3. SCREENING FOR ADDITIVES FOR THEIR ABILITY TO ENHANCE STABILITY OF JACALIN AGAINST THERMAL AGGREGATION

3.1 INTRODUCTION

The biological function of a protein relies on its conformational stability. The information required for a protein to fold into its three-dimensional, native structure is encoded in its unique amino acid sequence. The tertiary structure of proteins are stabilized by non-covalent interactions such as hydrophobic interactions, van der Waals forces, ionic interactions and hydrogen bonds while disulfide bonds are the only stabilizing covalent interaction [95]. The native conformation of a protein gets destabilized when these forces are perturbed. Under accustomed in vivo conditions, while most proteins maintain their completely folded structure, some of them are misfolded due to several reasons [192]. Such partially unfolded proteins or misfolded are more susceptible to form protein aggregates. Aggregation is perceived to be an alternate pathway by which a protein folds [193]. During aggregation, intermolecular interactions among partially unfolded and misfolded protein states are favored over intramolecular interactions [194,195]. Under specific conditions, changes that alter the secondary conformation of proteins are known to assist in formation of intermolecular interactions amongst the β strands which in turn result in the formation of amyloid fibrils [196,197]. In vivo, aggregation results in the formation of amyloid fibrils that are correlated with a many diseases such as Alzheimer’s, Creutzfeldt-Jakob, and other polyglutamine diseases [198-201].

The native proteins are misfolded or unfolded due to several surrounding environmental factors such as pH, ionic strength, and temperature or due to genetic mutation. In vitro, most proteins which are unfolded due to extreme pH, increase in temperature and due to the presence of chemical denaturants have the ability to refold to their native state when they are back in their normal environments [202]. In other cases, the proteins that are irreversibly denaturated, will eventually lose their biological functions
To understand the conformational stability structure-function relationship of a protein, it is important to study the denaturation and unfolding of that protein.

3.1.1 Thermal denaturation of proteins and protein aggregation

Increased temperature can have a direct impact on the conformation of polypeptide chains leading to thermal-induced unfolding. The thermal characteristics of proteins are prone to factors such as protein concentration, pH, salt concentration, sugar additives, etc. [204,205]. Information regarding the thermal stability of proteins is crucial in pharmaceutical, food and biological industries. The melting temperature ($T_m$) that refers to the midpoint of the unfolding transition is a measure of thermal stability of a protein. The $T_m$ varies among proteins and usually range from 40-80°C, with higher $T_m$ values denoting greater conformational stability.

Aggregation is ascertained to be an alternate pathway of protein folding. It remains to be a major concern in pharmaceutical and clinical research as it can interfere with purification, production, characterization and also during shipping of commercial proteins [206], as it is one of the major reasons which prevents a denatured protein from refolding to its native, three-dimensional structure. Further, lyophilisation or rehydration of proteins may also result in aggregate formation [207,208]. There are a number of aggregation pathways that differ among proteins and the aggregation process can result in the formation of soluble aggregates and/or insoluble aggregates.

The extent of aggregation depends on many parameters including temperature, pH, presence of cosolutes such as denaturants, chaotropes, ligands and the presence or absence of molecular chaperones [209,210]. These parameters determine whether a protein will aggregate or not and also the rate of aggregation. As pH controls the electrostatic interactions on the surface of the proteins through charge distribution, it can have a strong influence on the rate of aggregation [211].
Thermal-induced aggregation is a process, wherein, the exposed hydrophobic or free SH-groups will eventually fold back in such a way that they form aggregates as the temperature increases. The aggregates are formed mainly due to the disulfide and non-covalent bonds that are formed between the exposed groups [212]. If aggregation can be prevented, there is a possibility that the unfolded protein may refold to form an intermediate state or native conformation [213].

3.1.2 Effects of additives on protein aggregation

The chemical additives that include the ionic surfactants, osmolytes and water-soluble polymers have been extensively used in the prevention of aggregation [214]. Small molecular additives including polyamines, sugars amino acids and amines are known to reduce and sometimes collectively prevent the protein aggregate formation. Most of these additives are biocompatible and during protein refolding, they are anticipated to act like chemical chaperones in hindering the interaction between residues that are prone to aggregation [215]. For instance, arginine is a widely used additive which prevents formation of protein aggregates, without destabilizing the native conformation [216]. On the contrary, some of the additives have been reported to enhance protein aggregation. Such additives exert their effects depending on the concentration of both the protein and the additive. For instance, osmolytes such as glycine and Trimethlyamine-N-oxide (TMAO) have been shown to increase Guanidine hydrochloride (GdnHCl)-induced aggregation of phosphorylase b [214].

