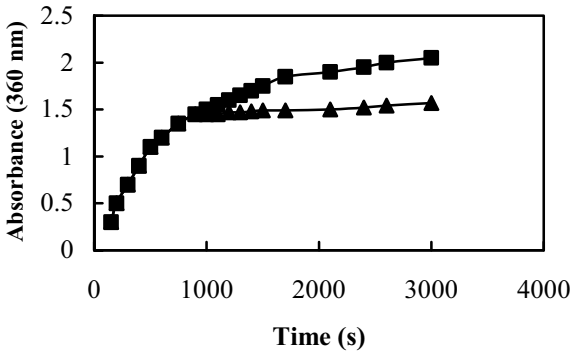


Fig. 4. Aggregation of lysozyme in the absence (■) and presence of β-CyD at the middle point of aggregation (▲).

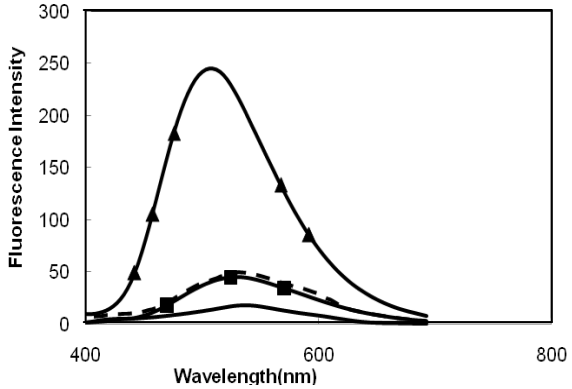


Fig. 5. ANS fluorescence spectra of lysozyme in the absence (■), presence (▲) of β-CyD, ANS with β-CyD (dash) and ANS alone (—) incubated at 37 °C for 50 min.

among these hydrophobic patches resulted in protein aggregation and deactivation of the enzyme. To test the hydrophobic effects under our study conditions we monitored ANS fluorescence (Fig. 5). ANS exhibits weak fluorescence in aqueous solutions, however, in non-polar environments its quantum yield increases [49]. Hence, it was used to detect the non-polar region in proteins by binding to such regions and increasing fluorescence. The results obtained from the extrinsic fluorescence of lysozyme revealed that there were interactions between the exposed hydrophobic patches of β-CyD and exposed hydrophobic patches of the protein (Fig. 5). Thus, β-CyD prevented the direct interactions between protein hydrophobic patches and reduced protein aggregation. The presence of β-CyD as a chaperone molecule prevented these hydrophobic-hydrophobic interactions in the enzyme. The exposed hydrophobic patches of the enzyme occurred upon interaction with hydrophobic patches of β-CyD which prevented hydrophobic-hydrophobic interactions diminishing the aggregation of the enzyme and resulting in the constant activity of lysozyme [50]. Thus, the ascending specific activity of lysozyme occurred due to a disaggregation phenomenon.

The effects of α-Cyd, γ-Cyd and 2-hydroxypropyl-β-CyD on the protein refolding and suppression of lysozyme aggregation have been previously reported [51]. Unfolding globular proteins normally involves the exposure of buried hydrophobic side chains [21]. The binding of CyDs (α and β) to these exposed residues should destabilize native conformations chain [21]. The study of differential scanning calorimetry showed that CyDs (α and β) reduce the mean unfolding temperature (Tm) and also reduce the thermal stability for globular proteins [21].

It has been previously shown that the enthalpies of the solute-solvent (lysozyme + β-CyD in the present case) interactions in the aqueous solvent (β-CyD + water in the present case) system, can be accounted for quantitatively in terms of three factors: preferential solvation by the components of a mixed solvent, weakening or strengthening of solvent-solvent bonds by the solute, and the change in the enthalpy of the solute-solvent interactions [52]. This treatment leads to:

$$\Delta H = \Delta H_{\max} x'_B - \delta^{\theta}_A (x'_A L_A + x'_B L_B) - (\delta^{\theta}_B - \delta^{\theta}_A)(x'_A L_A + x'_B L_B) x'_B \quad (1)$$

