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Analysis of structural stability of human prosecretory mitogenic lacritin by circular dichroism

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Analysis of Structural Stability of Human Prosecretory Mitogenic Lacritin by Circular
Dichroism

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Integrated Science and Technology
James Madison University

by Anna Patricia Desmarais and
Casey Quinn Ramirez Cortes

Accepted by the faculty of the Department of Integrated Science and Technology, James Madison University, in partial fulfillment of the requirements for the Honors College.

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Preface

To complete this thesis, co-authors Annie Desmarais and Casey Ramirez Cortes collaborated during all phases of the study. For initial data collection, Annie and Casey worked together to prepare samples and obtain appropriate concentrations. Annie then completed CD procedure for unmutated lacritin and all salt bridge mutants while Casey completed CD procedure for control samples and hydrophobic mutants. The accumulated data was then put together for analysis.

Abstract

Purpose: Lacritin is a human tear glycoprotein that has high thermal stability. When cleaved, lacritin has antimicrobial activity resulting from the C-terminus amphipathic alpha helical region. The alpha helices contain three salt bridges; ionic bonds between neighboring oppositely charged amino acids. The purpose of this research was to investigate the hypothesis that the salt bridges within the alpha helices contribute to the high thermal stability.

Methods: To determine the role of salt bridges in the thermal stability of lacritin, point mutants were prepared for each salt bridge by site directed mutagenesis that replaced the oppositely charged amino acids with serine. The point mutants were expressed in *E. coli* and purified. Western blot analysis confirmed the identity of lacritin proteins. Circular dichroism (CD) was used to study conformational changes in the secondary structure of these mutants compared to unaltered lacritin along with two controls, bovine serum albumin (BSA) and lysozyme. Data collected was analyzed with the alpha helix formula to determine the percent alpha helix structure at ten degree increments from 25-85⁰C, using poly-l-lysine as the standard.

Results: The mutated proteins reacted with lacritin specific antibodies in Western blot analysis. Under thermal denaturation conditions, the control proteins both had a significant decrease in alpha helical structure while alpha helical structure of normal lacritin increased slightly. At 25⁰C, the mutants had 12-25% less alpha helix than unaltered lacritin. Increasing the temperature did not have a significant impact on alpha helix structure.

Conclusions: The salt bridges play a role in formation of the alpha helices but not in overall thermal stability of lacritin.

Introduction

Discovery, Structure, and Function of Lacritin

Dr. Gordon Laurie discovered and named lacritin, a human tear glycoprotein produced in the lacrimal gland in 2001 (Sanghi *et al*, 2001, Ma *et al*, 2008). Following this discovery, a consortium was formed to further the characterization of lacritin and explore the possibilities of developing new therapeutics for various ocular diseases.

Lacritin is secreted from the lacrimal gland via acinar secretory granules and ducts and emerges on the surface of the eye as a component of tears (Sanghi *et al*, 2001, Ma *et al*, 2008). McKown *et al* found that lacritin is mitogenic, prosecretory, and, when cleaved, displays antimicrobial activity (McKown *et al*, 2014). The structure of lacritin plays a crucial role in how it functions in tears. It is 119 amino acids long and 12.3 kDa in size with two alpha helices between base pairs 65 to 88, and 93 to 113, respectively (Figure 1) (Zhou *et al*, 2006). Both alpha helices are amphipathic, being nonpolar and hydrophobic on one side and polar and hydrophilic on the other (Figure 1). These helices contain three salt bridges; ionic bonds between neighboring positively and negatively charged amino acids (McKown *et al*, 2014). The first salt bridge is between residues K66 and E70, the second is between residues K95 and E99, and the third is between E103 and K107.

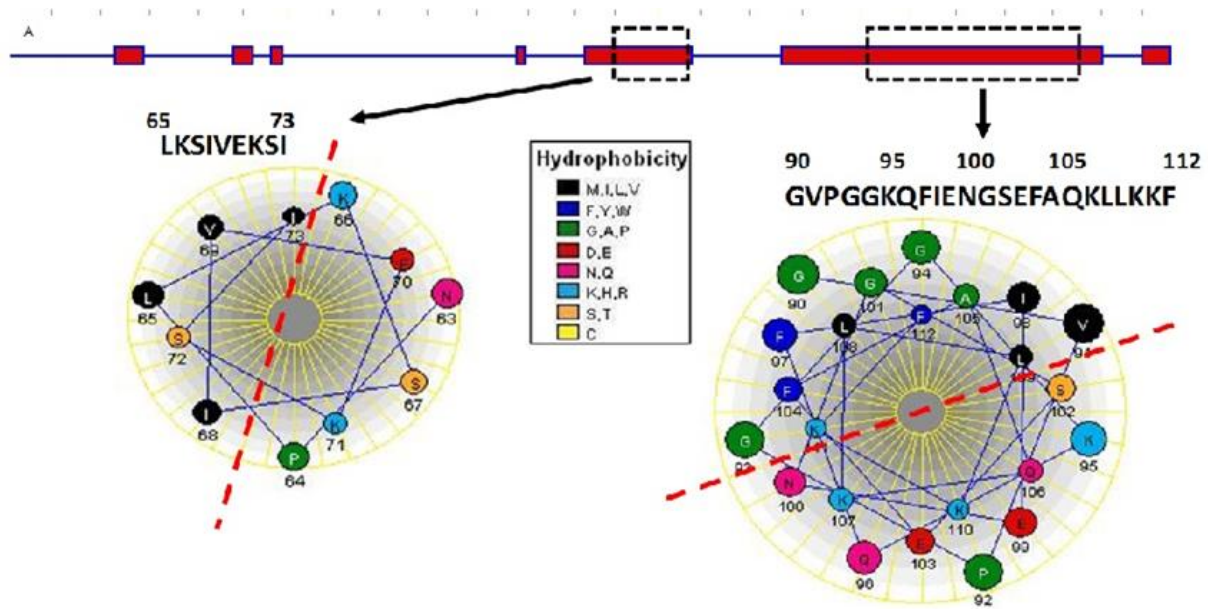


Figure 1. Helical wheel diagram of the two alpha helices found in lacritin with the amino acids labeled by number from the N-terminus to the C-terminus and the relative hydrophobicity by color coding. The dotted line represents the division between the hydrophobic side, top left, and the hydrophilic side, bottom right.

Purpose of the Structural Analysis of Lacritin

McKown *et al* found that lacritin is thermally stable up to 100⁰C, indicating that it does not denature at high temperatures as other proteins typically do (McKown *et al*, 2014). The reason behind this unusual thermal stability has not been determined. A structural analysis of lacritin could lead to the understanding of not only its thermal stability but also other functions such as its antimicrobial activity. It is hypothesized that the salt bridges in the alpha helices contribute to the thermal stability of lacritin.

Circular Dichroism Spectroscopy

Circular Dichroism (CD) is a spectroscopy technique that uses right-hand and left-hand circular polarized light to study the secondary structures of proteins (Fasman, 1996). CD can collect spectra in the far ultraviolet region, which spans from 178 to 260 nm. In this region, the spectra are produced from the amide bond of the protein absorbing a specific wavelength

producing a unique spectrum. The spectra produced represent the secondary structure of the protein and can be used to study conformational changes in a single protein caused by external factors such as temperature (Figure 2). Generally, negative ellipticity values at 222 and 208 nm represent an alpha helix and increases or decreases in the ellipticity value indicate a conformational change in the alpha helix (Greenfield, 2006). CD was chosen to analyze the structure of lacritin because its alpha helical structure is easily observed through the generated spectra. The temperatures used to study thermal stability ranged from 25⁰C to 85⁰C. The salt bridges were analyzed by site directed mutagenesis, removing a positive or negative amino acid and replacing it with a nonpolar amino acid to destroy one salt bridge at a time. For double mutants, several positive/negative amino acids were removed to break two salt bridges simultaneously. The mutant lacritin was then exposed to the same temperature range to see if the alpha helix underwent a conformational change. The primary objective of this study was to confirm that the thermal stability of lacritin, found via CD thermal denaturation, is mainly a factor of the salt bridges in the second alpha helix.

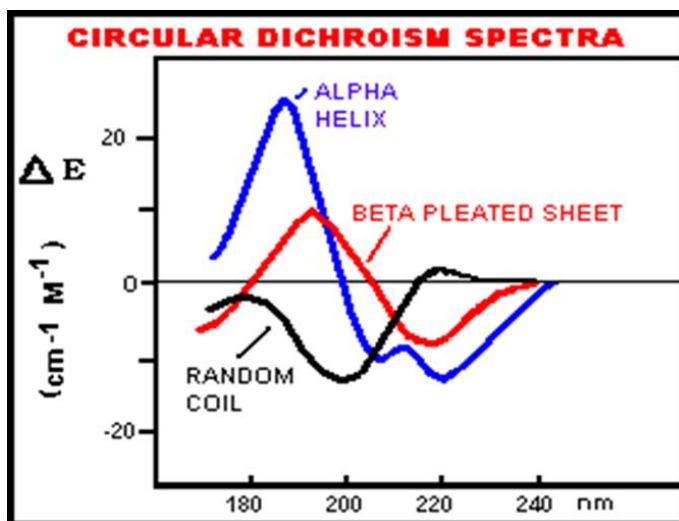


Figure 2. An example of what different secondary structures look like on CD spectra. The magnitude of the blue line at the specified wavelengths, 208 and 222 nm, illustrates relative amount of alpha helical structure.

Materials and Methods

Circular Dichroism Analysis

All samples used had a concentration of 0.2 mg/mL or greater. CD spectra were obtained using the Jasco 810 CD/ORD with Fluorescence Monochrometer in a 1 mm quartz cell from Starna Cells, Inc. The spectra were obtained at 25°C from 250 nm to 190 nm in continuous scanning mode (scanning speed, 100 nm/min; data pitch, 0.1 nm; bandwidth, 1 nm; response time, 4 s) with a nitrogen flow rate of 100 mL/min. An average of 3 spectra were obtained for each sample. Thermal denaturation was performed starting at 25°C increasing in 10°C increments to 85°C. Degree of rotation (mdeg) at 222 nm was recorded for each sample. At 222 nm, the value in mdeg of poly-l-lysine in NaOH buffer gives the value for 100% alpha helix and the value in HCl buffer gives the value of 0% alpha helix. These values were used in the alpha helix formula to calculate structural composition of other proteins (Figure 3).

$$\frac{(\theta_{222 \text{ nm sample}}) - (\theta_{222 \text{ nm } 0\% \alpha \text{ helix poly-l-lysine}})}{(\theta_{222 \text{ nm } 100\% \alpha \text{ helix poly-l-lysine}}) - (\theta_{222 \text{ nm } 0\% \alpha \text{ helix poly-l-lysine}})} = \% \alpha \text{ helix}$$

Figure 3. The formula used to calculate percent alpha helix. The theta represents the degree of rotation in mdeg at 222 nm (Greenfield, 2004).