Osmolytes are small, organic, intracellular molecules that protect the cells against environmental stresses [217]. There are various classes of osmolytes including those that completely stabilize the protein, others that have a moderate effect on protein stability, denaturing osmolytes and counteracting osmolytes [218]. While, denaturing osmolytes such as urea shifts the equilibrium towards the unfolded protein state, protecting osmolytes are known to increase the stability of proteins [219]. Several osmolytes have been shown to protect proteins against denaturation and aggregation that
occurs as a result of certain stressed environmental conditions. The commonly used stabilizing osmolytes include sugar, polyols, neutral polymers, polysaccharide and amino acids and their derivatives [220]. During protein denaturation, osmolytes are known to delay the aggregation process by influencing the energetic barrier that exists between the native and intermediate protein states [221]. The interaction of osmolytes with the unfolded protein state results in an unfavorable free energy transfer that is primarily responsible for protein stabilization [222]. For most proteins, the increase in $T_m$ was found to be directly proportional to the concentration of the osmolytes used [223].

3.1.3 Effects of detergents on protein aggregation

Triton X 100, SDS and tween 20 are some of the detergents that have been effectively used in the prevention of protein aggregation. These detergents are usually known to denature the proteins. Thus, it is crucial to figure out the optimum concentration of detergents that can effectively prevent the aggregation of proteins. Moreover, the detergents prevent formation of protein aggregates only by altering the aggregation behavior and not by stabilizing the native state. Thus, in most cases the biological activity of the proteins are not restored even after they fold back [224, 225]. The interaction of proteins with surfactants including cetyltrimethylammonium bromide (CTAB) and SDS has been made use of in biological and pharmaceutical industries. The ionic surfactants prevent aggregation of proteins by weakly binding to them and stabilizing them [210]. SDS, an anionic surfactant interacts with proteins through ionic and hydrophobic interactions and possess the ability to stabilize as well as destabilize the proteins [226, 227]. The stabilization effects of detergents can be attributed to their weak binding to proteins thereby partially or completely blocking the hydrophobic, aggregation-prone sites that are exposed to the surface [228]. In case of a strong binding, the detergents are known to induce protein denaturation resulting in enhanced protein aggregation [229].
This chapter provides a detailed account of purification of jacalin from the seeds of jackfruit by affinity chromatography a matrix that contains cross-linked guar gum, followed by an investigation of the effects of various additives on thermal stability of jacalin. Considering the potential applications of jacalin, it was deemed important to sustain the functional stability of the protein during storage, transport and longer use. Among the additives studied, SDS was observed to prevent jacalin from forming aggregates, thereby, providing resistance against thermal denaturation. Thereupon, the focus of the study was on the protective effects of SDS.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Locally available jackfruit seeds were obtained as and when required. Copper sulfate, crystalline bovine serum albumin (BSA), disodium hydrogen phosphate, dihydrogen monosodium phosphate, epichlorohydrin, galactose, guar gum, sodium chloride, sodium hydroxide, acrylamide, sorbitol, tween 20 were purchased from Himedia; tris buffer, tris-HCl, Folin-Ciocalteu phenol reagent, N,N-methylene bis acrylamide, ammonium sulphate, ammonium per sulfate (APS), TEMED, glycerol, 2-mercaptoethanol, glacial acetic acid, methanol, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and coomassie Brilliant Blue were obtained commercially from Sisco Research Laboratories (SRL). Pierce centrifuge columns (89898) was purchased from Thermo Scientific company. Betaine, GdnHCl, glycine, sarcosine and SDS were purchased from Sigma.

3.2.2 Purification of jacalin

(i) Activation of guar gum (affinity matrix)

1.5 ml of epichlorohydrin was mixed with 25 ml of 3N sodium hydroxide and a fine emulsion was prepared. To this, 10 g of guar gum was added with continuous stirring until the mixture solidified. The solidified mixture was kept at 40°C in a water bath for about 24 h. The mixture was occasionally stirred and was then kept in a hot air oven at a temperature of 70°C for about 8-12 h.
The activated guar gum was then soaked in about three times the volume of water. The pH was made neutral by repeatedly washing with distilled water and finally with PBS (pH 7.4).

