Chapter 3

Validation of *Leishmania donovani* citrate synthase as drug target through biophysical studies

Abstract

Citrate synthase serves as the inaugural enzyme of the TCA cycle, essential for its continuation within the mitochondria, as well as for the synthesis of fatty acids and cholesterol in the cytosol. Despite its potential as a therapeutic target, information regarding this enzyme in leishmanial parasites remains limited. In this study, we cloned and expressed *Leishmania donovani* citrate synthase (LdCS) in a bacterial system, followed by purification and characterization using biophysical techniques. Circular dichroism analysis of LdCS at physiological pH revealed a properly folded protein structure predominantly comprised of alpha helices. Thermal analysis indicated a melting temperature of 44°C, suggesting moderate stability. Furthermore, tertiary structure analysis via intrinsic fluorescence spectroscopy demonstrated that LdCS maintained its native folded state and conformational stability. Quenching studies indicated that most tryptophan residues are buried within the protein's hydrophobic core. The protein exhibited moderate tolerance to urea and GnHCl. Collectively, these findings lay the groundwork and offer potential for utilizing citrate synthase as a target for leishmaniasis treatment.

Keywords: Citrate synthase, Circular dichroism, Intrinsic fluorescence, Quencher, Leishmaniasis

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3.1 Introduction

Even though leishmaniasis was discovered a century ago, there is still a severe threat to global health. Every year, between 700,000 and 1 million new cases and 26,000 to 25,000 deaths occur worldwide. Although this disease is primarily found in tropical and subtropical regions, its distribution pattern has recently changed, and it is now found in a new territory. This has been attributed to a number of variables, including co-infections with other diseases, particularly HIV, as well as environmental factors, human behavior, demographics, and climate change [Sasidharan and Saudagar, 2021]. The other issue that has occurred in recent times is the parasites' growing resistance to the marketed chemotherapy medications. This arises can be an adaptation to climate changes and also co-evolving vectors to new hosts [Butt et al., 2020]. These days, in the wake of such a challenge, the search for novel therapeutic targets is becoming imperative. By exploring specific enzymes and their structural and functional characteristics, biochemical and biophysical research are becoming more and more prominent.

In all living cells, the tricarboxylic acid (TCA) cycle serves as a vital source of intermediates necessary for amino acid production, gluconeogenesis, ketogenesis, and lipogenesis while also providing two reducing equivalents for the electron transport chain (Wiegand and Remington, 1986). Among the enzymes involved, citrate synthase stands out as the sole catalyst in the cycle that forms carbon-carbon bonds, mediating the Claisen condensation of acetyl-CoA and oxaloacetic acid to yield citrate. Citrate serves dual roles, acting as a substrate for the citric acid cycle within mitochondria and as an acetyl donor for acetyl-Coenzyme A synthesis via ATP-citrate lyase outside the mitochondria in the cytoplasm, following transport across the mitochondrial membrane (Wiegand and Remington, 1986). Acetyl-CoA plays a crucial role in cholesterol and fatty acid synthesis, facilitated by acetyl-CoA carboxylase (ACC). Even in Leishmania, the synthesis of extended fatty acids, triglycerides, and phosphor-glycerides occurs due to the presence of 12-tandemly linked

homologous genes and fatty acid desaturases, contributing to energy and structural functions (Opperdoes and Michels, 2008; Ranjan and Dubey, 2023). Mitochondrial citrate synthase, encoded by nuclear DNA, is translated into a precursor in the cytoplasm, transported into mitochondria, and localized on the inner membrane. Citrate synthase is classified into type-I and type-II based on its oligomeric condition and an additional β-sheet at the N-terminal region. Type-I CS exists as homo-dimers, found in gram-positive bacteria, archaea, and eukaryotes, while type-II CS are hexameric with an extra β-sheet at the N-terminal region, present in gram-negative bacteria (Lee et al., 2019; Ge et al., 2019). A notable pharmacological distinction between the two forms lies in their inhibition by NADH, with type-I CS typically regulated by competitive inhibition and type-II CS by non-competitive inhibition, with some exceptions (Lee et al., 2019). In *Toxoplasma gondii*, three varieties of citrate synthase—CSI, CSII, and CSIII—are identified, with CSI located in the mitochondrion (Liu et al., 2017). Biochemical characterization studies of citrate synthase have been conducted in various organisms, including cyanobacteria *Microcystis aeruginosa* PCC7806 and Anabaena sp. PCC 7120, gram-positive bacteria *Streptomyces diastaticus* no.7 strain M1033 and Synechocystis, and archaea *Metallosphera sedula* (Ge et al., 2015, 2019, 2020; Ito et al., 2019; Lee et al., 2019). However, information about this enzyme in *Leishmania donovani* remains scarce.

Hence, in this study, we have reported the cloning, expression, and purification of the citrate synthase from *Leishmania donovani* (LdCS) and then its characterization with the help of biophysical methods. This provides essential insights into the structural and functional characteristics of the enzyme to consider LdCS as a potential drug target.

