Research Article

Secondary Structural Conformation of Lysozyme at Lipid Membrane Interface Using Circular Dichroism Spectroscopy

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Abstract | This study employs the Circular Dichroism Spectroscopy (CDS) to unfold the possible changes in secondary structural conformation of lysozymes in different pH, temperature and lipid bilayer interfaces. The choice of solvents for the proteins functionality is remained a challenge for biochemical applications, hence there is always a need to understand the effects of solvents on secondary structural conformation. Biophysical investigations about the changes in secondary structure conformation of lysozymes were performed with CDS using them in water and buffer. Membrane bilayer mimics were prepared from dimyristoylphosphatidylcholine (DMPC) through rehydration method. CDS identified that secondary structure was maintained in saline phosphate buffer (PBS) pH (6.4, 7.4 and 8.0), similar to that of water. Temperature based experiments depicted the resistance due to changes in the secondary structural conformation up to 50 °C, however such resistance was reversible after the cooling to 20 °C. In both solvents (PBS and water) lysozyme was predominantly folded to α-helical conformation. At 50 °C, helices were altered to turns. However, in presence of dimyristoylphosphatidylcholine (DMPC) lipid bilayer interfaces, lysozyme adopted β-sheet conformation. This study proposed that hydrophobic groves of lysozyme could be a key in changes of structural conformation while interacting with lipid membrane bilayers. Such protein membrane interactions would facilitate the membrane perturbation. Here, we confirmed the supporting evidence that lysozyme could perturb the lipid bilayer and conforming the sheet structure. Overall, this research suggests that lysozyme could be a tool for designing the novel drug delivery vehicles along its already published potential application.

Received | April 13, 2021; **Accepted** | March 02, 2022; **Published** | June 20, 2022
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Citation | Majid, A., F. Naz, N. Laghari, S. Abbasi, S. Lal, S. Ujjan. 2022. Secondary structural conformation of lysozyme at lipid membrane interface using circular dichroism spectroscopy. *Journal of Innovative Sciences,* 8(1): 29-35.
DOI | https://dx.doi.org/10.17582/journal.jis/2022/8.1.29.35
Keywords | Lysozyme, Secondary structure conformation, Membrane-protein interaction, Circular dichroism spectroscopy, Helices

1. Introduction

A range of experimental techniques have been published to investigate the processes of folding and unfolding of proteins, most of them are relying on a spectroscopic analysis), such as circular Dichroism, tryptophan fluorescence, and a specific denaturation method in which protein folding and unfolding

process initiate, either by using thermal or chemical denaturing agents (McGill *et al.*, 2019). *In vivo,* such folding patterns take place too quickly (in seconds) and more efficiently without producing any by-product such as aggregate of proteins. The efficient way to examine the folding process of protein is to study the refolding way after unfolding (Samelson *et al.*, 2018). According to Samelson *et al.* (2018) for the smaller proteins rate and efficiency of refolding is similar *in vitro* and *in vivo*, while the larger proteins with multi-domains have low rate and efficiency of refolding resulting in the formation of aggregates is common. *In vivo*, however, there are certain others factors that increase the rate and efficiency of folding of proteins.

Different techniques have been established such as high resolution NMR, X-ray crystallography and CD which provide the structural information of thousands of proteins. Among these CD has certain advantages in the measurement of protein structure. Studies on the far-UV region (especially 260nm to 180nm) can be useful for the qualitative analysis and quantification of secondary structure content (Chen *et al.*, 2021). The analysis on far-UV CD spectra is much easier and can be assumed more confidently. With various biological samples in various buffers, it is often difficult to obtain a good quality CD data at below 190nm wavelength due to this region the compound have higher absorbance, however if data is apparently good in quality at 200nm and above, it is possible to get idea of secondary structure of proteins reliably from the ellipticities 208nm and 222nm for helicity and 215nm for ß-sheets. Far-UV CD spectra are used to assess the thermal stability pH, ionic effect and solution structure of proteins and peptides (Micsonai *et al.*, 2021). Some proteins on denaturation process such as elevated temperature, extreme pH and high or low concentration of ions form aggregates or precipitates when unfolded, specifically in irreversible unfolding. Reversible unfolding of proteins can be examined by changing the sample again on the starting conditions in order to see the folding effect in duplicates. Hence, the conformational stability of proteins is directly related to these conditions. However, the protein aggregates in presence of high temperature and different pH the unfolding is irreversible. The relation of unfolding is analysed by width and shape of peaks of CD spectra qualitatively (Chen *et al.*, 2021; McGill *et al.*, 2019). When protein could not refold properly, it causes certain disorders.