Sample Preparation

Poly-L-Lysine

Poly-l-lysine was the standard protein used to compare percent alpha helix to lacritin and other proteins. Poly-l-lysine samples were made at a concentration of 0.3 mg/mL from a stock solution of 10 mg/mL from Sigma. NaOH buffer was prepared by adding 2.0 g (0.05 mol) of NaOH to 500mL of deionized H₂O to give a final concentration of 0.1 M. HCl buffer was prepared by adding 4.13 mL (4.91g, 0.135 mol) of 12 M HCl to 500mL of deionized H₂O to give

a final concentration of 0.100 M. Poly-l-lysine was analyzed by CD in 0.1 M NaOH and then in 0.1 M HCl with a concentration of 0.3mg/mL each time.

Lysozyme

Lysozyme from chicken egg white was used as a comparison protein for lacritin. Lysozyme samples with a concentration of 0.3 mg/mL were made from a stock solution of 10 mg/mL from Sigma. Phosphate-buffered saline (PBS) solution was prepared from a pre-packaged sample from Sigma, giving a final concentration of 0.01 M. Lysozyme was analyzed by CD and the mdeg value at 222 nm was compared to that of poly-l-lysine to give a percent alpha helix using the formula (Figure 3).

BSA

Bovine serum albumin (BSA) was used as a second comparison protein for lacritin. BSA samples were made to a concentration of 0.300 mg/mL from a stock solution of 10 mg/mL from Sigma. The procedure and concentrations used to make the NaOH and HCl buffers is the same as for poly-l-lysine. BSA was analyzed by CD and the mdeg value at 222 nm was compared to that of poly-l-lysine to give a percent alpha helix using the formula (Figure 3).

pLAC (Recombinant lacritin made in E. coli.)

pLAC was used to establish a baseline for percent alpha helix of lacritin. A concentration of 520 µg/mL was made from a stock solution of 10 mg/mL. PBS solution was prepared from a pre-packaged sample from Sigma, giving a final concentration of 0.01 M. pLac was analyzed by CD and the mdeg value at 222 nm was compared to that of poly-l-lysine to give a percent alpha helix using the formula (Figure 3).

Salt Bridge Mutants

A total of four different mutants, each missing a salt bridge within the alpha helices, were used and analyzed by CD to study alpha helix formation and protein structural stability. The first mutant, K66S/E70S, replaced amino acids K66 and E70 with serines at a total concentration of 357 $\mu\text{g}/\text{mL}$. The second mutant, K95S/E99S, replaced K95 and E99 with serines for a total concentration of 163 $\mu\text{g}/\text{mL}$. K95S/E99S was dialyzed using a dialysis membrane in 1x PBS to bring the total protein concentration up to 250 $\mu\text{g}/\text{mL}$, making it high enough to obtain an effective signal for CD analysis. The third mutant, K66S/E70S E103S/K107S, replaced amino acids K66, E70, E103, and K107 with serines, and was made to a concentration of 280 $\mu\text{g}/\text{mL}$. The fourth mutant, E103S/K107S, is missing a salt bridge in the larger alpha helix due to replacement of amino acids E103 and K107 with serine and was made to a concentration of 289 $\mu\text{g}/\text{mL}$.

Hydrophobic Mutants

Five different mutants that were within the hydrophobic region of the alpha helices were prepared so that they could be analyzed by CD. The first mutant replaced amino acids L108, L109, and F112, located in the larger alpha helix, with serine. This mutant, referred to as L108S/L109S/F112S, had a total concentration of 530 $\mu\text{g}/\text{mL}$. The second mutant replaced amino acids V91 and L109 in the larger alpha helix with serine was referred to as V91S/L109S. For effective CD analysis, V91S/L109S required dialysis with a dialysis membrane in 1x PBS overnight to bring the total concentration up to 300 $\mu\text{g}/\text{mL}$. The third mutant replaced amino acids I68 and I78 with serine, referred to as I68S/I78S and had a total concentration of 205 $\mu\text{g}/\text{mL}$. The fourth mutant analyzed replaced amino acid F112 with serine, referred to as F112S, and had a total concentration of 292 $\mu\text{g}/\text{mL}$. The fifth mutant analyzed replaced amino acid L109

with serine, referred to as L109S. L109S required dialysis with a dialysis membrane in 1x PBS overnight to bring the total concentration up to 306 $\mu\text{g}/\text{mL}$.

Western Blot Analysis

SDS-PAGE was used to determine protein purity and Western blot analysis was used to confirm the protein identity. Sodium dodecyl sulfate polyacrylde gel electrophoresis (SDS-PAGE) was performed using samples of interest. Samples were then boiled for five minutes and centrifuged at 1300 rpm for ~5 s and each lane was loaded with 20 μL of the corresponding sample. The Precision Plus Protein Kaleidoscope Standard marker (8 μL) was added into the final lane. The gel was run for 40 minutes at 200v.

Results

Determination and Confirmation of Standard Values for Formula

Before circular dichroism analysis could be completed or data could be analyzed, standard alpha helical composition values were established using Poly-L Lysine in NaOH and HCl buffers (Figure 4). The standard values were -52.47 mdeg for 100% alpha helix and 3.16 mdeg for 0% alpha helix. When compared to published data, the standard values obtained using these Poly-L Lysine samples were very consistent.

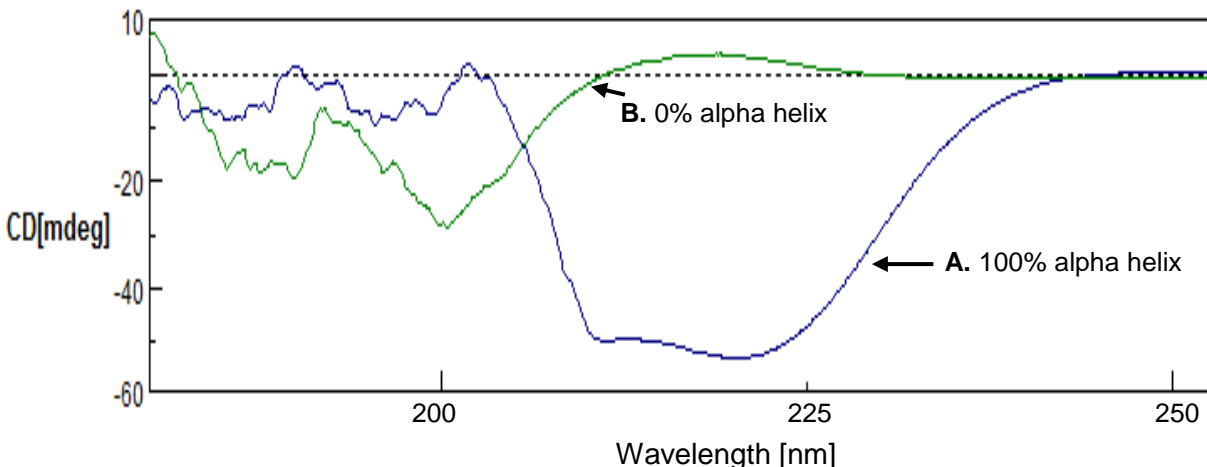


Figure 4. CD spectra of Poly-L Lysine standards. 0.3mg/mL Poly-L Lysine in A) 0.1 M NaOH and B) 0.1 M HCl. Values for mdeg of rotation at 222 nm were -52.47 mdeg and 3.16 mdeg, respectively.

To further confirm these standard values, bovine serum albumin (BSA) and lysozyme were tested. At 25° C, BSA was determined to have an alpha helical structure of 45.8% (Figure 5, Table A2). As the experimental temperature was increased to 25° C, alpha helical structure decreased to 19.9% (Table A2).

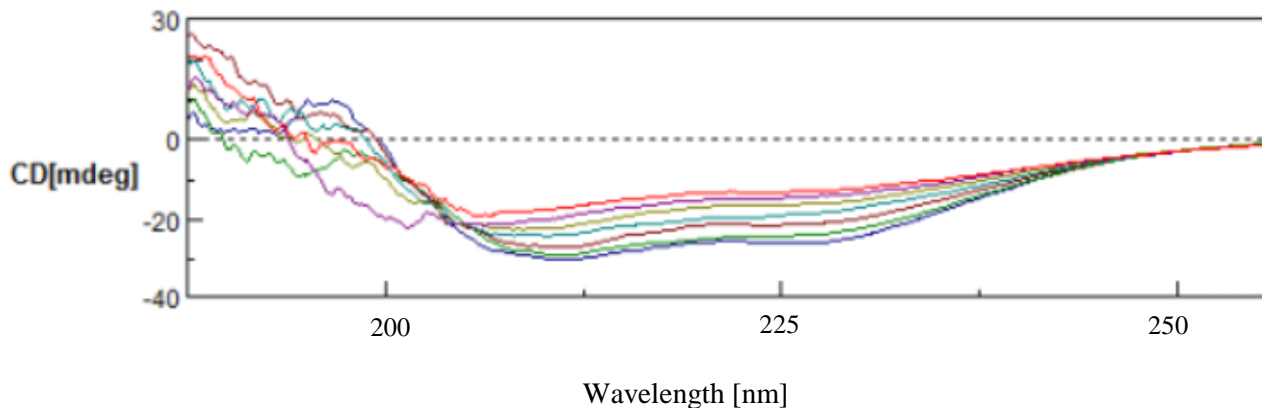


Figure 5. CD spectra of BSA. Thermal denaturation of 0.3 mg/mL BSA in 0.1 HCl, spectra were taken from 25-85°C in increments of 10°C. Black is 25°C, Brown is 35°C, Teal is 45°C, Neon green is 55°C, Purple is 65°C, Green is 75°C and Red is 85°C.

At 25° C, Lysozyme was determined to have a rotation of -19.53 mdeg, resulting in 33.1% alpha helix structure (Figure 6). This alpha helical structure dropped to 17.4% as the experimental temperature increased from 25° C to 85° C (Figure 6, Table A1).