3.2 Materials & Methods

3.2.1 Materials

Sodium acetate, Potassium phosphate dibasic, Potassium phosphate monobasic, Glycine, Tris-

HCl, PMSF, Potassium chloride, Sodium chloride, Glycerol, Imidazole, Lysozyme, Tween-20, 8-Anilionnapthalene-1-sulphonic acid (ANS), Acrylamide, Potassium iodide, Urea and other chemicals of analytical grade were purchased from SRL and HI media, India.

3.2.2 Molecular Cloning, Expression and Purification of LdCS

The Ldcs gene was amplified from genomic DNA of *Leishmania donovani* AG 83, with the use of gene-specific forward (5'GGATCCAGATGGCGTCATCGGTATTG3') and reverse (5'AAGCTTATCACGTTCACGCCGTC CGC3') primers including restriction sites EcoRI and HindIII and directionally cloned into the pET28a vector. The gene size was confirmed by colony PCR, restriction digestion, and Sanger sequencing. For overexpression, constructs of LdCS: pET28a were transformed into *E. coli* BL21 (DE3) and transformed cells were grown at 37 °C until the optical density reached the exponential phase. It was induced with 0.5 mM of IPTG overnight at 18 °C and 180 rpm. After incubation, cells were pelleted down at 10,000 rpm and resuspended into lysis buffer (50 mM Tris-HCl pH 8, 1mM PMSF, 200 mM NaCl, 10% Glycerol, 0.2% Tween 20, 10 mM imidazole and10mg/ml lysozyme) and then incubated for an hour at 4°C. Following incubation, the cells were subjected to a 25-minute sonication cycle with a 5-s pulse on and 7-s-pulse off. The supernatant was separated from the lysate by centrifugation at 11,500 rpm for 35 min at 4°C and loaded into a pre-equilibrated Ni-NTA bead containing column (with 10 mM imidazole in 50 mM Tris-HCl pH 8 and 200 mM KCl). It was further incubated for half an hour at 4°C. After that, LdCS protein was purified using wash buffers I and II containing 50 mM Tris-HCl pH 8, 200 mM KCl, 10% Glycerol, 30 mM, and 50 mM imidazole, followed by elution with 150 mM imidazole. The purified protein was dialyzed with buffer 50 mM Tris-HCl pH 8 and 200 mM KCl using an ultrafiltration device (Vivaspin 20, 30-kDa filter cut-off; Satorius, Gottingen, Germany). The size and purity of the protein were confirmed through 12% sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE), and its concentration was quantified with Bradford assay using BSA as the reference [Raj et al., 2019a].

3.2.3 CD Measurements

The secondary structure of recombinant LdCS was evaluated using a CD spectrophotometer (JASCO 1500) connected with a Peltier-type temperature controller and a continuous nitrogen flow. The CD spectra were recorded in the far UV range (190-260 nm) at a 100 nm/min scanning speed using a quartz cell with a path length of 2.0 mm. In this study, 0.5μM of LdCS protein was employed in 25 mM concentration of different pH buffers such as sodium acetate buffer (pH 4), potassium phosphate buffer (pH 7), Tris-HCl (pH 8) & Glycine-NaOH buffer (pH 10). The thermal denaturation of LdCS was carried out by continuously measuring ellipticity at 222 nm throughout a temperature range of 20 to 70 °C with a linear increment of 1 °C/min. Using the origin 2021, the acquired data was fitted into Boltzmann's sigmoid equation. Every experiment was done twice [Murtas et al., 2021].

3.2.4 Intrinsic and Extrinsic Fluorescence Studies of LdCS Protein at Different pH

The intrinsic fluorescence study was performed with the help of a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) to determine the folding dynamics of LdCS in its native state at 25°C using 50 mM of each buffer sodium acetate (pH 4-5), Potassium phosphate (pH 6-7), Tris-HCl (pH 8-9), and Glycine-NaOH (pH 10) (pH 4 to 10) and 0.5 mg/ml of the protein. The sample was excited at 280 nm, and using a scanning rate of 100 nm/min, the emission spectra were recorded from 300 to 400 nm. Moreover, the unfolding behavior of LdCS at different pH values using buffer i.e., sodium acetate (pH 4-5), Potassium phosphate (pH 6-7), Tris-HCl (pH 8-9), and Glycine-NaOH (pH 10) (pH 4 to 10) was evaluated using hydrophobic cluster binding dye ANS dye. The 0.5 mg/ml (14.5uM) protein was mixed with a more than 100 times molar excess of ANS dye and incubated for 30 minutes at room

temperature in the dark, then it was excited at 380 nm, and emission spectra were recorded between 400 and 600 nm [Jakkula et al., 2021].