The parameters $\delta^{\theta}_A = (\alpha n + \beta N)^{\theta}_A$ and $\delta^{\theta}_B = (\alpha n + \beta N)^{\theta}_B$ are the indexes of the lysozyme stability as a result of interaction with β-CyD in the low and high β-CyD concentrations, respectively. These will result in the formation of a cavity wherein n solvent molecules become the nearest neighbors of the solute and βN reflecting the enthalpy change from strengthening or weakening of solvent-solvent bonds of N solvent molecules ($N \geq n$) around the cavity ($\beta < 0$ indicates a net strengthening of solvent-solvent bonds). The constants α and β represent the fraction of the enthalpy of water + β-CyD interaction associated with the cavity formation or

restructuring, respectively. x'_B can be expressed as follows:

$$x'_B = \frac{px_B}{x_A + px_B} = \frac{\nu}{g} \quad (2)$$

x_B is the fraction of the β-CyD needed for the saturation of the binding sites, and $x_A = 1 - x_B$ is the fraction of unbounded β-CyD. We can express x_B fractions as the total β-CyD concentrations divided by the maximum concentration of the β-CyD upon saturation of all lysozyme as follows:

$$\chi_B = [\beta\text{-CyD}]_T / [\beta\text{-CyD}]_{max} \qquad x_A = 1 - x_B \quad (3)$$

$[\beta\text{-CyD}]_T$ is the total concentration of β-CyD and $[\beta\text{-CyD}]_{max}$ is the maximum concentration of the β-CyD upon saturation of all lysozyme. In general, there will be "g" sites for the binding of β-CyD per lysozyme molecule and ν is defined as the average moles of bound β-CyD per mole of the total lysozyme. L_A and L_B are the relative contributions of unbound and bound β-CyD to the enthalpies of dilution with the exclusion of lysozyme and can be calculated from the enthalpies of dilution of β-CyD in buffer, ΔH_{dilut}, as follows:

$$L_A = \Delta H_{dilut} + x_B \left(\frac{\partial \Delta H_{dilut}}{\partial x_B} \right)$$
$$L_B = \Delta H_{dilut} - x_A \left(\frac{\partial \Delta H_{dilut}}{\partial x_B} \right) \quad (4)$$

The enthalpies of β-CyD + lysozyme interactions, ΔH, were fitted to Eq. (1) over the whole β-CyD compositions. δ_A^θ and δ_B^θ parameters have been also optimized to fit the data. The optimized δ_A^θ and δ_B^θ values are recovered from the coefficients of the second and third terms of Eq. (1). The small relative standard coefficient errors and the high r^2 values (0.99999) support the method. Φ is the fraction of lysozyme molecule undergoing complexation with β-CyD which can be expressed as follows:

$$\Phi = \frac{\Delta H}{\Delta H_{max}} \quad (5)$$

ΔH_{max} represents the heat value upon saturation of lysozyme. The equilibrium constant values, K_a, as a function of β-CyD concentration can be calculated as follows:

$$K_a = \frac{\Phi}{(1-\Phi)[\beta - CyD]_F} = \frac{\Phi}{(1-\Phi)[\beta - CyD]_T (1 - x_B)} \quad (6)$$

The appearance of association equilibrium constants, K_a, for successive replacement of the water molecules by β-CyD ions, are as follows:

$$K_a = x_A^{\,g} - \sum_{i=1}^{g} K_i \frac{x_B^i}{x_A^{\,i-g}} \quad (7)$$

In fact K_a values are the equilibrium constants for the equilibria:

lysozyme + β-CyD ⟶ lysozyme-β-CyD (E1)

K_a values obtained from Eq. (6), were fitted to Eq. (7) using a computer program for nonlinear least-square fitting. Therefore, we can approach to "g" value easily (g = 2 in this work). K_i's are the equilibrium products for the individual equilibrium in the equilibria E1. ν Values can be calculated at any concentration of β-CyD from Eq. (3). The Gibbs free energies as a function of β-CyD concentration can be obtained as follows:

$$\Delta G = -RTLnK_a \quad (8)$$

where K_a is the association equilibrium constant as a function of β-CyD concentration. Gibbs energies, ΔG, calculated from Eq. (8) are summarized in Table 1.

The results of ITC showed that the heat of complex formation between β-CyD and lysozyme in the first and second binding sites are 112.84 and 225.75 kJ mol^{-1}, respectively (see Table 1). The positive values of transition enthalpy indicate that the reaction between β-CyD and lysozyme was an endothermic reaction. The endothermic process for lysozyme upon interaction with 2-hydroxypropyl-β-CyD has been previously reported by differential scanning calorimetry (DSC) technique [53]. The binding of cyclodextrins to the exposed side-chain on unfolded polypeptides will destabilize the native folded form of the protein and will lead to denaturation at lower temperatures

Table 1. Binding Parameters for Lysozyme-β-CyD Determined from Eqs.1 and 2 at pH 7. The Small Negative Values of δ_A^θ or δ_B^θ Indicated Minimal Structural Changes in Lysozyme Upon Interactions with β-Cyclodextrin

Parameters	First binding sites	Second binding sites
g_i	1.86 ± 0.01	4.22 ± 0.01
K_d (mM)	299.7	680.70
ΔH (kJ mol^{-1})	112.84	225.75
ΔG (kJ mol^{-1})	-1702.55	-543.46
$T\Delta S$ (kJ mol^{-1})	1815.40	656.31
δ_A^θ	-0.04	
δ_B^θ		-0.04

[21].