(ii) **Affinity column packing**

The epichlorohydrin activated guar gum was packed in a column and equilibrated with PBS (pH 7.4).

(iii) **Sample preparation (Preparation of crude extract)**

Briefly, jackfruit seeds were washed, cut into small pieces and soaked overnight in sodium phosphate buffer, pH 7.4, along with 150 mM NaCl (PBS). The seeds were then homogenized and stirred for about 8 h at 4°C. After filtration, the extract was subjected to centrifugation at 5,000 rpm for 30 mins at 4°C. The pellet was discarded and the supernatant was stored at -80°C.

(iv) **Sample loading**

The supernatant was loaded onto the cross-linked guar gum column, pre-equilibrated with PBS and the flow through (F1) was collected. A 1 ml aliquot of F1 was separately stored at -80°C for SDS-PAGE analysis. The F1 was reloaded onto the column and the flow through so obtained was labeled as F2.

(v) **PBS wash**

The column was washed with 80 ml of PBS (10X the height of the column) to remove the unbound proteins. 10 ml aliquots were collected in 15 ml tubes and were labeled as wash 1 (W1), wash 2 (W2), etc. The A260 value of last wash (WL) should be ≤0.05.

(vi) **Elution of jacalin**

Jacalin bound to the column was eluted using 200 mM galactose. The purified protein was dialyzed against PBS and filtered using a 0.45 µm syringe filter. The protein was stored at -80°C until use.
3.2.3 Estimation of protein concentration by Lowry’s method

(i) Preparation of solutions

**Solution A** (2% sodium carbonate in 0.1 N NaOH)

2 g of sodium carbonate and 0.4 g of NaOH were added to 80 ml of deionized water and completely dissolved by mixing. The final volume was brought to 100 ml.

**Solution B** (0.5% copper sulfate solution)

25 mg of copper sulfate was added to 3 ml of deionized water by gentle heating. The solution was made up to 5 ml.

**Solution C** (1% sodium potassium tartrate solution)

50 mg of sodium potassium tartrate was dissolved in 3 ml of deionized water and the solution was made up to 5 ml.

**Lowry’s solution**

48 ml of solution A was mixed with 1 ml each of solution B and solution C.

**Folin-Ciocalteu reagent (1N)**

The Folin-Ciocalteu reagent (2N) was diluted with equal volumes of deionized water. The reagent was diluted prior to use.

(ii) Methodology

1 mg/ml stock solution of BSA was prepared using PBS. Various concentrations of standard solutions (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/ml) were prepared from the BSA stock solution and the total volume was made up to 1 ml with PBS. 1 ml of PBS alone served as the blank. To all the tubes, including the blank, 4.5 ml of Lowry’s solution was added and incubated at RT for 10 mins. Later, 0.5 ml of Folin-Ciocalteu reagent was added to all the tubes and were incubated for 30 mins in dark. Later, OD at 660 nm was measured. As
shown in figure 3.1, a standard graph was plotted and the concentration of proteins in the unknown solution were estimated from the standard plot.

**Table 3.1** Estimation of jacalin concentration by Lowry’s method

<table>
<thead>
<tr>
<th>S.No</th>
<th>Volume of BSA (ml)</th>
<th>Concentration of BSA (mg/ml)</th>
<th>Volume of PBS (ml)</th>
<th>OD at 660 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.1</td>
<td>0.1</td>
<td>0.9</td>
<td>0.21</td>
</tr>
<tr>
<td>2.</td>
<td>0.2</td>
<td>0.2</td>
<td>0.8</td>
<td>0.37</td>
</tr>
<tr>
<td>3.</td>
<td>0.3</td>
<td>0.3</td>
<td>0.7</td>
<td>0.52</td>
</tr>
<tr>
<td>4.</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
<td>0.67</td>
</tr>
<tr>
<td>5.</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.78</td>
</tr>
<tr>
<td>6.</td>
<td>0.6</td>
<td>0.6</td>
<td>0.4</td>
<td>0.87</td>
</tr>
</tbody>
</table>

4.5 ml of Lowry’s solution was added and incubated for 10 mins

0.5 ml of Folin-Ciocalteu reagent was added and incubated for 30 mins in dark

y = 1.3354x + 0.1026

R² = 0.9899

**Figure 3.1** BSA Standard curve
3.2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

(i) Preparation of solutions

**Acrylamide: bisacrylamide solution**

15 g of acrylamide and 0.4 g of bis-acrylamide were dissolved in 40 ml of deionized water. The final volume was made up to 50 ml. The solution was stored away from light at 4°C.