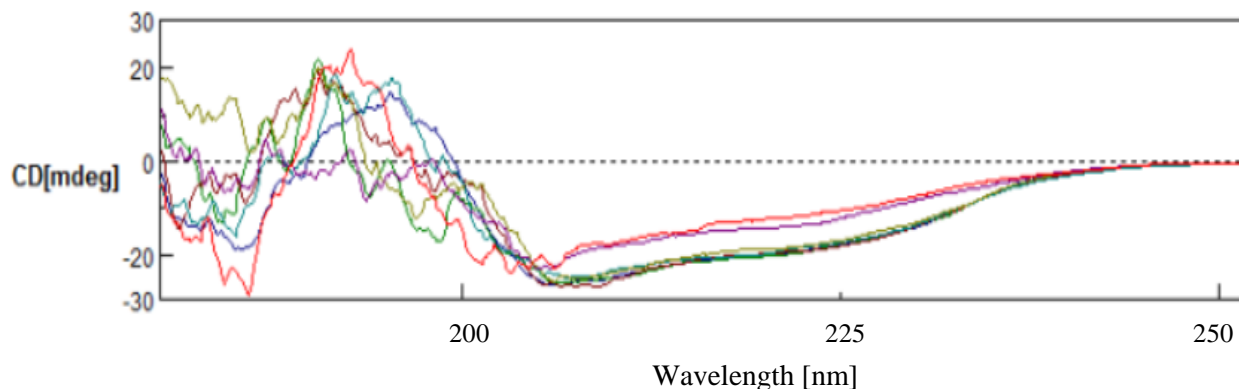


Figure 6. CD spectra of Lysozyme. Thermal denaturation of 0.3 mg/mL Lysozyme in PBS, spectra were taken from 25-85°C in increments of 10°C. Black is 25°C, Brown is 35°C, Teal is 45°C, Neon green is 55°C, Purple is 65°C, Green is 75°C and Red is 85°C.

Western Blot Analysis

SDS gel and Western Blot of both salt bridge and hydrophobic mutants were performed to confirm the viability of the mutants. The 20 kDa marker is the predicted weight of glycosylated recombinant lacritin. The hydrophobic mutant, V91S/L109S, was not expressed as indicated by the lack of this band in both the SDS gel and Western Blot. A band was visible at 20 kDa for all other mutants (Figure 7).

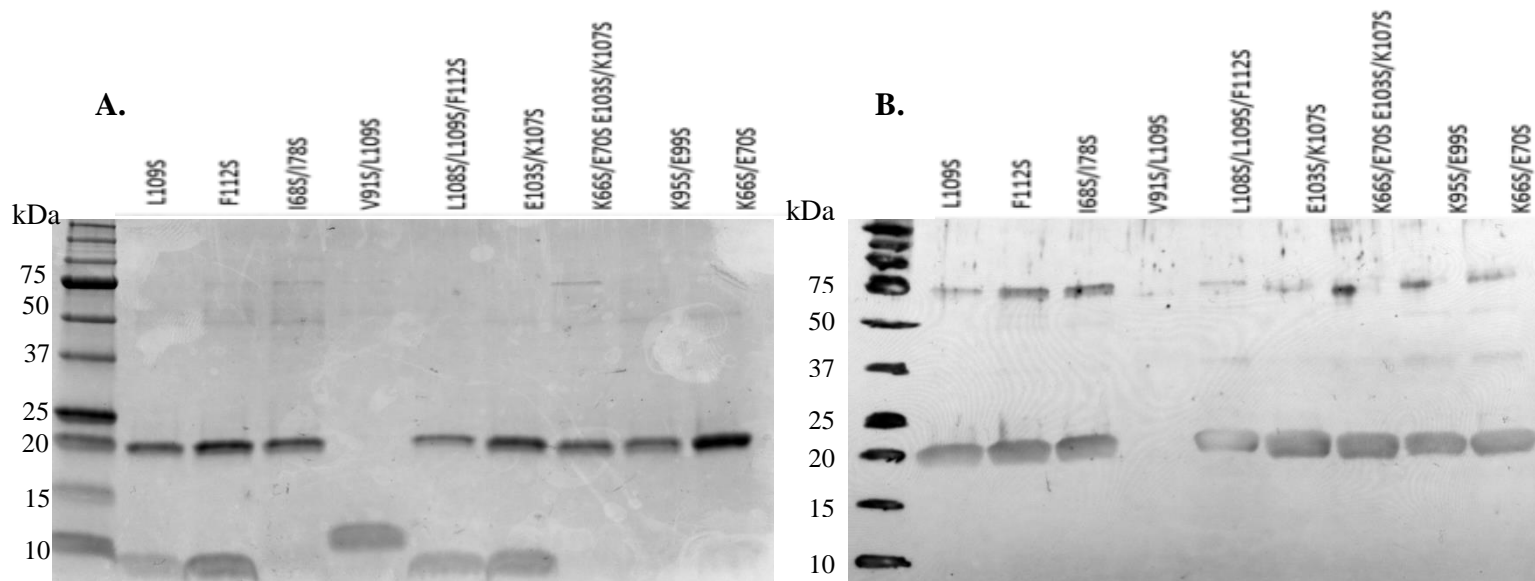


Figure 7. A) SDS-PAGE gel and B) Western Blot were conducted to confirm the presence of the mutants. The marker lane is on the far left with the mutants in each corresponding lane. All mutants were observed at the expected 20 kDa marker except V91S/L109S.

Thermal Denaturation of Lacritin Mutants

Unaltered lacritin was first analyzed at 25 degrees and the resulting graph served as a basis for comparison. Exposed to increasing temperature, the alpha helical structure of Lacritin increased from 33.5% at 25° C to 43.6% at 85° C (Figure 8).

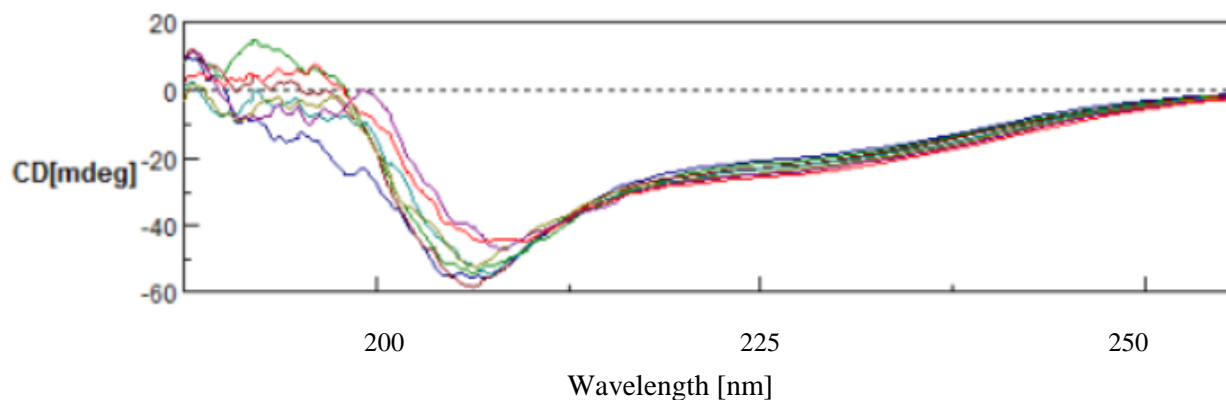


Figure 8. CD spectra of Lacritin (pLAC). Thermal denaturation of 520 µg/mL of pLAC in PBS spectra were taken from 25-85°C in increments of 10°C. Blue is 25°C, Green is 35°C, Black is 45°C, Teal is 55°C, Neon Green is 65°C, Purple is 75°C and Red is 85°C.

Lacritin mutants were also tested using CD thermal denaturation. Salt bridge mutants were observed first (Figure 9). When K66S/E70S was exposed to increasing temperature, the percent helical structure increased from 19.07% at 25° C to 24.75% at 85° C (Table 1). The percent helical structure of K95S/E99S increased as temperature increased, changing from 11.6% 25° C to 16.8% at 85° C (Table 1). K66S/E70S E103S/K107S had an overall decrease in percent helical structure, dropping from 7.51% at 25° C to 4.26% at 85° C, while E103S/K107S had an overall increase in percent helical structure, increasing from 11.8% at 25° C to 16.4% at 85° C (Table 1). Thermal denaturation data for all salt bridge mutants was compared in tandem to determine significance and examine overall trend in alpha helical nature relative to experimental temperature (Figure 9).

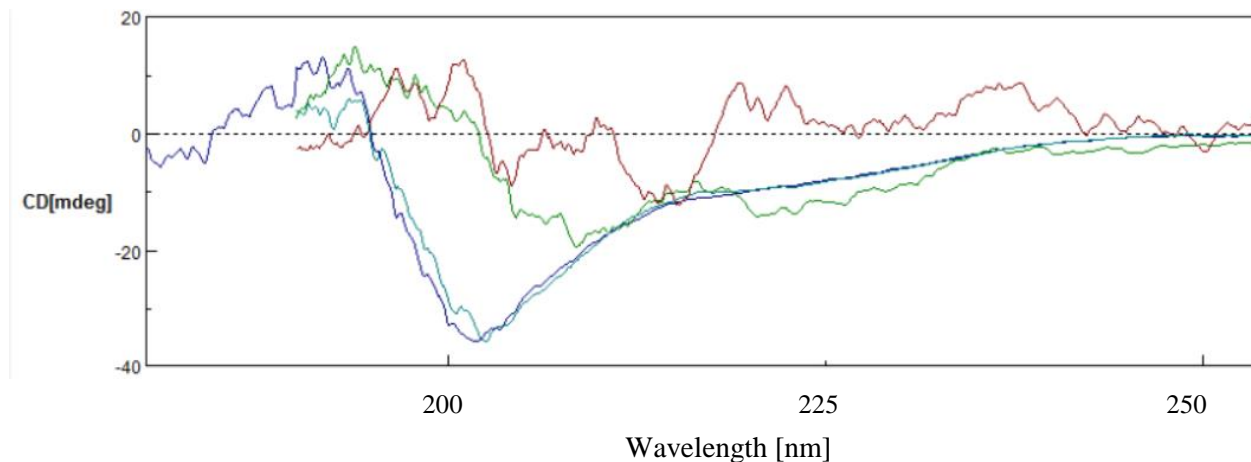


Figure 9. Overlay of the salt bridge mutants at 25°C. The K66S/E70S is in blue, the K95S/E99S is in green, the K66/E70 E103/K107 is in red, and the E103/K107 is in teal.

Table 1. Percent alpha helix relative to temperature. Alpha helical nature of lacritin and salt bridge mutant was measured every ten degrees as temperature increased from 25° C to 85° C.