There are a huge number of diseases in which protein or peptide does not properly folded to its native structural confirmation and form aggregates. These abnormal conditions are called "*protein misfolding diseases*" (Tang *et al.*, 2021). The globular proteins in condition for example increased temperature, pressure, lower pH, or even presence of organic solvent showed higher tendency to aggregate (Han *et al.*, 2022). Self-assembling proteins and peptides have potential to form their aggregates, and these aggregates are referred as amyloid fibrils. Amyloid formation is key element of various diseases such as various neurological or other systematic and non-systematic disorders, where this protein aggregates deposits *in vivo*.

Moreover, mis-folded or often partly folded globular proteins in some cases also produces linear structures, however these linear (fibril) structures of aggregated proteins are irreversible. The whole scenario of unfolded globular protein that undergo on heat induced aggregation is still unclear. Especially, amyloid fibrillar formation needs more research to understand the complete thermodynamics and kinetics of globular proteins

Human lysozyme has been extensively studied in the formation of amyloid fibrils *in vitro* (Han *et al.*, 2022). Prabhu and Sarkar (2022) have studied the folding mechanism of lysozyme. In amyloid diseases, the human lysozyme is also one of key responsible. Hen egg white lysozyme is simple protein containing 129 residues has been chosen because it is extensively studied and well characterised and it is homologous human lysozyme (Kummer *et al.*, 2021). The aggregation into fibril structure has been found at lower pH 1.6 to 2 upon incubation and at higher temperature (above 55^0C) (Sawaya *et al.*, 2021). The studies on 3D structure, thermal stability, and mechanism of folding leading it to more suitable for the studies of fibril formation. Stability curves against various temperatures also provide useful information that relates to the equilibrium parameters between folded and unfolded states. Thermodynamic curves are mostly used to get idea for the globular protein structures in a particular environment. To understand the folding mechanism of globular proteins and requires great deal of protein characterisation techniques. In circular dichroism studies the unequal absorption of left-handed and right-handed circularly polarised light is measured. The aim of this paper is to study the structural conformation of lysozyme

in different pH, solvents and temperatures, such structural conformations are correlated to variety of biological applications, such as in drug delivery, antimicrobial activity and cellular toxicity.

2. Materials and Methods

Hen egg white lysozyme was purchased from (Merck, Germany) and used without further purification. The pH of buffer was maintained with addition of monobasic and/or dibasic solution using standard pH meter. All solutions were prepared in Milli-Q water with 18 MΩ·cm resistances. All other chemicals were purchase from (Sigma-Aldrich UK) otherwise stated and prepared freshly.

2.1 Lipid bilayer preparation

Lipid membrane bilayers (small unilamellar vesicles) were synthesised according to method (Majid *et al.*, 2020). DMPC at final concentration of 1 mM were rehydrated in 0.1 M PBS buffer for 4 hours after dissolving in chloroform-ethanol mixture. The hydrated suspension was sonicated for 30 minutes in ultrasonic bath.

2.2 Circular dichroism spectroscopy

CD spectra of lysozyme were recorded on a J-815 spectrpolarimeter (JASCO, UK). Far-UV CD spectra of lysozyme were recorded in water and 10 mM PBS at pH of 6.4, 7.4 and 8, secondary structure was determined using methodology previously described by Majid at al. (2020). Lysozyme sample was dissolved in water and PBS of pH (6.4, 7.4 and 8) to final concentration of 1.7 µM. 10 mm path length cuvette was used for each CD spectrum and four scans were averaged over a wavelength of 260 to 180 nm, band width 2 nm, data pitch 0.5 nm, scanning speed 100 nm/min and response time 2 second. For each spectra acquired before running the sample the baseline of instrument was measured. All results were shown in molar residue weight ellipticities after subtracting the control solution in the absence of lysozyme. The temperature was controlled using JASCO Peltier Temperature Controller at 20 ˚C except where suggested. In order to investigate the thermal stability of secondary structure of lysozyme the sample was analysed at temperature range of 10° – 90°C at 10°C interval and 10 seconds interval for equilibration before reaching to target temperature, while all other instrument parameters were kept same and all experiments were repeated four times.