**Lower tris buffer (1.5 M, pH 8.8)**

9.1 g of tris base was dissolved in 40 ml of double distilled water, the pH was adjusted to 8.8 and the final volume was made up to 50 ml. The buffer was stored at 4°C.

**Upper tris buffer (0.5 M, pH 6.8)**

3.03 g of tris base was added to 40 ml of double distilled water, the pH was adjusted to 6.8 after which the final volume was brought to 50 ml. The prepared buffer was stored at 4°C until use.

**10% ammonium persulfate (APS) solution**

0.05 g of APS was dissolved in 0.5 ml of deionized water. APS solution was made fresh prior to use.

**10% SDS**

1 g of SDS was added to 5 ml of deionized water. The solution was made up to 10 ml and stored at RT.
5X Sample loading buffer

### Table 3.2  Preparation of 5X sample loading buffer

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M tris, pH 6.8</td>
<td>1</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.8</td>
</tr>
<tr>
<td>10% SDS</td>
<td>1.6</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>0.4</td>
</tr>
<tr>
<td>0.25% bromophenol blue</td>
<td>0.4</td>
</tr>
<tr>
<td>Deionized water</td>
<td>3.9</td>
</tr>
</tbody>
</table>

10X running buffer

14.4 g of glycine, 3.03 g of tris base and 1 g of SDS were added to 80 ml of deionized water and the final volume was made up to 100 ml. The buffer was stored at 4°C.

Stain/destain solution

0.1 g of coomassie brilliant blue R-250 was added to a mixture of 40 ml of methanol, 10 ml of glacial acetic acid and 40 ml of deionized water. The solution was stored at RT.

The destain solution was the same as stain solution, minus the coomassie brilliant blue.
(ii) Preparation of separating and stacking gels

15% Resolving gel (lower gel)

Table 3.3 Preparations of 15% resolving gel

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>2.2</td>
</tr>
<tr>
<td>Acrylamide: bisacrylamide solution</td>
<td>5</td>
</tr>
<tr>
<td>Lower tris buffer</td>
<td>2.6</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
</tr>
</tbody>
</table>

5% stacking gel (upper gel)

Table 3.4 Preparation of 5% stacking gel

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>2.97</td>
</tr>
<tr>
<td>Acrylamide: bisacrylamide solution</td>
<td>0.67</td>
</tr>
<tr>
<td>Upper tris buffer</td>
<td>1.25</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
</tr>
</tbody>
</table>

(iii) Sample preparation

20 µl of protein sample was mixed with 5 µl of 5X sample loading buffer and was heated to 100°C for 10 mins. After heating, the mixture was subjected to centrifugation at 12,000 rpm for 5 mins to pellet down the cell debris.
(iv) **Running a gel**

The electrophoretic tank was filled with fresh 1X running buffer and the prepared sample supernatant was carefully loaded into the wells. The gel was initially run at 60 V. Once the dye front reached the running gel, the power was increased to 100 V. The run was continued until the dye front reached the bottom of the gel.

(v) **Staining and destaining**

After the run was over, the resolving gel was stained overnight. The gel was then washed with deionized water, destained for 1 h and the gel images were acquired using ChemiDoc MP Imager (Bio-Rad).

### 3.2.5 Hemagglutination assay

The lectin activity was determined by haemagglutination assay against human erythrocytes. 1 ml of blood was collected and the red blood cells (RBCs) were separated by centrifuging at 1700 rpm for about 10 mins at 25°C and suspended in PBS, pH 7.4 so as to obtain 5% RBC suspension.

Jacalin was diluted two-fold in series with PBS (pH 7.4) in a 96 well microtiter plate. Equal volumes of 5% erythrocyte suspension was added to all the wells and the microtiter plate was mixed by gentle swirling. The mixture was incubated for 60 mins at RT after which the haemagglutination activity was measured visually. The procedure was repeated using BSA (non-hemagglutinating protein control) and PBS (negative control).