Sample	25° C	35° C	45° C	55° C	65° C	75° C	85° C
pLAC	33.5%	35.3%	37.5%	38.1%	40.2%	42.0%	43.6%
K66S/ E70S D	19.1%	18.7%	22.9%	22.3%	21.1%	25.6%	24.8%
K95S/E99S D	11.6%	12.4%	13.4%	14.8%	15.0%	14.8%	16.8%
K66S/E70 E103S/K107S	7.5%	6.9%	6.5%	7.3%	6.4%	3.3%	4.3%
E103S/K107S	11.8%	12.8%	14.6%	15.2%	16.4%	15.6%	16.4%

Thermal Denaturation of Hydrophobic Mutants

The hydrophobic lacritin mutants were examined under thermal denaturation conditions (Figure 10). Mutants 2, 3 and 7 required dialysis prior to circular dichroism in order to obtain enough volume at the correct concentration. Hydrophobic mutant 3 did not contain enough protein following dialysis to continue with analysis. Throughout thermal denaturation, the alpha helical structure of all hydrophobic mutants remained relatively constant (Table 2).

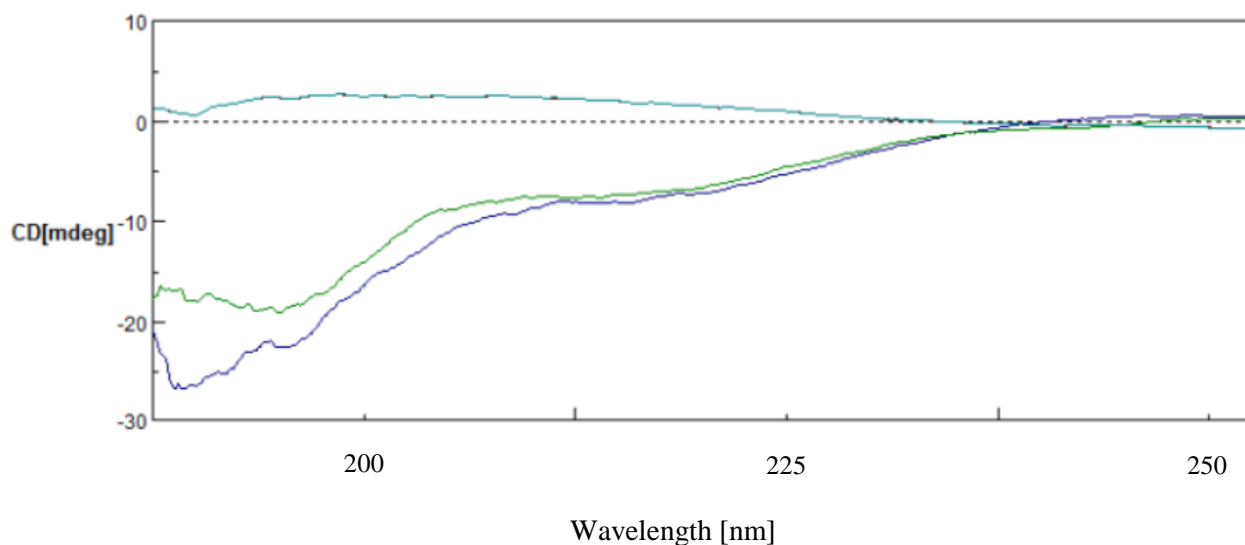


Figure 10. Hydrophobic mutants overlay at 25 degrees. Overlay of the hydrophobic mutants at 25°C. Purple is F112S, Green is I68S/I78S, Red is L109S, Teal is L108S/L109S/F112S.

Table 2. Percent alpha helix relative to temperature. Alpha helical nature of each hydrophobic mutant was measured every ten degrees as temperature increased from 25° C to 85° C.

Mutant	25° C	35° C	45° C	55° C	65° C	75° C	85° C
L108S/L109S/F112S	2.1%	2.1%	2.1%	2.1%	2.1%	2.1%	2.1%
V91/L109 D*	2.1%	2.2%	2.1%	2.1%	2.1%	2.1%	2.1%
I68S/I78 D	8.3%	8.9%	10.1%	9.3%	9.9%	10.8%	10.5%
F112S D	9.7%	12.4%	11.6%	12.0%	13.6%	15.4%	14.4%
L109S D	2.2%	2.0%	2.0%	2.2%	2.0%	2.0%	2.0%

Discussion

At 25⁰C, BSA has more alpha helical structure than either lacritin or lysozyme as shown by the more negative mdeg value of BSA at 222 nm (Figure 11); however, once at 85⁰C lacritin has more alpha helical character than BSA or lysozyme (Figure 11). The decrease in alpha

helical character of BSA and lysozyme at 85^oC indicated that these proteins denature at the higher temperature, while lacritin remains unchanged. The thermal stability of lacritin is evidenced by no significant change in mdeg value at 222 nm.

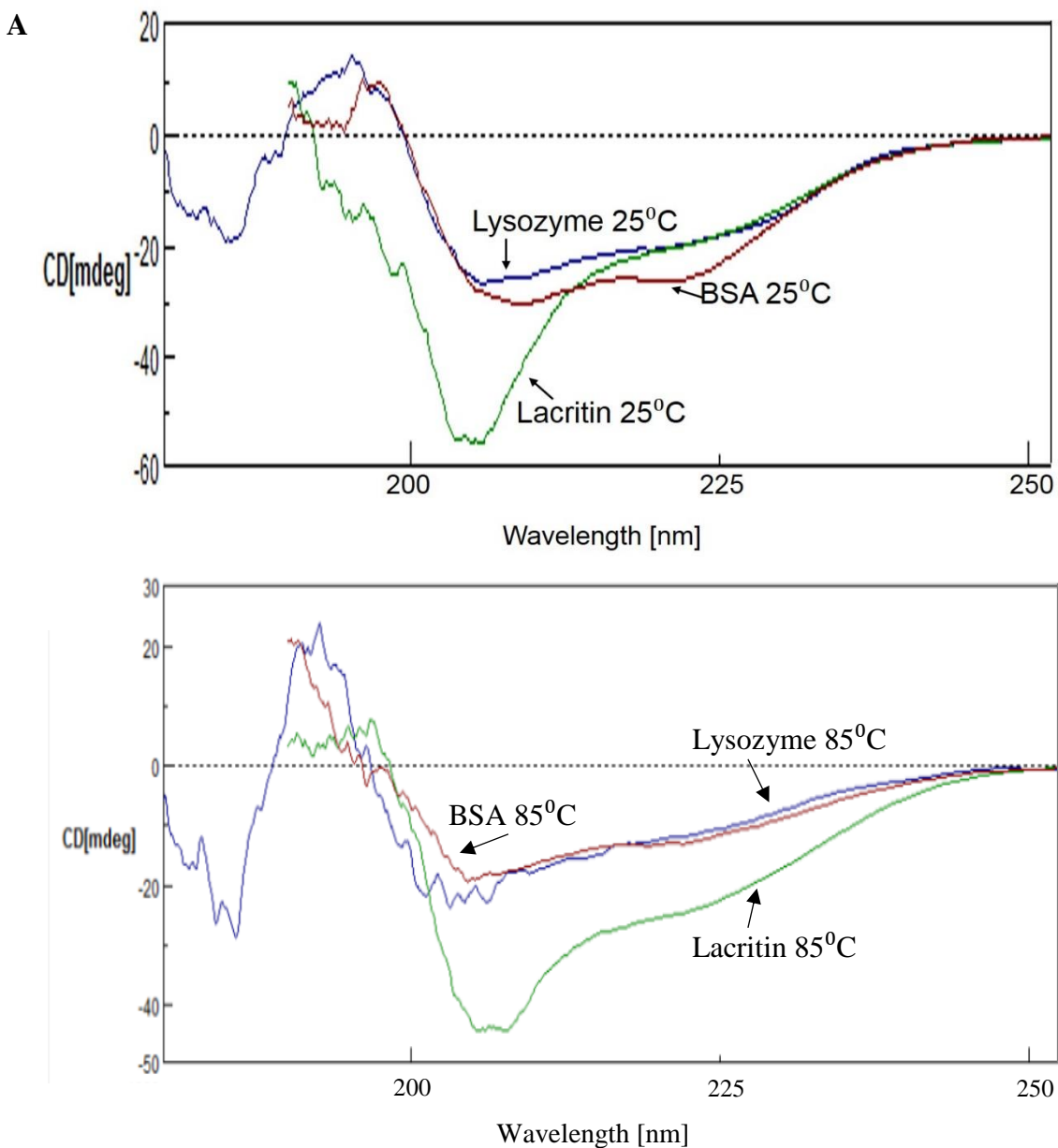


Figure 11. Impact of increasing temperature on secondary structure of lacritin, BSA and Lysozyme. Blue is lysozyme, Green is lacritin, red is BSA. A) 25°C B) 85°C

Lacritin has been observed to have significantly higher thermal stability than other similar proteins (McKown *et al*, 2014). To examine this characteristic, lacritin, BSA, and lysozyme underwent thermal denaturation. Lacritin increased slightly in percent alpha helix as temperature increased from 25° C to 85° C, while BSA and lysozyme significantly decreased in percent alpha helix (Figure 12). This indicated the significant difference in thermal stability between lacritin and other proteins (Figure 12).