2.3 Data analysis

The CD spectra were analysed using the Dichroweb online server to calculate the percentage of secondary structure of lysozyme (Whitmore and Wallace, 2004) where CDSSTR method developed by Compton and Johnson Jr (1986) was selected with reference dataset 3 (optimised for 185nm-240nm) this method was also adopted (Majid *et al.*, 2020). The data of changes in secondary structure percentage was further analysed using SPSS 20 with One Way ANOVA based on the null hypothesis where percentage helicity is not different and significance level was set ρ- 0.05.

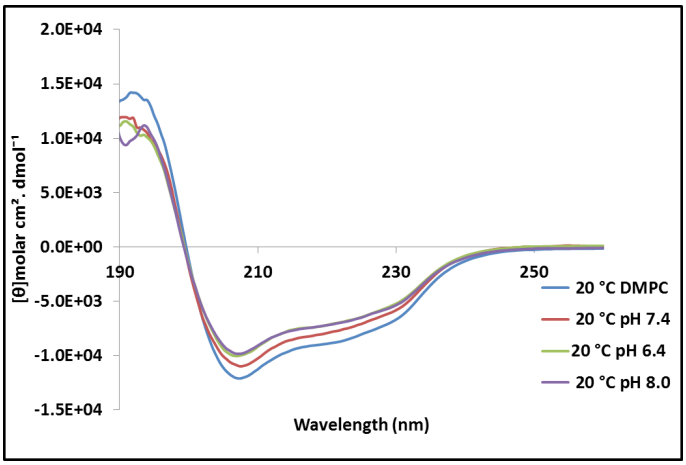

Figure 1: Far-UV CD spectra of lysozyme in different pH and lipid membrane model.

3. Results and Discussion

3.1 Effect of pH

Figure 1 show a maxima at 192 nm and two minima at around 208 nm and 222 nm which are characteristics of α- helix in the lysozyme spectra at pH 6.4, 7.4, 8.0 and lipid membrane interfaces. The pH analysed was lower than isoelectric point 11.35, the spectra showed no significant variations in term of identification of secondary structure. The CDSSTR analysis of the far-UV DC data with Reference data set 3 (Figure 2) indicates that when pH increases towards alkalinity, the content of helicity of the lysozyme structure increases, indicating that the helicity is favoured when the protein possesses more positive electrostatic charges. At pH 6.4 the helicity content was above 30% and trend similarly increases to 40% and 50% for pH 7.4 and pH 8.0, respectively, and statistical tests were also performed using an ANOVA tests, which confirmed our results that the increase in pH is also significantly increases the percentage helicity ($F=11.961$; $\rho - 0.003$). Furthermore, the post-hoc Tukey test revealed that there was a significant

difference (ρ = 0.035) between pH6.4 and pH 7.4. pH 6.4 and pH 8.0 also showed significant difference (ρ = 0.002) while pH 7.4 and pH 8.0 did not show significant difference (ρ = 0.2). The molar residue weight degrees at 208nm also showed similar co-operativity against the different pH (data not shown). The data suggesting that helicity content is a function of pH and rises as pH became more alkaline. Turns and unordered showed overall same trend containing nearly 40 % and in PBS pH 8.0 it is more than 30%, similarly ANOVA also showed the no significant changes in turns and unordered (F=0.624; ρ- 0.557) this trend remained same using pot-hoc Tukey test for ß – sheets and turns and unordered showing no significant differences in all pH ranges analysed.

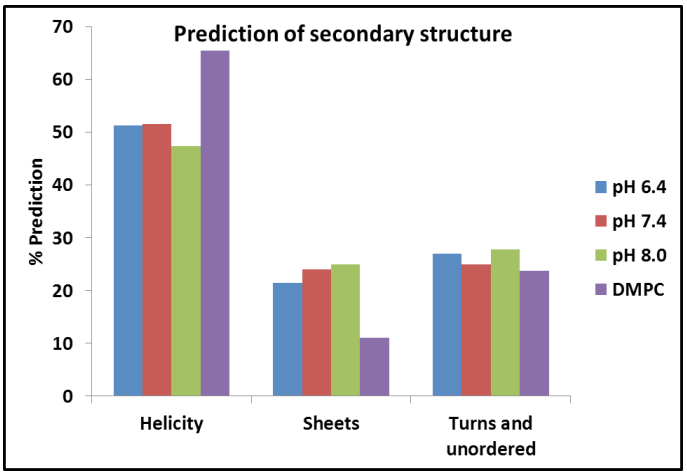

Figure 2: Prediction of secondary structure lysozyme in different pH and lipid membrane model.