3.2.5 Evaluation of tryptophan position in LdCS protein using Potassium iodide and Acrylamide.

To assess the tryptophan residue locations in the LdCS, the various concentrations of Acrylamide (0 to 0.3 M) and Potassium Iodide (0 to 0.4 M) were used. These quenching agents were incubated with 0.5 mg/ml of a protein for an hour, and then their emission spectra were detected at 300 to 400 nm after excitation at 280 nm using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). The Stern-Volmer graph was plotted between the quencher concentration on the X-axis and F0/F1 on the Y-axis, and the quencher constant (Ksv) was calculated from the slope of the Stern-Volmer equation. The Stern-Volmer equation is given as: **F0/F1= 1+ Ksv [Q]**

Where F0 = Fluorescence intensity in the absence of quencher; F1= Fluorescence intensity in the presence of quencher; Ksv = Stern-Volmer quenching constant; Q = Concentration of the quencher.

3.2.6 Unfolding studies of LdCS protein using Urea and Guanidine Chloride.

The unfolding studies of LdCS protein were done with intrinsic fluorescence spectroscopy. In this study, the 0.5 mg/ml of LdCS protein was incubated with different concentrations of urea (0 to 5 M) and Guanidine chloride (GnHCl) (0 to 4M) for an hour, and then their emission spectra were observed at 300-400 nm after excitation at 280 nm. Further, the unfolded fraction was estimated using the following equation:

fD = (y- yN) / (yD – yN)

Where fD = fraction denatured or unfolded; y = value of wavelength for any given concentration of urea or GnHCl; yN = value of wavelength of native protein; yD = value of

wavelength when protein is completely unfolded (or, wavelength at highest denaturant concentration)

The unfolded fraction values were normalized and fitted into the Boltzmann equation using a non-linear regression method. The unfolding concentration of the LdCS protein was calculated from the sigmoid curve [Narsimulu et al., 2022].

3.2.7 Statistical analysis

Each quantitative experiment was carried out in triplicate with two different experiments, and a corresponding blank correction was applied each time. All graphs and their statistical analysis were done using Origin Pro 2021 software, and its data were presented as the mean of the triplicate ± SEM.

3.3 Results

3.3.1 Cloning, expression, and purification of *Leishmania donovani* Citrate synthase

The Ldcs gene with 924 bp was amplified and directionally cloned into a pET28a expression vector, which was confirmed through gene amplification, double restriction digestion through insert release as illustrated in Figure 3.1 (A), and finally through the Sanger sequencing method. The plasmid containing Ldcs-pET28a construct was transformed into BL21(DE3) cells to express their enzymatically active solubilized protein and then purified using Ni-NTA affinity chromatography, and 12% SDS-PAGE gel was used to measure its size and purity. The purified LdCS protein exhibits a single band on SDS-PAGE gel that is roughly 34 kDa, consistent with the protein's expected size, as shown in Figure 3.1 (B).

3.3.2 Determination of the secondary structure of LdCS and its thermal stability

The purified LdCS protein secondary structure at different pH and its thermal stability were examined by Far-UV circular dichroism (CD) spectroscopy. The CD spectra of the LdCS at pH 7 revealed one positive peak at 193 nm and two negative peaks at 208 and 222 nm, as shown in Figure 3.2(A), which suggests that alpha helix is a major secondary structure in the protein

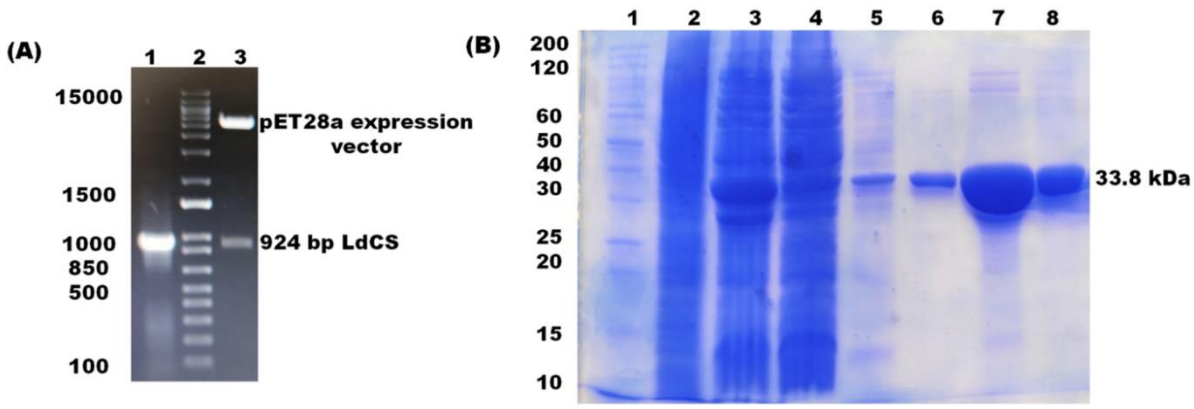

Figure 3.1: Cloning, Expression, and Purification of *Leishmania donovani* Citrate synthase (LdCS)
(A) Confirmation of pET28aLdCS construct. Lane 1: shows the amplification of the LdCS gene by PCR method; **Lane 2:** shows the 1kb^{+} DNA Ladder; and **Lane 3:** shows the release of insert (LdCS) from pET28aLdCS construct using the restriction digestion method. **(B) Expression and Purification of LdCS protein. Lane 1:** Unstained Protein marker; **Lane 2:** soluble fraction of uninduced LdCS transformed in *E. coli* BL21(DE3), **Lane 3:** soluble fraction of induced LdCS with 0.5M IPTG, **Lane 4:** Flow through fraction of LdCS protein, **Lane 5&6:** wash I and wash II fraction of LdCS protein, **Lane 7&8:** purified fraction of LdCS protein (~ 33.8 kDa) which had done by affinity chromatography using Ni-NTA column.