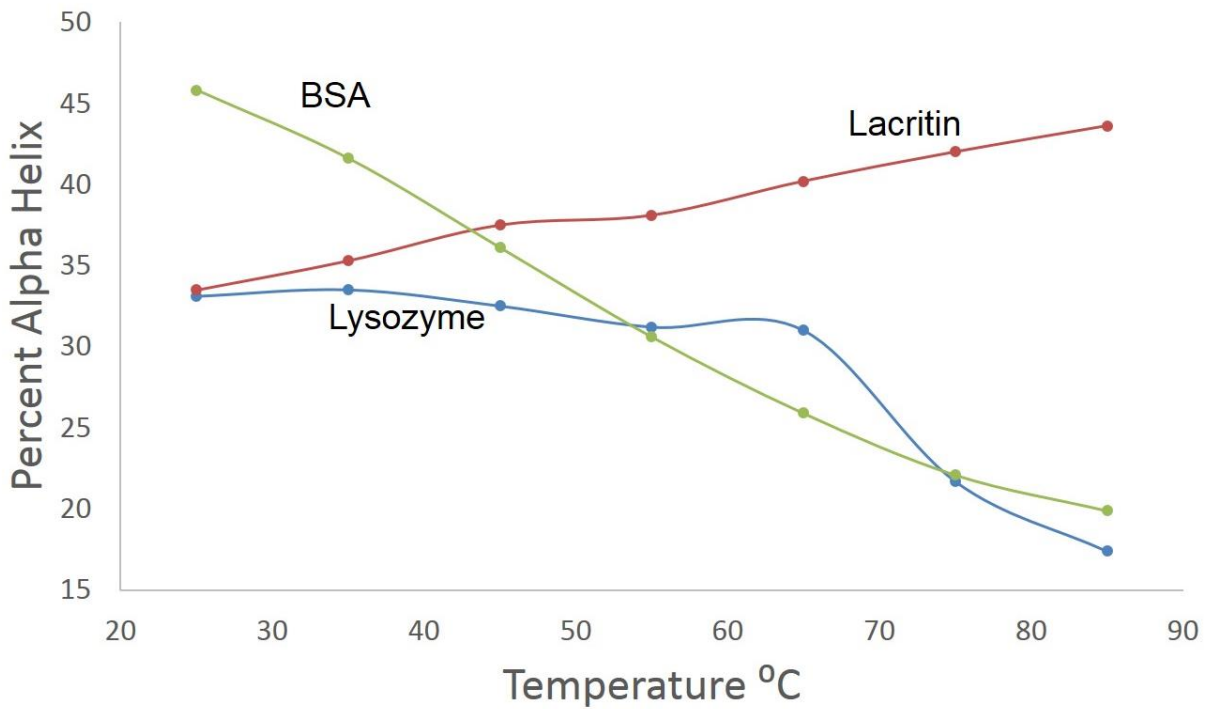


Figure 12. Thermal denaturation data of lacritin (red), BSA (green), and lysozyme (blue) over the temperature range of 25-85°C.

It was hypothesized that the high thermal stability of lacritin resulted from salt bridges within the alpha helices. Thermal denaturation spectra did not support the hypothesis that the salt bridges played a role in thermal stability (Figure 13). However, it did support the conclusion that the salt bridges played a role in the initial formation of the alpha helices (Figure 13). This was indicated by the significant decrease in percent alpha helix of the point mutants at 25°C, but no further significant change in structure as temperature increased by 60° C (Figure 13).

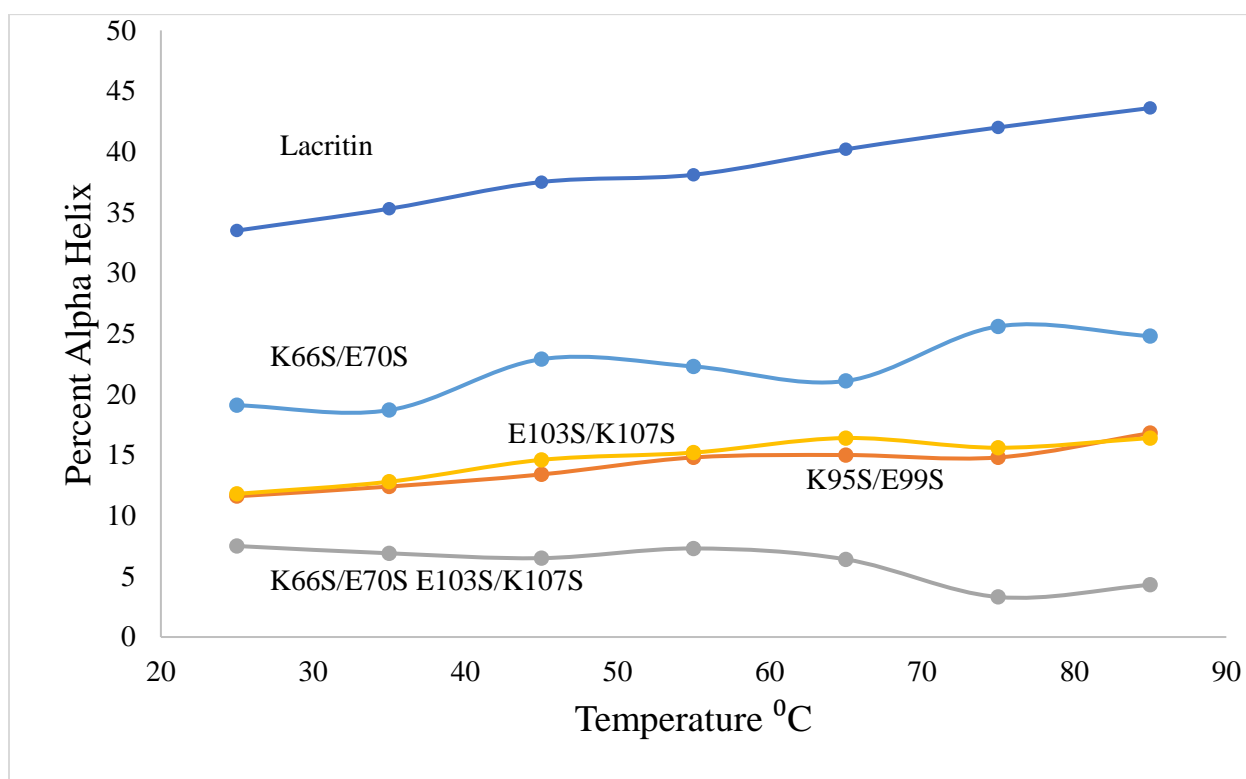


Figure 13. Comparison of Lacritin salt bridge mutants. Thermal denaturation data, collected every ten degrees, for each salt bridge mutant was used to calculate percent alpha helix. Data was graphed in comparison to experimental temperatures. Lacritin is dark blue, K66S/E70S is teal, K95S/E99S is orange, E103S/K107S is yellow, and K66S/E70S E103S/K107S is gray.

The conclusion that salt bridges do not play a role in thermal stability was further confirmed by plotting the CD mdeg at 222 nm versus the temperature to give a melting point of lacritin. If the salt bridges were involved in thermal stability then there would be a significant change in the mdeg values at the steepest part of the line, the identifier of melting temperature. Mdeg values for the salt bridge point mutants did not change significantly, indicating they are not a major player in thermal stability (Figure 14).

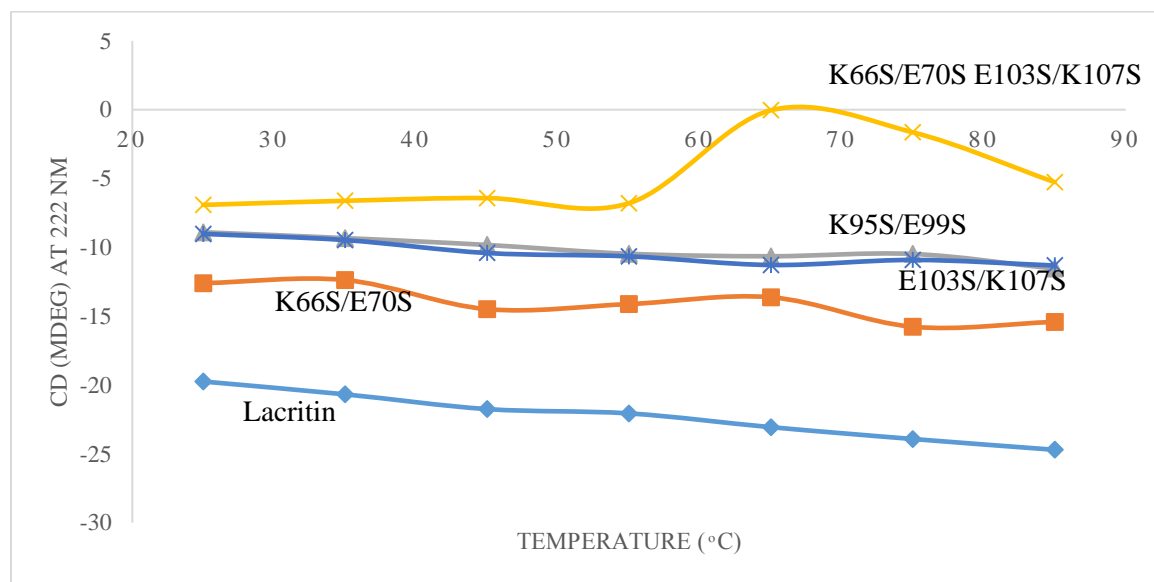


Figure 14. The salt bridge point mutants melting temperature profile. pLac (diamonds) does not display an increase in mdeg as temperature increases, indicating it does not melt. K66S/E70S (squares), K95S/E99S (triangles), K66S/E70S E103S/K107S (x's), and E103S/K107S (asterisks) also do not show significant change.

To further analyze structure, the hydrophobic residues on the amphipathic alpha helices were examined. Mdeg values for the hydrophobic point mutants also did not change significantly (Figure 15). The formation of the alpha helices was impacted but thermal stability was not. This was indicated by a significant decrease in percent alpha helix at 25°C with no further significant decrease as temperature increased (Figure 15).

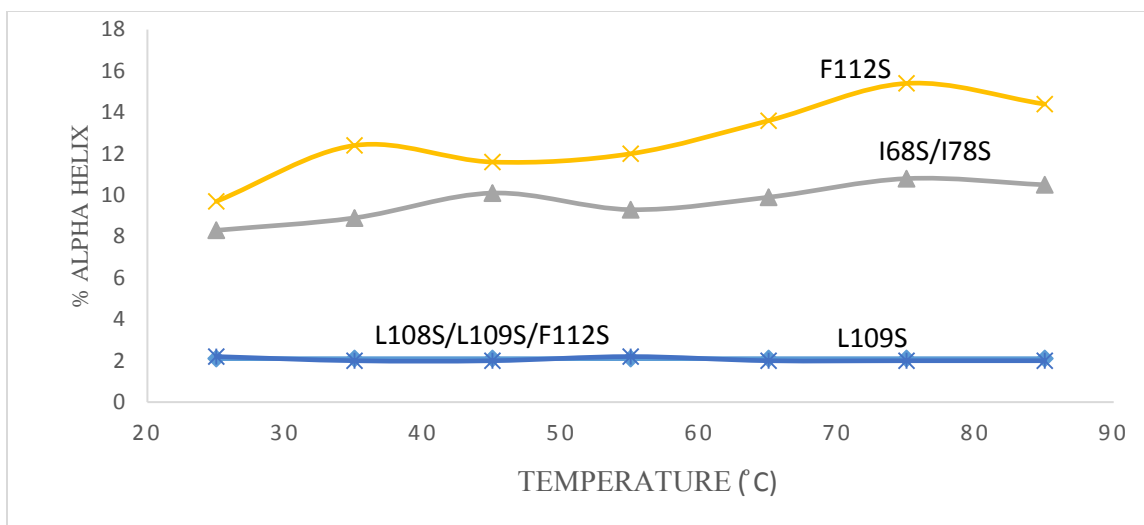


Figure 15. Comparison of lacritin hydrophobic mutants. Thermal denaturation data, collected every ten degrees, for each hydrophobic mutant was used to calculate percent alpha helix. Data was graphed in comparison to experimental temperatures. F112S (x's), I68S/I78S (triangles), L108S/L109S/F112S (diamonds), and L109S (asterisks). L108S/L109S/F112S and L109S displayed almost identical % alpha helices and are overlaid.