3.2 Effect of temperature

The far-UV CD spectra (190–260 nm) of lysozyme in water and in PBS buffer (pH 7.4) were recorded at 10 °C, after thermal unfolding at 90 °C and cooling again to 10°C as shown in Figure 3. At initial 10 °C, the CD spectra display two strong minima at 208 and 222 nm both are characteristics of α-helices. Also CD ellipticities at 208 nm have been extensively reported for the estimation percentage helicity of proteins (Miles *et al.*, 2021). The changes appeared in CD spectra of lysozyme against thermal treatment as shown in Figures 3 and 4. A slower decrease in ellipticity at 208 nm was found for lysozyme till 60 °C, when it was reached at 90 °C, indicated a complete loss of the helicity. It seems the denatured structures showed ß-sheets and turns. B-sheets show one minima at 218 nm and a maxima at 195 nm. CD spectra data provide two state transitions of folded (native) and unfolded state in reversible pattern. Thermal denaturalization of lysozyme in water seems to be a reversible process to a certain extent, because there is a difference of the native and heated-cooled lysozyme spectra shown in Figure 3. The cooperation of reversibility of unfolding and refolding due to temperature effect of the far-UV CD spectra was monitored. If the data of unfolding/ refolding is reversible, the thermo-stabilities studies in sense of equilibrium thermodynamics are more applicable. To see the thermal effect on lysozyme in water and PBS solution CD spectra were also collected by heating-cooling cycle, on every 10 °C from 10° to 90 °C and the change in secondary structure was analysed using the CDSSTR program and reference data set 3 methods (see Figure 4) (Miles *et al.*, 2021).

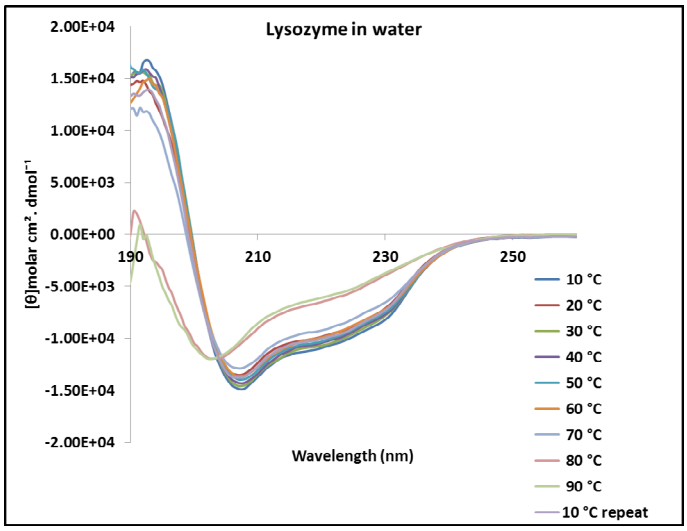

Figure 3: Far-UV CD spectra of lysozyme at different temperature.

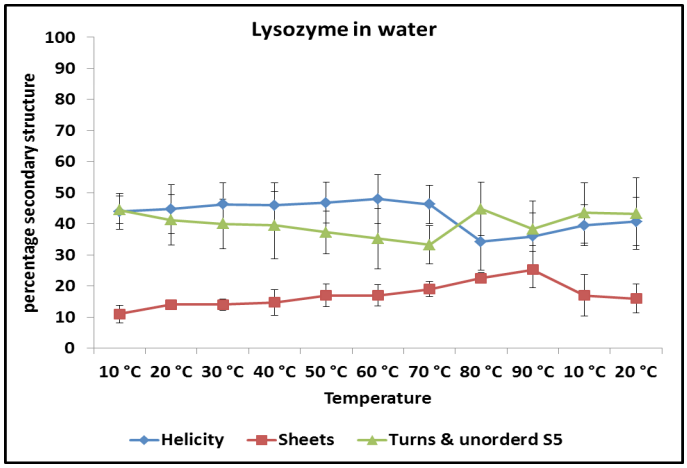

Figure 4: Prediction of secondary structure of lysozyme in different temperature.

The effect of pH on the thermal denaturalisation of lysozyme secondary structure was analysed. Similarly heating-cooling cycles were made as in water in a pH

7.4, interestingly irreversible unfolding of thermal denaturalization was obtained (Figure 4).