and also show a high level of protein ellipticity, indicating appropriate protein folding. At pH 8, LdCS shows almost similar levels of ellipticity. However, at both low and high pH, the ellipticity of the protein is significantly reduced, which indicates the significant loss of secondary structure of LdCS. The DichroWeb server subsequently predicted the secondary structure of the protein at different pH, as shown in Table 3.1, consistent with the theoretical secondary structural components suggested by the modeled structure, which primarily consisted of α-helices. The digenetic life cycle of leishmania parasites fluctuates between 25°C and 37°C. To comprehend LdCS's thermal stability, CD spectroscopy was conducted at a temperature between 20 to 70°C. According to Figure 3.2(B), the thermal denaturation plot showed the cooperative unfolding with the rise in temperature after the stages of folding, transition, and unfolding from 20 to 30, 30 to 50, and 50 to 70°C, respectively. It was discovered that LdCS has a thermal melting point (Tm) of around 44°C.

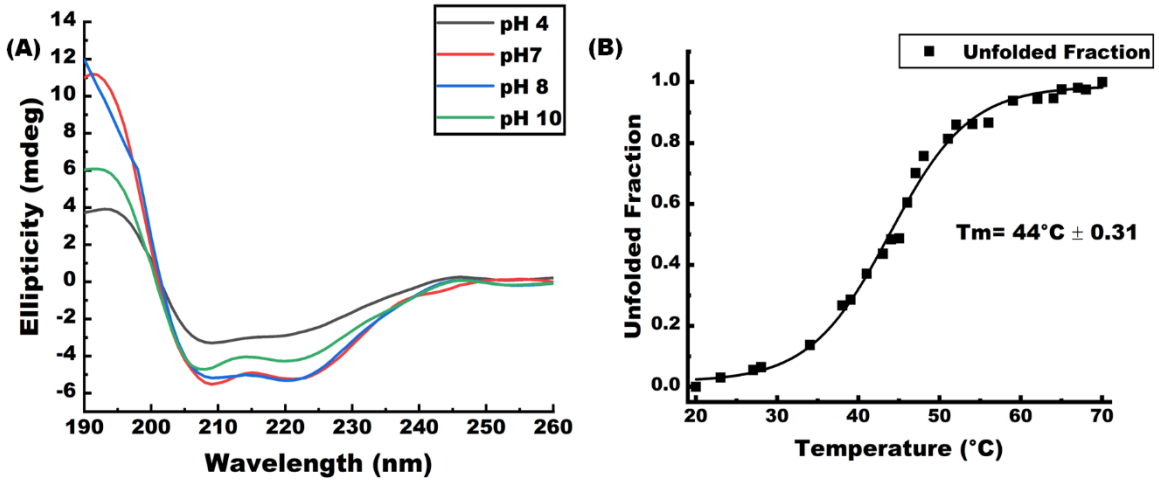

Figure 3.2: CD spectra of LdCS at varying pH and temperature. (A) Far UV-CD spectra of purified protein from 260 to 190 nm at different pH. **(B)** Normalized thermal denaturation plot of purified protein at different temperatures.

Table 3.1: Secondary structure contents of the LdCS at different pH.

pH	α- helices	β- sheets	Random coils	Turns
4	25.2	43.6	24.5	6.7
7	43.9	28.5	27.6	0.0
8	39.0	32.0	24.8	4.2
10	28.7	34.3	29.7	7.3

3.3.3 Intrinsic and extrinsic fluorescence analysis of LdCS at different pH

The conformational change of protein can be estimated through its intrinsic fluorescence, which depends on its fluorophore residues. In the case of LdCS, three tryptophan and ten tyrosine residues are present. The effect of different pH on the conformation of the LdCS protein was monitored through the change in its fluorescence spectra. When increasing the pH from 4 to 10, there was a gradual decrease in fluorescence intensity without any significant

change in its emission maxima, as shown in Figure 3.3 (A), which illustrates the minor structural changes in the tryptophan microenvironment. The decrease in pH lead to a red shift from 331 nm to 333 nm, as shown in Figure 3.3 (B), which shows that the tryptophan residues were more accessible to the solvent environment. Additionally, an extrinsic fluorescent dye 8-anilino-1-naphthalenesulfonic acid (ANS) was used to monitor the unfolding state of LdCS protein at various pH from 4 to 10. An approximately threefold increase in fluorescence intensity was observed at pH 4 compared to pH 7, which depicts the protein aggregate formation at acidic pH, as shown in Figure 3.3 (C).