The conclusion that the alpha helices do not play a role in thermal stability was further supported by a melting temperature profile for the hydrophobic mutants (Figure 16). Similarly to the salt bridge mutants, there was no significant change in the melting point determining region of the plot, indicating no significant correlation between the hydrophobic mutants and thermal stability (Figure 16).

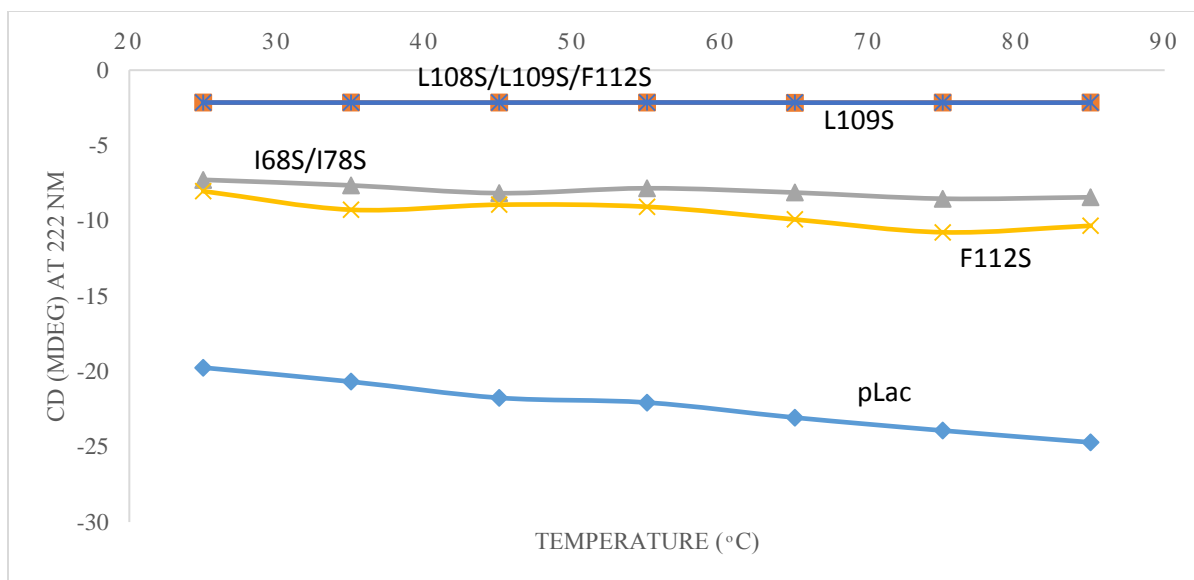


Figure 16. The hydrophobic point mutants melting temperature profile. pLac (diamonds) does not display an increase in mdeg as temperature increases, indicating it does not melt. L108S/L109S/F112S (squares), I68S/I78S (triangles), F112S (x's), and L109S (asterisks) also do not show significant change.

The SDS gel image was further analyzed to examine the predicted self-splicing nature of Lacritin. The location of splice sites and the resulting functional components have been previously studied using SDS and Western blot technique to diagram the protein (Figure 17). In this study, the lower band on the SDS gel, at approximately 8 kD, was visible in lanes for all samples except K66S/E70S, K95S/E99S, K66S/E70S E103S/K107S, and I68S (Figure 7). This indicates that amino acid residues K66, I68, E70, K95 and E99 play a significant role in the self-splicing nature of lacritin.

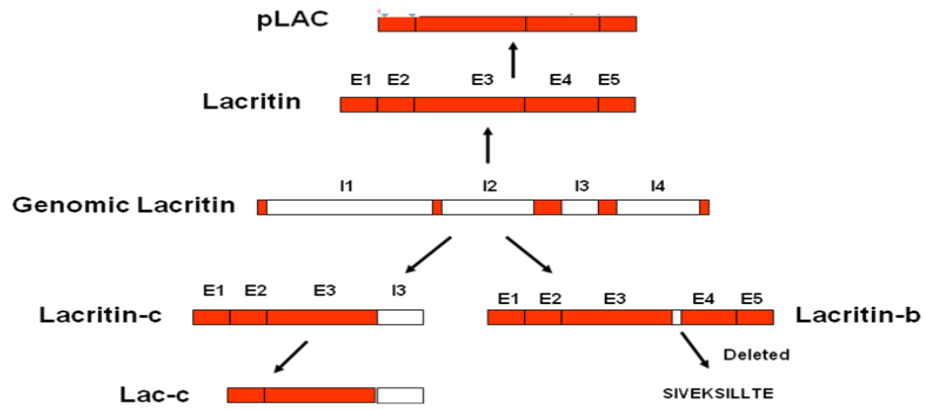


Figure 17. Lacritin splice sites diagram for Western Blot analysis. Genomic structure of genomic lacritin with the predicted splice sites.

Appendix A- Circular Dichroism Mechanism

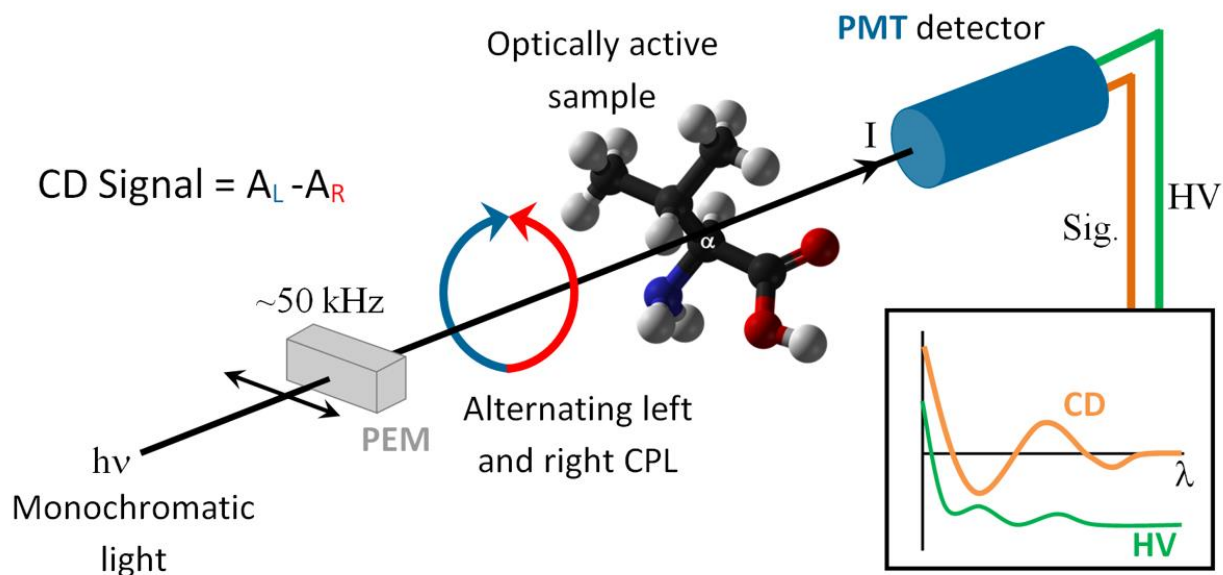


Figure A. Mechanism of circular dichroism. Diagram of circular dichroism procedure including light direction, detector, and output sample.

Appendix B- Western Blot Mechanism

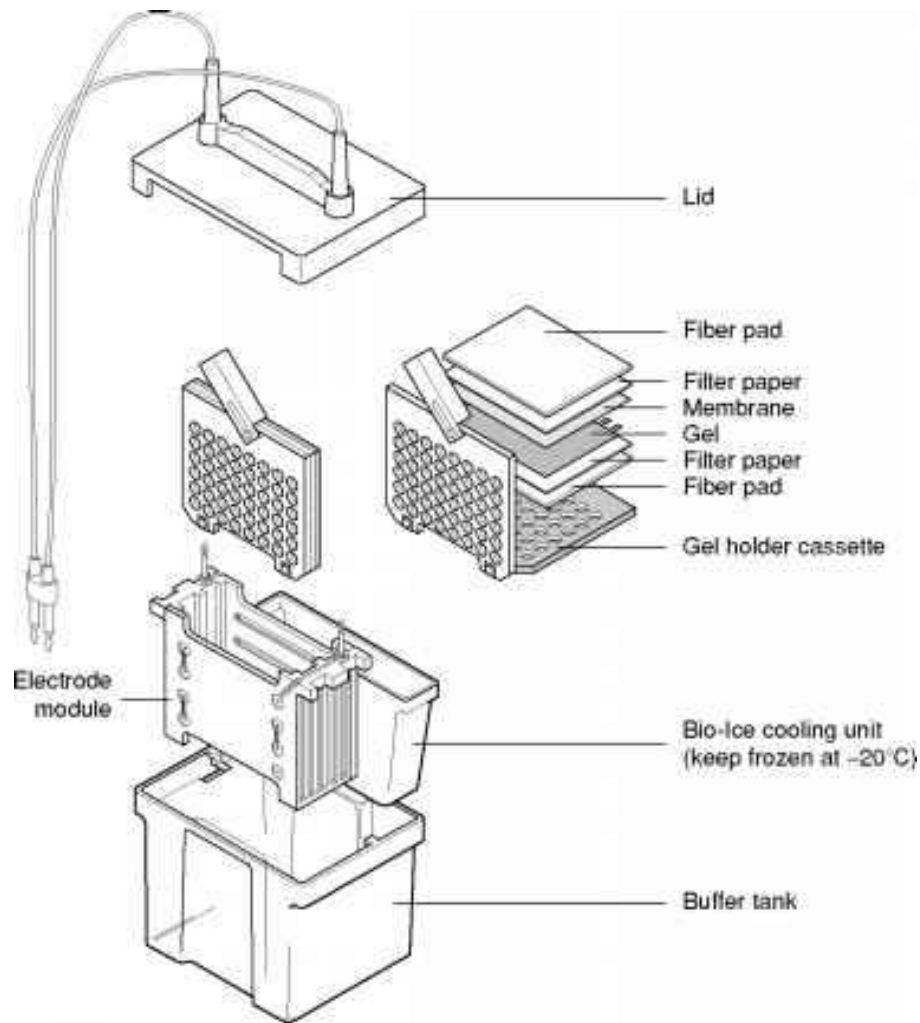


Figure B. Western blot mechanism. Diagram of the materials and technique used for western blot procedure.