Protein folding is dependent on certain factors, such as pH values and more likely the function of temperature, hence the stability of proteins varies significantly against different pH conditions and changes in temperatures, however in our studies we used pH ranges where lysozyme is in its active form (pH 6-9). It has been reported (Attri *et al.*, 2021) the mechanisms and rate of folding may be different in the variation in the pH. At the alkaline pH, all acidic side chains of lysozyme ionized either in native or unfolded states. While at low pH such as 1.5, all the basic chains of lysozyme are fully protonated in both native and unfolded states; and in the pH range of 1.5–5, several groups of lysozyme would be protonated (Ashrafi *et al.*, 2022; Attri *et al.*, 2021).

Our studies also revealed that the helicity content was slightly increased as pH goes higher at 8.0. During heat treatment of lysozyme, the secondary structure remained same when the temperature was increased to 60°C. In endothermic transition globular proteins undergo unfolding and on subsequent exothermic protein stacking induces the aggregation process. The changes in secondary structure of lysozyme were seen gradually, however a critical temperature actually exists. The CD spectra of lysozyme at 20 °C showed three characteristics shifts one maxima at 192 nm and two minima at 208 nm and 222 nm both are characteristic of α-helix. When the temperature was reached at 60 °C, the intensity of spectra at 208 nm and 222 nm were disappeared and shifted to 200 nm and maxima at 192 nm was also significantly decreased these data are in good agreement with (Mine *et al.*, 1990), where they suggested that the egg white proteins are going to start the denaturation process when they reach to 60 °C and this trend continues as temperature goes higher. Overall the intensities in the spectra were shifted with increasing temperature. As the temperature was increased from 60 °C, the new minima at 200 nm appeared. Upon denaturation of lysozyme, the major changes observed in the characteristics in the spectra of protein secondary structure were a disappearance of the both minima at 90 °C which is attributed to identification α-helical structure and this trend suggesting the complete loss of secondary structure of lysozyme. As temperature decreased from 90 to 10 °C, the major conformational changes were again observed in the spectra, the minima at 200 nm again shifted to 208 nm. On cooling the heated lysozyme solution, the CD spectra of lysozyme were retained almost on the same intensities, this suggesting that lysozyme remained in its native form and that folding was a reversible process because the difference in fresh and heated cooled spectra are not significantly observed. Our studies have confirmed the findings of (Arnaudov and de Vries, 2005). As temperature increases the spectrum of lysozyme is completely changed, however the helicity decreases, sheets and turns and unordered increased on the same trend. Another structure "Molten" that is partly unfolded and likely to be more flexible than in the native form may be formed during heat denaturation. It is interesting that in the pH 7.4 and in water the denaturation curves of heat-treated lysozyme showed the good relation, although in water the helicity prediction was lower than that in pH 7.4, the trend of changes in structure remain same. It is suggesting that the unfolded stable structure was formed by the heat treatment of lysozyme. Our results are in agreement with the results published in Kato and Takagi (1988) whish shows ß-sheet structure is formed during heat treatment of ovalbumin.

Conclusions and Recommendations

In summary, the CD data suggesting that the secondary structures of lysozyme in PBS and water resembles. Temperature effect studies show that the secondary structure of lysozyme in water unfolds the lysozyme to apparently on same temperature as in PBS, in case of water the percentage helicity content is lower compared to PBS pH 7.4 and 8. Moreover, thermal unfolding of lysozyme is reversible. The lysozyme secondary structure distribution is highly depending on the temperature conditions and also depends on pH. Lipid membrane interface data revealed that lysozyme could have great importance for therapeutic effects and drug delivery applications.

Acknowledgements

The first author is thankful to Institute of Microbiology, Shah Abdul Latif University, Khairpur, Sindh (Pakistan), where this research work was done.

Novelty Statement

The structural conformations of lysozyme in different pH, solvents, temperatures, and in vitro membrane

interfaces of dimyristoylphosphatidylcholine have not been fully reported in a single study. This study reports the structural conformation of lysozymes for variety of biological applications, such as in drug delivery, antimicrobial activity, and cellular toxicity.

Author's Contribution

This work was carried out in collaboration among all authors. Author AM provided conception and design of the study. Authors FN and NL performed Testing and lab experiments. Authors SL and SA did analysis and interpretation of the data. Authors AM and FN wrote draft of the manuscript.Author SU did critical revision of the article for important intellectual content. Author SU revised thearticle. Author AM approved the final draft and guarantor of the article. All authors read and approved the final manuscript.

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There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

Consent

A written consent from dental practices has been collected and preserved by the authors.

Ethical approval

A written ethical approval from University has been obtained and preserved by the authors.

Conflict of interest

The authors have declared no conflict of interest.

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