### 3.2.6 Buffers

20 mM of the following buffers were used: Glycine-HCl (pH 2.0), sodium acetate (pH 4.0), sodium phosphate buffer (pH 6.0 and 7.0), tris-HCl (pH 8.0) and glycine-NaOH (pH 10.0 and 12.0). Stock solutions of glycine, betaine, sarcosine, sodium chloride, ammonium sulfate, SDS were dissolved in sodium phosphate buffer (pH 7.0), just prior to performing the experiments. All the
buffers and the stock solutions were filtered through a 0.45 µm syringe filter and stored at 4°C.

3.2.7 Thermal denaturation of jacalin

The heat denaturation of 3 µM and 5 µM jacalin was monitored at a wavelength of 276 nm by increasing the temperature from 20°C to 85°C at 1°C/min. The gradual change in absorbance of the protein was recorded using a Jasco V730 UV-Vis spectrophotometer.

To assess the reversibility of thermal transition, the temperature was decreased to 20°C at 1°C/min. 5 µM jacalin was incubated at RT for approximately 18 h, prior to performing the experiments so as to attain equilibrium. The temperature was maintained by a water bath connected to the spectrophotometer.

3.2.8 Turbidity measurements

The increase in absorbance at wavelength of 350 nm signifies an increase in turbidity. The presence of aggregates will result in decrease in the amount of light transmitted, thereby resulting in light being scattered.

Hence, the turbidity was assessed by measuring the absorbance of jacalin at $\lambda = 350$ nm using a Jasco V730 UV-Vis spectrophotometer.

(i) Measure of turbidity as a function of pH

The effects of pH on aggregation of jacalin was monitored by heating 5 µM jacalin suspended in buffer solutions of varying pH (2-12) for 1 h at 70°C. After overnight incubation at RT, absorbance of the incubated samples were recorded at 350 nm.

(ii) Concentration-dependent changes in turbidity of jacalin

Jacalin (0-10 µm) was subjected to 70°C for 1 h. After incubation, the tubes were left overnight at RT. The absorbance of the samples at 350 nm were measured using a Jasco V730 UV-Vis spectrophotometer.
(iii) **Temperature-dependent changes in turbidity of jacalin**

The effects of temperature on the turbidity of jacalin was assessed by increasing the temperature from 20°C to 85°C at 1°C/min. The gradual increase/decrease in absorbance of samples at 350 nm was measured. The UV absorbance was recorded using a Jasco V730 UV-Vis spectrophotometer.

(iv) **Time-dependent changes in turbidity of jacalin**

The absorbance at 350 nm was measured as jacalin was continuously heated at a specific temperature for 30 mins, so as to determine if turbidity of jacalin increases with time. 5 and 10 µM jacalin were subjected to heating at 55, 60 and 70°C for 30 mins and the UV absorbance was recorded using a Jasco V730 UV-Vis spectrophotometer.

3.2.9 **Effects of denaturants on thermal unfolding of jacalin**

5 µM jacalin was incubated with different concentrations of urea and Guanidine hydrochloride (GdnHCl). The tubes were incubated for about 18 h at RT to attain equilibrium. The thermal denaturation of the samples were then assessed at 276 nm by increasing the temperature from 20 to 85°C at 1°C/min. As $T_m$ is a measure of protein stability, the difference in $T_m$ were also recorded. The gradual change in UV absorbance and the $T_m$ were recorded using a Jasco V730 UV-Vis spectrophotometer.

3.2.10 **Effects of stabilizers on thermal unfolding of jacalin**

5 µM jacalin was incubated with different concentrations of glycine, betaine, sarcosine, sodium chloride, ammonium sulfate, sucrose, trehalose, sorbitol, SDS, DMSO and glycerol. The tubes were incubated for about 18 h at RT to attain equilibrium. The thermal denaturation of the samples were then assessed at 276 nm by increasing the temperature from 20 to 85°C at 1°C/min. As $T_m$ is a measure of protein stability, the difference in $T_m$ were also recorded. The gradual change in UV absorbance and the $T_m$ were recorded using a Jasco V730 UV-Vis spectrophotometer.
3.2.11 Aggregation study at different concentrations of SDS and jacalin

5 µM jacalin, in the presence of high (1-8 mM) and low (0-1mM) concentrations of SDS, was incubated for 1 h at 70°C. The tubes were left overnight at RT and the absorbance of the samples were measured at 350 nm. Images of microcentrifuge tubes were taken prior to recording the UV absorbance at 350 nm.