Appendix C- Supplemental Data, Standards and Controls

	25°C	35°C	45°C	55°C	65°C	75°C	85°C
[Θ]222 (mdeg)	-25.8	-23.7	-21.0	-18.3	-16.1	-14.1	-13.0
% alpha helix	45.8	41.6	36.1	30.6	25.9	22.1	19.9

Figure C1. Thermal denaturation of BSA. Circular dichroism measurements and alpha helix calculations for BSA during increase in thermal stress. Measurements were recorded every ten degrees from 25° C to 85° C.

	25°C	35°C	45°C	55°C	65°C	75°C	85°C
[Θ]222 (mdeg)	-19.5	-19.7	-19.2	-18.6	-18.5	-13.9	-11.8
% alpha helix	33.1	33.5	32.5	31.2	31.0	21.7	17.4

Figure C2. Thermal denaturation of Lysozyme. Circular dichroism measurements and alpha helix calculations for Lysozyme during increase in thermal stress. Measurements were recorded every ten degrees from 25° C to 85° C.

Appendix D: Supplemental data salt bridge mutants

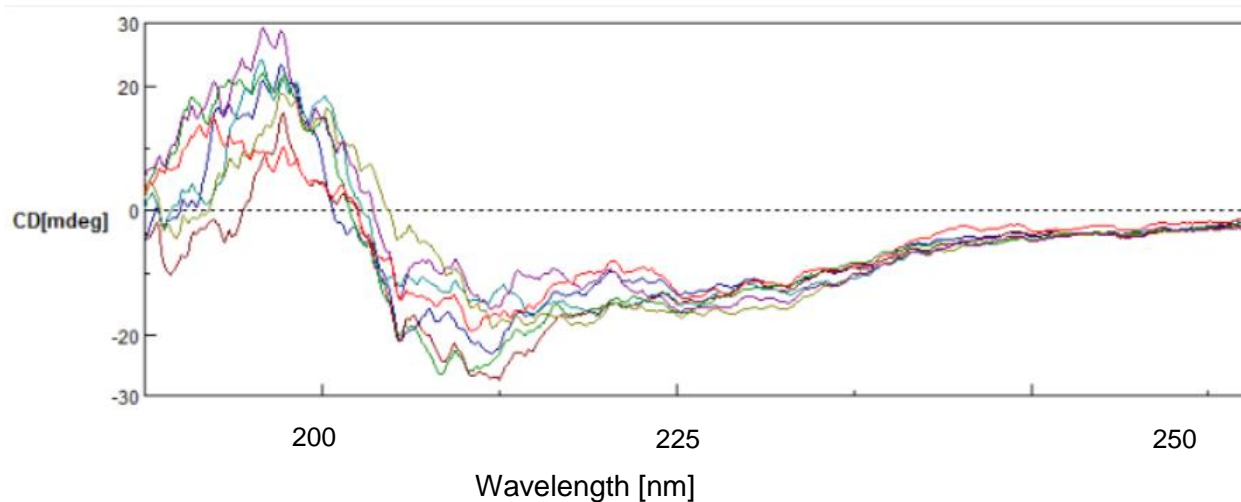


Figure D1. Thermal denaturation of K66S/E70S, in PBS. Spectra were taken from 25-85°C in increments of 10°C. Blue is 25°C, Green is 35°C, Brown is 45°C, Teal is 55°C, Neon green is 65°C, Purple is 75°C and Red is 85°C.

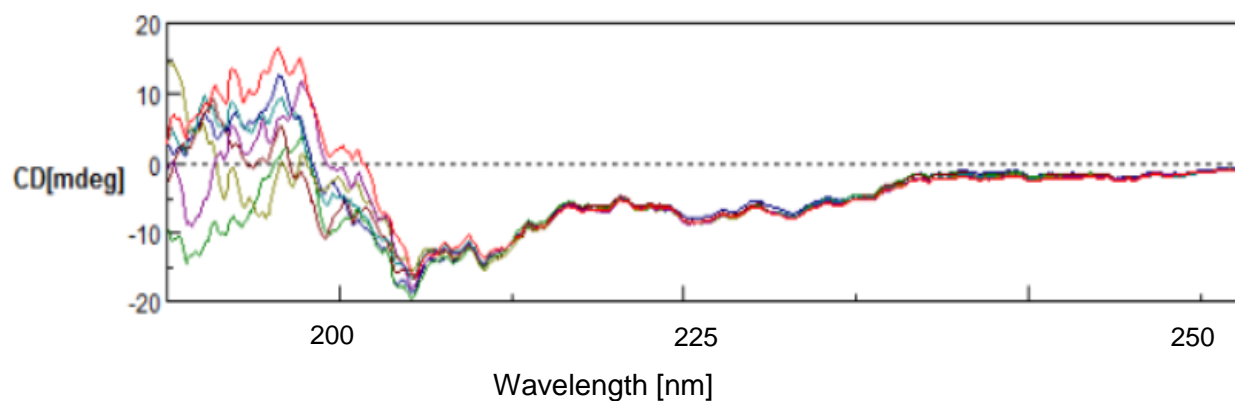


Figure D2. Thermal denaturation of K95S/E99S, in PBS. Spectra were taken from 25-85°C in increments of 10°C. Blue is 25°C, Green is 35°C, Brown is 45°C, Teal is 55°C, Neon green is 65°C, Purple is 75°C and Red is 85°C.

Appendix D: Supplemental data salt bridge mutants

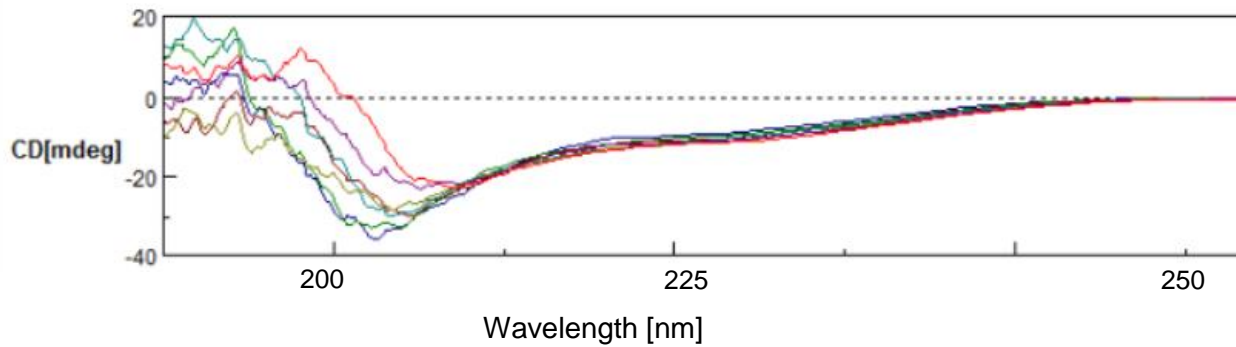


Figure D3. Thermal denaturation of E103S/K107S, in PBS. Spectra were taken from 25-85°C in increments of 10°C. Blue is 25°C, Green is 35°C, Brown is 45°C, Teal is 55°C, Neon green is 65°C, Purple is 75°C and Red is 85°C.

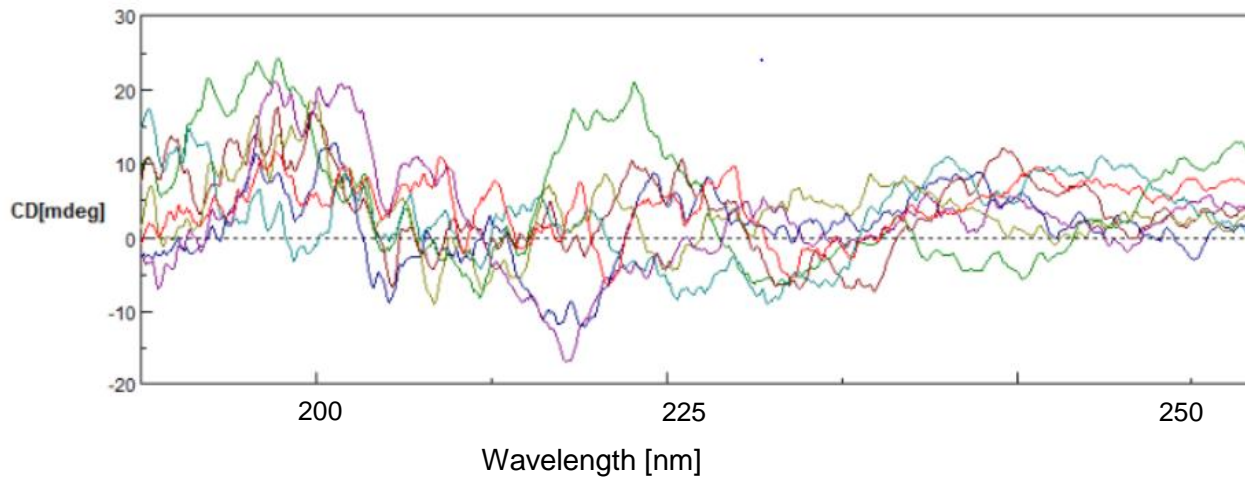


Figure D4. Thermal denaturation of salt bridge mutant, K66S/E70S E103S/K107S, in PBS. Spectra were taken from 25-85°C in increments of 10°C. Blue is 25°C, Green is 35°C, Brown is 45°C, Teal is 55°C, Neon green is 65°C, Purple is 75°C and Red is 85°C.