The most common mechanism of protein aggregation involves protein denaturation via hydrophobic interfaces, and often results in loss of biological activity. It is possible to introduce a correlation between a change in δ_A^θ and an increase in the stability of proteins. The δ_A^θ value reflects the hydrophobic property of lysozyme, leading to the enhancement of water structure. The greater the extent of this enhancement, the greater the stabilization of the lysozyme structure and the greater the value of δ_A^θ. δ_A^θ value (Table 1) for lysozyme + β-CyD interaction is -0.04 (Table 1), indicating that in the low concentration of β-CyD the lysozyme structure is also partially destabilized. The δ_B^θ value in high concentration of β-CyD was -0.04, indicating that the lysozyme structure was also partially destabilized by β-CyD in this region.

The ITC results revealed that the native conformation of protein was slightly destabilized. The binding of β-CyD to lysozyme reduced thermodynamic stability (see Table 1) and inversely increased its kinetic stability (Fig. 1). This was due to the position of the active site and its Trp amino acids. Trp 62 and 63 (in the pdb file: 2ZYN) are in the core protein. When the lysozyme is unfolded partially, Trp 62 and 63 are more available. The β-CyD binds to Trps 62 and 63 (images 1-2) and increases the intensity of the florescence (Fig. 5). Based on the results obtained from images 1-2, the increase in the activity is due to the partial unfolding of the protein which leads to the active site being more available for substrate

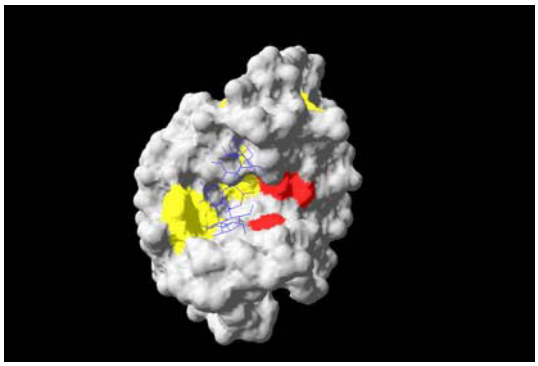


Image 1. Trp 62, 63 and 111 are yellow (in the online system, light in the print version). Active site is red color (in the online system, dark in the print version). The image indicates β-CyD binding to Trps 62 and 63 in the protein core.

binding. The Eckenhoff study showed that destabilization of a protein by a ligand indicates that the ligand binds preferentially (either at more sites or with higher affinity) to a partially unfolded protein. Our results are in a good agreement with this study (see Fig. 5 and Table 1). Such effects are characteristic of nonspecific interactions, in that the nonspecific ligand binds weakly to many different groups at the protein/water interface [54]. From our results, we can

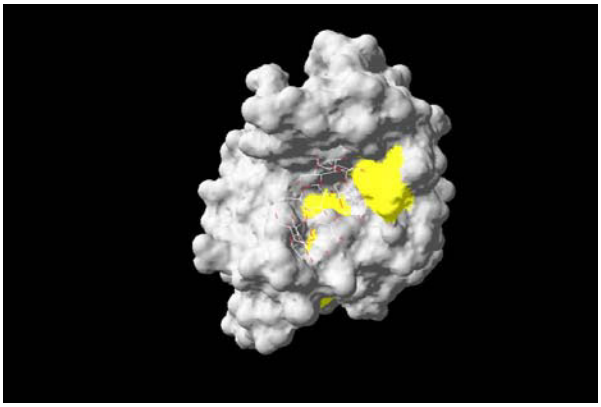


Image 2. Lysozyme-βCyD surface area. All Trp residues bolded by yellow color (in the online system, light in the print version). The active site grove also indicated Trps 62 and 63 (pdb file 2zyn) are embedded in protein core.

conclude that the interaction of β-CyD results in lysozyme conformational changes indicating a partial protein unfolding at high β-CyD concentration, resulting in the increased lysozyme activity.

CONCLUSIONS

In thermodynamic terms, β-CyD, both in high and low concentrations, partially destabilized lysozyme structure due to the conformational changes that led to partial protein unfolding. Thus, the active site was more available to the substrate, and the binding of β-CyD to lysozyme increased the kinetic stability and activity of lysozyme. Alternatively, the presence of β-CyD as a chaperone molecule prevented the hydrophobic-hydrophobic interactions in the enzyme, resulting in the ascending specific activity of lysozyme occurring due to a disaggregation phenomenon.

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