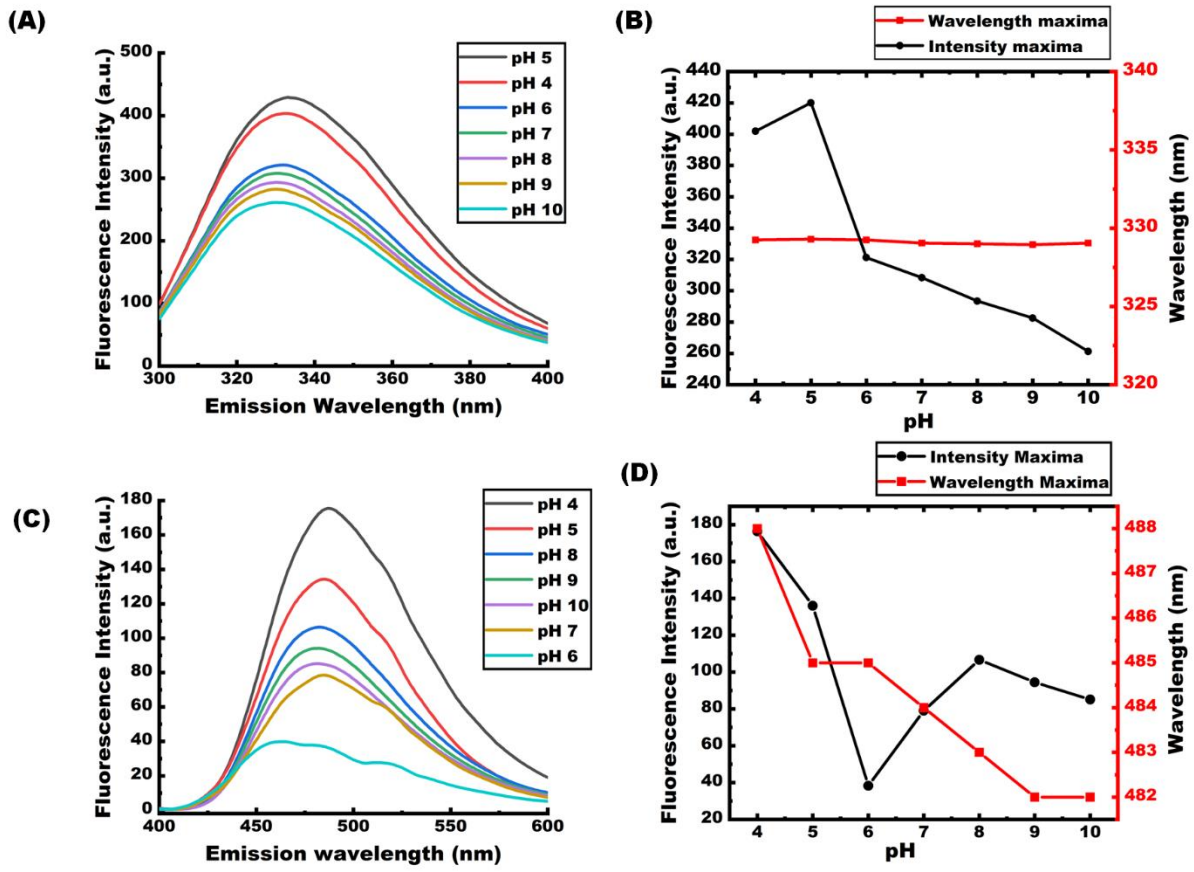

Figure 3.3: Fluorescence emission spectra of LdCS.
(A) Intrinsic fluorescence spectrum of LdCS at different pH. **(B)** Fluorescence intensity and wavelength maxima at different pH. **(C)** Extrinsic fluorescence spectrum of LdCS at various pH with ANS. **(D)** Fluorescence intensity and wavelength maxima at different pH.

Additionally, the wavelength maxima change from 482 to 488 nm, which shows a redshift, advocating the protein's partially or fully unfolded state, as shown in Figure 3.3 (D).

3.3.4 Assessment of tryptophan location in LdCS protein using Potassium Iodide and Acrylamide.

To assess the tryptophan location in LdCS, fluorescence quenching studies were conducted with increasing acrylamide and potassium iodide (KI) concentrations. As the quencher concentration increases, the fluorescence intensity gradually decreases. The fluorescence the intensity was reduced by 3.0 and 1.14 times at 300 mM acrylamide and 400 mM potassium iodide concentrations, respectively, as illustrated in Figure 3.4 (A, C), which suggests that a neutrally charged molecule acrylamide quenched the tryptophan residues hidden inside the