Appendix E- Supplemental data hydrophobic mutants

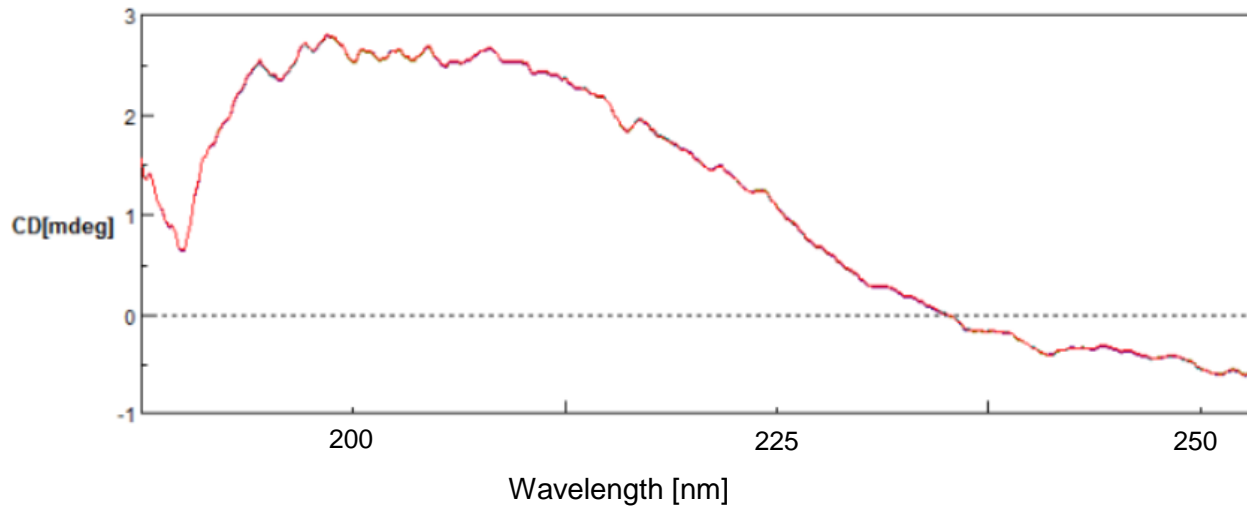


Figure E1. Thermal denaturation of hydrophobic mutant, L108S/L109S/F112S in PBS. Spectra were taken from 25-85°C in increments of 10°C. Black is 25°C, Green is 35°C, Brown is 45°C, Teal is 55°C, Neon green is 65°C, Purple is 75°C and Red is 85°C.

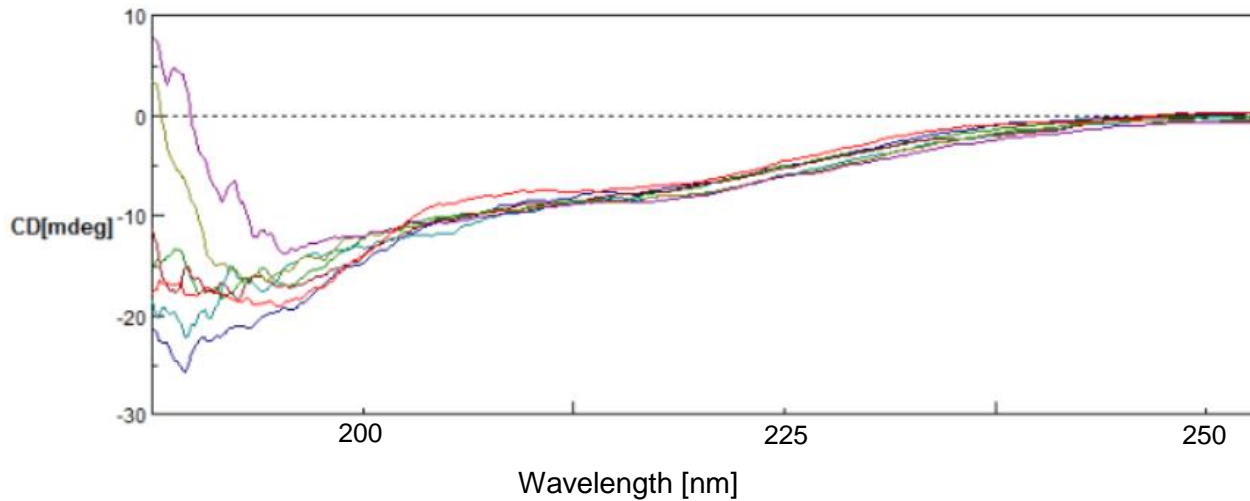


Figure E2. Appendix E. Thermal denaturation of hydrophobic mutant, I68S/I78S in PBS. Spectra were taken from 25-85°C in increments of 10°C. Bue is 25°C, Green is 35°C, Brown is 45°C, Teal is 55°C, Neon green is 65°C, Purple is 75°C and Red is 85°C.

Appendix E- Supplemental data hydrophobic mutants

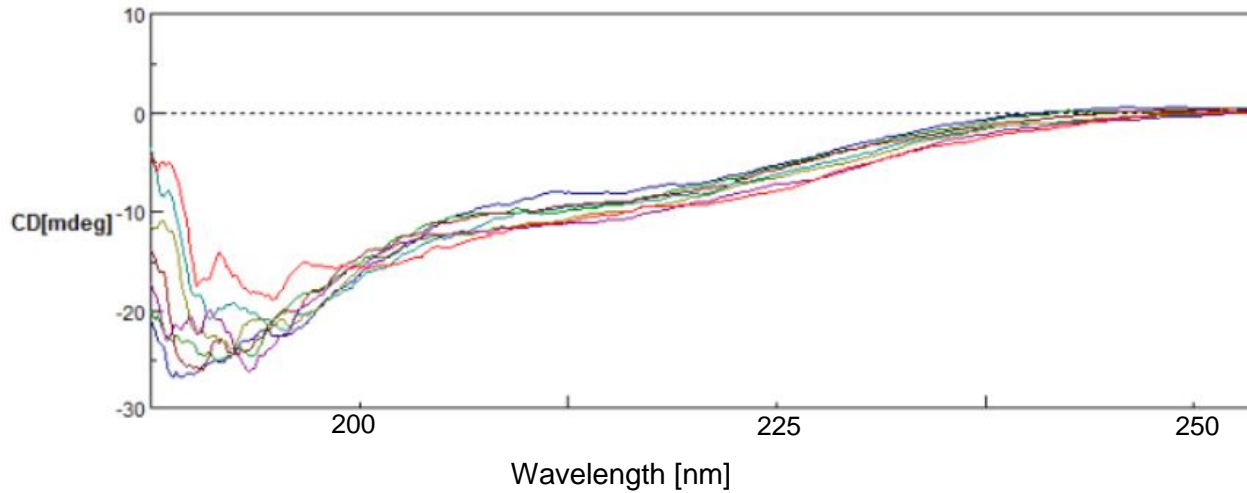


Figure E3. Thermal denaturation of hydrophobic mutant, F112S in PBS. Spectra were taken from 25-85°C in increments of 10°C. Black is 25°C, Green is 35°C, Brown is 45°C, Teal is 55°C, Neon green is 65°C, Purple is 75°C and Red is 85°C.

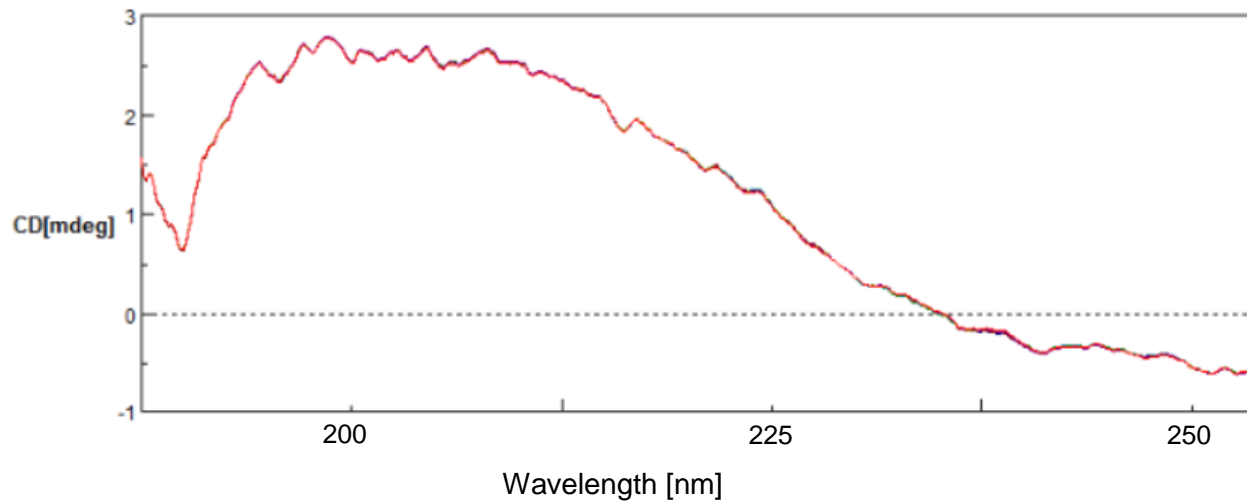


Figure E4. Appendix E. Thermal denaturation of hydrophobic mutant, L109S in PBS. Spectra were taken from 25-85°C in increments of 10°C. Black is 25°C, Green is 35°C, Brown is 45°C, Teal is 55°C, Neon green is 65°C, Purple is 75°C and Red is 85°C.

References

- Fasman, G.D. (Ed.). (1996). *Circular Dichroism and The Conformational Analysis of Biomolecules*. New York, New York: Plenum Publishing Corporations.
- Greenfield, N. (2004) Circular Dichroism for Protein-Protein Interactions. *Methods in Molecular Biology*, **261**, 55-77
- Ma, P.; Wang, N.; McKown, R. L.; Raab, R. W.; & Laurie, G.W. (2008) Focus on molecules: Lacritin. *Experimental Eye Research*, **86**, 457-458.
- McKown R.L.; Coleman Frazier E.V.; Zadrozny K.K.; Deleault A.M.; Raab R.W.; Ryan D.S.; Sia R.K., Lee J.K.; Laurie G.W. (2014) A cleavage-potentiated fragment of tear lacritin is bactericidal. *J Biol Chem*. **289**, 22172-82.
- McKown, R. L., Wang, N., Raab, R. W., Karnati, R., Zhang, Y., Williams, P. B., et al. (2008). Lacritin and other new proteins of the lacrimal functional unit. *Experimental Eye Research*, **86**, 457-458.
- Sanghi, S.; Kumar, R.; Lumsden, A.; Dickinson, D.; Klepis, V.; Trinkaus-Randall, V.; Frierson, H.F.; Laurie, G.W. (2001) cDNA and genomic cloning of lacritin, a novel secretion enhancing factor from the human lacrimal gland. *Journal of Molecular Biology*, **310**, 127-139.
- Zhou, L.; Beuerman, R. W.; Foo, Y.; Liu, S.; Ang, L. P.; Tan, D.T; (2006). Characterization of human tear proteins using high-resolution mass spectrometry. *Annals of the Academy of Medicine, Singapore*, **35**, 400-407.