Similarly, 5 and 10 µM jacalin, at neutral pH were heated along with 3mM SDS for 1 h at 70°C and left overnight at RT. Images of the microcentrifuge tubes were taken prior to monitoring the variation in absorbance at 350 nm.

3.2.12 Effects of SDS on temperature and time-dependent aggregation of jacalin

3 and 5 µM of jacalin at neutral pH was heated from 20°C to 85°C at 1°C/min, in the presence of 3 mM SDS and the variations in absorbance of the samples at 350 nm were analyzed using Jasco V730 UV-Vis spectrophotometer.

5 and 10 µM of jacalin along with 3 mM SDS, were heated at 55, 60 and 70°C for 30 mins. The variations in absorbance of the samples at 350 nm were analyzed using Jasco V730 UV-Vis spectrophotometer.

3.2.13 Rayleigh light scattering measurements

The Rayleigh light scattering intensity of proteins is another technique that can be used to identify the presence of aggregates. 5 and 10 µM of jacalin were incubated with and without SDS, overnight at RT, prior to performing the experiment. The samples were excited at 350 nm and the emission spectra were recorded at a wavelength range of 360 to 450 nm, using a 1 cm path length cuvette. The experiment was performed on a Perkin Elmer LS45 spectrofluorimeter.
3.2.14 Circular-dichroism studies

The circular dichroism studies helps to ascertain modifications in the secondary structure of proteins [230]. 5 and 10 μM of jacalin were incubated overnight at RT, in the absence and presence of SDS, prior to performing the experiment. The experiment was performed on an Applied photophysics Chirascan plus spectrometer. The spectra were taken at temperatures ranging from 20°C to 85°C at $\lambda = 190$-250 nm. A 0.1 cm path length quartz cuvette was used to load the samples.

The following equation was used to calculate the calculated mean residual ellipticity (MRE):

$$MRE[\theta]_{mrw} = MRW \times \theta (mdeg)/10 \cdot d \cdot c$$

Where, $\theta$ refers to the CD in millidegree (mdeg), d refers to the path length of the cuvette in cm, c refers to the concentration of protein in g/ml [231]. The mean residue weight (MRW) is 110.

The CD spectra were corrected for non-specific intensity as measured with buffer alone.

3.2.15 Effects of SDS on the Hemagglutination activity of thermally denatured jacalin

Haemagglutination assay was used to determine if jacalin that was thermally denatured with and without SDS was biologically active. Jacalin, with and without 3mM SDS was subjected to thermal denaturation at 70° for 1 h. Equal volumes of 2% erythrocytes diluted in PBS was added to the samples that were serially diluted in a 96 well 'U' bottom microtiter plate and was left at 37°C for about 1 h.
3.3 RESULTS AND DISCUSSION

3.3.1 Purification of jacalin

As shown in figure 3.2, two distinct bands at 14 and 16 kDa were observed when the purity of the jacalin was analyzed by SDS-PAGE under reducing conditions (figure 3.2). The intense 14 kDa band corresponds to the non-glycosylated α-chain while the 16 kDa band corresponds to the glycosylated α'-chain of jacalin. The crude extract (CE) which refers to the homogenized jackfruit seed mixture consists of a number of proteins as evident from multiple bands observed in the gel. Likewise, multiple bands were observed with F1, F2 and W1. As the unbound proteins are removed by multiple washes, no band was observed in WL.

![Figure 3.2](image)

Lane 1 - C (crude extract)  Lane 6 - E1 (elute 1)
Lane 2 - F1 (flow through 1)  Lane 7 - E2 (elute 2)
Lane 3 - F2 (flow through 2)  Lane 8 - E3 (elute 3)
Lane 4 - W1 (wash 1)  Lane 9 - E4 (elute 4)
Lane 5 - WL (wash last)  Lane 10 - low molecular weight protein marker

**Figure 3.2 Purification of jacalin.** The purity of the isolated jacalin was analyzed by SDS-PAGE, under reducing conditions.
3.3.2 Hemagglutination assay

Lectins are known to specifically bind to sugars present on the erythrocyte surface resulting in the formation of a cross-linked network. As shown in figure 3.3, the purified jacalin was found to be biologically active as determined by its ability to agglutinate the RBCs. Also, no agglutination was observed in case of the non-hemagglutinating protein control, BSA and the buffer control, PBS.