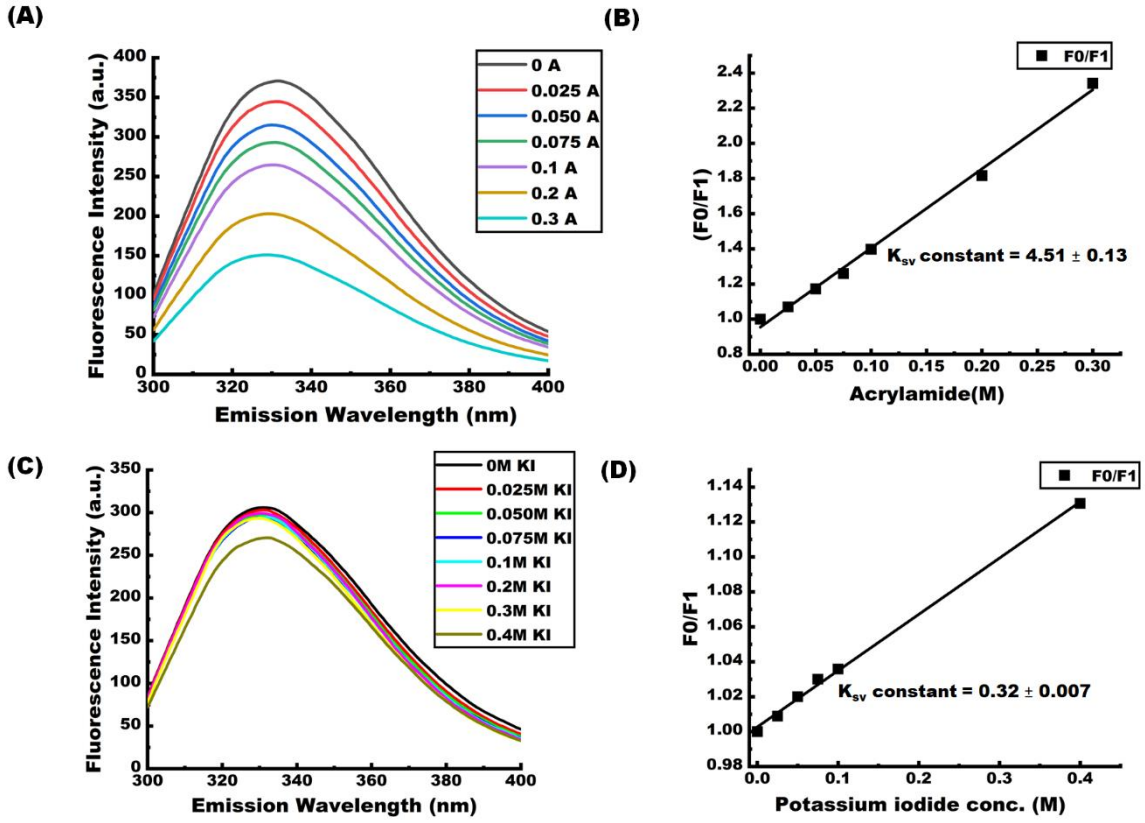

Figure 3.4: Intrinsic fluorescence quenching of LdCS.
Fluorescence spectra of LdCS at different concentration of **(A)** Acrylamide (0-0.3A) and **(C)** Potassium iodide (0-0.4KI) and their Stern-Volmer plots **(B) & (D)** which enumerate their quenching constant.

hydrophobic environment of the protein, while potassium iodide, a charged molecule, was unable to enter the protein and only quenched the tryptophan residues present on the surface of the protein. The quenching constant (Ksv) value of acrylamide and potassium iodide was found to be 4.51 ± 0.13 and 0.32 ± 0.007 $M^{-1,}$ respectively, as displayed in Figure 3.4 (B, D), indicating that the majority of the tryptophan residues of the LdCS were buried in the hydrophobic environment of the protein [Panigrahi et al., 2020].

3.3.5 Urea and Guanidine Chloride-induced unfolding studies of LdCS protein

The urea-induced unfolding of LdCS was studied by fluorescence spectra, where the change in the folding pattern of LdCS was observed at different urea concentrations through its change in fluorescence intensity and maximal fluorescence wavelength. The unfolding induced by urea manifestly displayed two transition states. During the first transition, when the urea concentration increased from 0 to 4 M, there was a red shift in the wavelength maxima and an increase in fluorescence intensity. These alterations suggested that an intermediate, distinct from the native LdCS in terms of fluorescence quantum yield, had formed. The fluorescence spectrum of this intermediate was sightly redshifted, suggesting that the tryptophan residue environments were not significantly changed. During the second transition, when the urea concentration increased from 4 to 5 M, an additional denaturation process was observed through its decrease in fluorescence intensity and a significant red shift from 320 nm to 330 nm, as indicated in Figure 3.5 (A). Further, no more noticeable changes were observed in wavelength maxima and fluorescence intensity with increased urea concentration. This fluorescence redshift indicated that the tryptophan residues became increasingly exposed to the polar environment as LdCS unfolded in urea. After normalizing the data and fitting them into a two-state equation, the mid-transition concentration was found to be 2.85 M, as shown in Figure 3.5 (B).

Similarly, a change in the folding pattern of LdCS was observed at different concentrations of GnHCl through a change in its fluorescence intensity. The unfolding induced by GnHCl also displayed the two-transition state. During the first transition, when GnHCl concentration was increased from 0 to 1 M, there was an increase in fluorescence intensity with no shift in wavelength maxima, suggesting that the protein was in its native state. During the second transition, when increased the GnHCl concentration was from 1 M to 3 M, there was a decrease in fluorescence intensity, and a large redshift from 338 nm to 352 nm was observed,

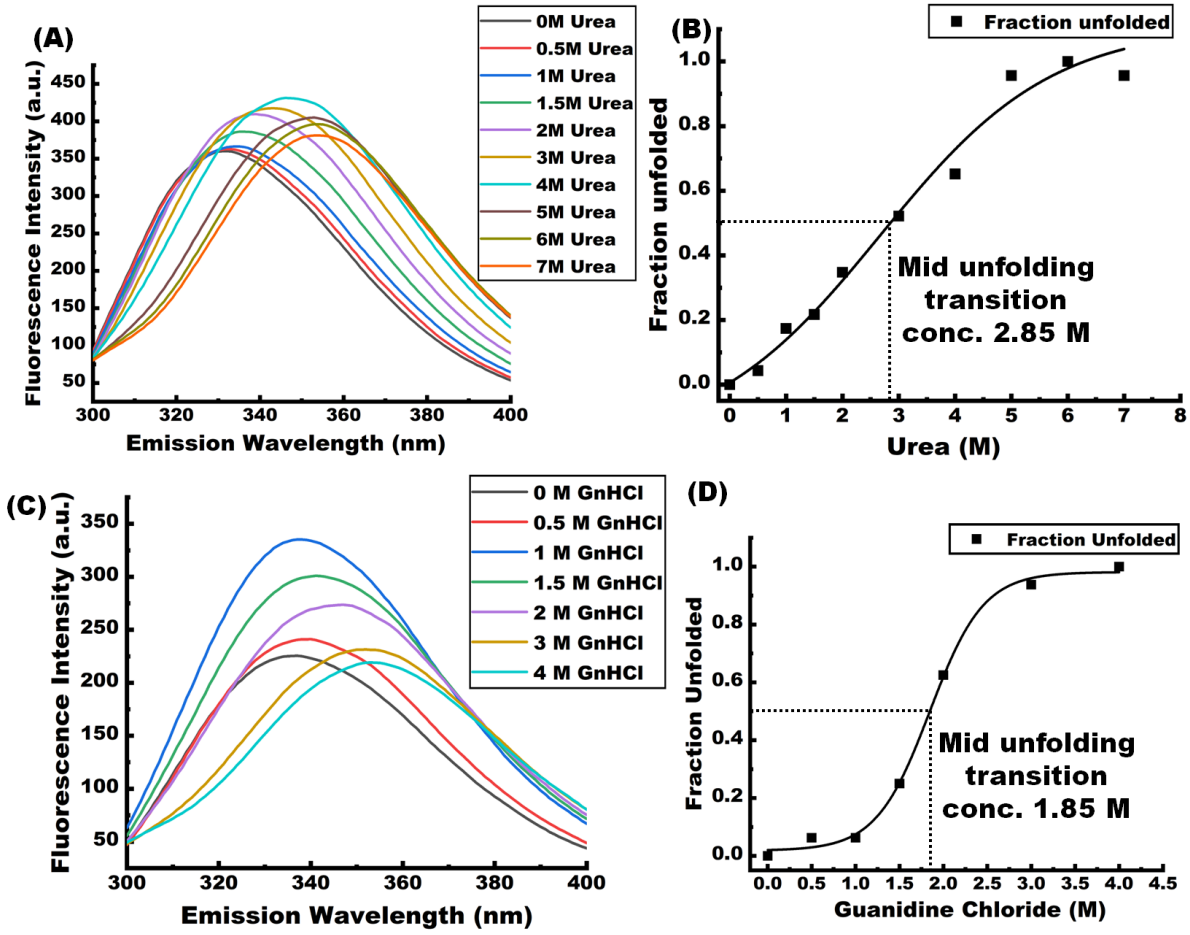

Figure 3.5: Intrinsic fluorescence unfolding studies of LdCS.
Fluorescence spectra of LdCS at increasing concentrations of **(A)** Urea (0 -7 M) and **(C)** Guanidine Chloride (0 – 4 M) and their unfolding plots **(B) & (D),** which enumerate their mid-unfolding transition concentration after fitted into two state equation.

as shown in Figure 3.5 (C), which indicate denaturation of protein and also exposure of tryptophan residues to the polar environment. However, no noticeable change was observed in wavelength maxima and fluorescence intensity when further increasing the GnHCl concentration from 3 M to 4 M. All data was normalized and fitted into the two-state equation; the mid-unfolding transition concentration was found to be 1.85 M, which is shown in Figure 3.5 (D).