Figure 3.3 Hemagglutination assay to assess the biological activity of purified jacalin.

Top panel: the hemagglutination activity of native Jacalin.
Middle panel: BSA - non hemagglutinating protein control
Last panel: PBS - buffer control
3.3.3 Thermal denaturation of jacalin

When jacalin was thermally denatured in the temperature range of 20-85°C, a characteristic thermal unfolding profile was observed. Upon thermal denaturation, an almost similar trend was observed at 3 and 5 µM (figure 3.4a). However, when the irreversibility of thermal unfolding of jacalin was assessed, it was observed that even after cooling, jacalin was not able to refold to its native state (figure 3.4b). This was in complete agreement with a previous report [232].

![Absorbance at 276 nm vs Temperature, °C graph]

**Figure 3.4a Thermal denaturation of jacalin.** The protein samples (3 µM and 5 µM jacalin) were left at RT for about 18 h to attain equilibrium. The thermal denaturation of jacalin at 276 nm was monitored from 20 to 85°C at 1°C/min. Jasco V730 UV–Vis spectrophotometer was used to record the absorbance at different temperature.
Figure 3.4b **Reverse scan of 5 µM jacalin.** Irreversibility of thermal transition of jacalin was analyzed by decreasing the temperature to 20°C at 1°C/min.

3.3.4 Concentration-dependent aggregation of jacalin

Different concentrations of jacalin were heated for 1 h at 70°C. The tubes were left overnight at RT. When analyzed, the turbidity at λ350 was found to increase linearly with increasing jacalin concentration (0 - 10 µM) [figure 3.5 (i)]. Further, as observed in figure 3.5 (ii), the amount of aggregates formed were shown to correspond to the concentration of jacalin. Thus, the turbidity of jacalin increased with increasing protein concentrations.
Figure 3.5 Concentration-dependent changes in turbidity of jacalin. 5 and 10 µM jacalin at neutral pH were subjected to 70°C for 1 h and incubated overnight at RT. Absorbance at 350 nm was recorded.

(i) Graph showing exponential increase in absorbance with increasing jacalin concentration
(ii) Representative images illustrate that the amount of aggregates correspond to the protein concentration.
3.3.5 Temperature-dependent aggregation of jacalin

When the effects of temperature on the turbidity of 3 and 5 µM jacalin was assessed, no change in absorbance at 350 nm was observed as the samples were heated from 20-50°C. However, at temperatures above 50°C, an exponential increase in turbidity was observed. This signifies increase in formation of protein aggregates. Further, as evident from increase in absorbance at λ₃₅₀, a remarkable increase in turbidity was observed at the temperature range 65-70°C. Also, no further increase in absorbance at temperatures higher than 70°C, signifies that a threshold had reached (figure 3.6).

![Graph showing temperature-dependent aggregation of jacalin](image_url)

**Figure 3.6 Temperature-dependent changes in turbidity of jacalin.** The effects of temperature on the turbidity of 3 and 5 µM jacalin were assessed by subjecting the protein samples to temperature ranging from 20°C to 85°C at 1°C/min. Prior to recording the spectra, the samples were left overnight at RT.
3.3.6 Time-dependent aggregation of jacalin

To monitor the time-dependent changes in turbidity of jacalin, the protein samples were subjected to a constant temperature, continuously for 30 mins and by recording the absorbance at 350 nm. When jacalin was subjected to heating at 55 and 60°C, the turbidity of both the concentrations of jacalin (5 and 10 µM) increased linearly with increase in time [figure 3.7 (i) & (ii)]. At 70°C, a significant increase in turbidity was observed within 2-3 mins of heating the protein which signifies the formation of enormous amounts of aggregates. Initially, the turbidity was shown to increase gradually as evident from increase in absorbance at $\lambda = 350$ nm. However, after some time, the absorbance of 5 µM jacalin remained unchanged, while absorbance of 10 µM jacalin was found to be decreased. The observed decrease in turbidity may be due to the settling down of protein aggregates that are formed.