3.4 Discussion

The leishmanial parasite undergoes a complex life cycle, encompassing the promastigote and amastigote stages, which exhibit resilience to pH and temperature fluctuations. These environmental changes prompt adaptations in the parasite's development within vectors, accompanied by structural alterations in parasitic proteins. Consequently, understanding the physical properties of these proteins and their roles in maintaining and disseminating the parasite at specific stages has become imperative (Narsimulu et al., 2022). In this study, the LdCS gene was cloned and expressed in an *E. coli* host, followed by purification from the organism. Additionally, far-UV CD and fluorescence spectroscopy were employed to elucidate the protein's secondary and tertiary structures. Circular dichroism spectroscopy provides insights into protein secondary structure and unordered conformations based on the magnitude and wavelengths of ellipticity bands (J. Greenfield, 2006). Analysis of LdCS revealed a predominant alpha-helical content with elevated ellipticity at physiological pH, indicating high protein folding, consistent with other leishmanial enzymes such as protein phosphatase 1 (Qureshi et al., 2019), peptidase T (Bhat et al., 2020), and ribulose-5-phosphate epimerase (RPE) (Narsimulu et al., 2022). Thermal denaturation studies conducted on LdCS demonstrated its resistance to structural changes upon unfolding. However, once denatured, the protein fails to revert to its original structure upon gradual cooling, resulting in irreversible denaturation and protein inactivation. This cooperative unfolding behavior resembles other

leishmanial proteins, such as protein phosphatase 2C (Qureshi et al., 2018) and RPE (Narsimulu et al., 2022).

Intrinsic fluorescence spectroscopy was employed to evaluate the molecular conformational changes of the tertiary structure of the protein based on their fluorescence spectra. The protein contains tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) fluorophore residue, specifically the indole ring containing tryptophan that helps in exploration of their conformation, dynamics and intermolecular interaction. The slight modification in the protein leads to relatively large variations in their fluorescence properties.

It is well known that the emission maxima of water-accessible tryptophan residue will be above 350 nm, and completely buried tryptophan residue in the hydrophobic environment will be near 330 nm [Khan, 2014]. In the case of LdCS, the emission maxima were found close to the buried tryptophan residues, implying that the LdCS protein is present in its native folded state and has retained its conformational stability. The fluorescence spectra of LdCS protein with increased pH from 4 to 10 show a gradual decrease in its intensity without any significant change in emission maxima, which means that the tryptophan residue interacted with the quenching agent either in solvent or in the protein itself [Khan et al., 2013]. Additionally, ANS, an extrinsic fluorescence dye, was used to check the folding pattern of the LdCS protein. This dye, when bound with a loosely packed, solvent-accessible hydrophobic core of the protein, exhibits high fluorescence with emission maxima in the range of 480-490 nm, whereas, in an aqueous solution, its fluorescence intensity will be minimal and emission maxima will be found above 500 nm [Lamba et al., 2009]. The several-fold increase in fluorescence of LdCS protein with a red shift in acidic pH revealed the exposed hydrophobic surfaces. This means the LdCS protein adopted a partially or fully unfolded state in an acidic environment. The other reason might be the protonation of negatively charged groups that facilitate the ANS binding. The insignificant change in fluorescence intensity at neutral pH

may be a well-buried hydrophobic region in the interior of the LdCS protein [Wahab, 2018]. Similar patterns were also seen in other leishmanial proteins, including pyridoxal kinase [Are et al., 2020] and 6-phosphogluconate dehydrogenase [Jakkula et al., 2021]. Further, to reveal the structural features of the LdCS protein, intrinsic fluorescence quenching studies were performed using quencher potassium iodide and acrylamide. The decrease in fluorescence intensity in the presence of acrylamide specifies the non-fluorescent complex formation near tryptophan residues. Compared to KI, the higher quenching constant of acrylamide revealed that most of the tryptophan residues are hidden inside the core of the protein and are accessible to the acrylamide [Panigrahi et al., 2020]. This is also observed in other leishmanial proteins like aspartyl-tRNA synthetase [Panigrahi et al., 2020], indicative of a folded protein.

The urea and GnHCl-mediated unfolding studies of LdCS were performed using intrinsic fluorescence spectroscopy. There was an increase in fluorescence intensity and minimal red shift in wavelength maxima observed when urea and GnHCl concentrations were used up to 4 M and 1M, respectively, which means that the protein was in either its native state or in transition state. However, when a further increase in both urea and GnHCl concentration, there was a decrease in fluorescence intensity, and a significant red shift in wavelength maxima was observed, which means that protein gets denatured or unfolded and tryptophan residues get exposed to the polar environment. Thus, LdCS had shown the two-state transition from the native to an unfolded state like other leishmanial protein ribulose-5- phosphate epimerase [Narsimulu et al., 2022]. Compared to folded proteins, unfolded proteins are more likely to be bound by the uncharged molecule urea [Siddaramaiah et al., 2017]; [Narsimulu et al., 2022].

3.5 Conclusion

In this investigation, we delved into the structural and functional attributes of LdCS and discovered that this enzyme maintains its folded state and catalytic activity within the bacterial expression system. Analysis revealed a substantial presence of alpha-helical structures in the

protein at physiological pH, with a determined melting temperature (Tm) of 44°C. Most fluorophore residues within LdCS are sequestered within the protein's hydrophobic core, inaccessible to solvents. Unfolding studies conducted in urea and GnHCl solution demonstrated the enzyme's moderate tolerance to urea and GnHCl. Overall, our findings shed light on the structural and biochemical characteristics of LdCS, suggesting its potential as a drug target.