Figure 3.7 Time-dependent changes in turbidity of jacalin. (i) 5 µM and (ii) 10 µM jacalin were subjected to denaturation at 55, 60 and 70°C for 30 mins and the gradual change in absorbance of was analyzed at a wavelength of 350nm.
3.3.7 Effects of different pH on aggregation of jacalin

When effects of different pH on aggregation of jacalin was analyzed, it was found out that jacalin formed thermal aggregates only at the pH range of 5-9 [figure 3.8 (i) & (ii)]. Upon denaturation at extreme acidic and alkaline pH, jacalin did not form any visible aggregates. The possible explanation for this may be that due to the charge repulsion that is bound to occur at extreme acidic and alkaline pH. Thus, the exposed hydrophobic residues are unable to interact and aggregate together. However, jacalin suspended in different pH buffers lost its biological activity upon thermal denaturation as assessed by its inability to agglutinate the RBCs [figure 3.8 (iii)].
Figure 3.8  Effects of pH on aggregation of jacalin. 5 μM jacalin suspended in buffers of different pH was incubated for 1 h at 70°C and left overnight at RT. Absorbance at 350 nm was recorded (i) Graph represents difference in absorbance at 350 nm. (ii) representative images illustrate the formation of aggregates when jacalin at neural pH was subjected to increasing temperature. At acidic (pH 2) and alkaline (pH 12) conditions, the samples do not form aggregates after heating. (iii) Hemagglutination assay.
3.3.8 Effects of denaturants on thermal-induced unfolding of jacalin

As expected of a typical denaturant, urea accelerated the denaturation process of jacalin as observed by decrease in $T_m$ of jacalin, when subjected to thermal denaturation with increasing concentrations of urea (figure 3.9a).

![Figure 3.9a Effects of urea on thermal-induced unfolding of jacalin.](image)

Figure 3.9a Effects of urea on thermal-induced unfolding of jacalin. 5 µM jacalin was incubated with 0 M, 0.5, 1 and 2 M urea and incubated at RT for about 18 h. The thermal denaturation of the samples were monitored by increasing the temperature from 20 to 85°C at 1°C/min. The absorbance at 276 nm was recorded using Jasco V730 UV–Vis spectrophotometer.
GdnHCl is another destabilizing salt that has been widely used to denature proteins. However, contrary to common perception, in the present study, GdnHCl was shown to exert a slight degree of stabilization as observed by the increase in $T_m$ of jacalin upon thermal denaturation with increasing concentrations of GdnHCl (figure 3.9b). Table 3.5 shows the changes in thermal unfolding transition temperature ($T_m$) of jacalin.

**Figure 3.9b Effects of GdnHCl on thermal-induced unfolding of jacalin.** 5 µM jacalin was incubated with 0, 0.5, 1 and 2 M GdnHCl and incubated at RT for about 18 h. The thermal denaturation of the samples were monitored by increasing the temperature from 20 to 85°C at the rate of 1°C/min. Jasco V730 UV–Vis spectrophotometer was used to record the absorbance at 276 nm.
### Table 3.5 Effects of denaturants on thermal unfolding transition temperature ($T_m$) of jacalin

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>Concentration (M)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>0.5</td>
<td>58.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>54.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>54</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>0.5</td>
<td>63.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>65.3</td>
</tr>
</tbody>
</table>
3.3.9 Effects of different additives (stabilizers) on thermal-induced unfolding of jacalin

It was observed that jacalin showed remarkable thermal stability in the presence of increased concentration of certain additives such as glycine, sarcosine, sucrose, trehalose, sodium chloride, ammonium sulfate, and sorbitol [figure 3.10 (i)-(vii)]. The additives were shown to stabilize jacalin to different extents with the largest degree of stabilization observed with sodium chloride and ammonium sulfate. In most of the cases, the $T_m$ was found to be directly related to the concentration of additive used. Among the polyols, sucrose and trehalose were shown to enhance the thermal stability of jacalin. Tween 20, a non-ionic surfactant also increased the $T_m$ of jacalin to some extent [figure 3.10 (viii)]. However, other common protectants such as betaine and glycerol did not have a remarkable effect on the $T_m$ of jacalin [figure 3.10 (ix) & (x)]. In fact, 5% and 10% glycerol was shown to decrease the $T_m$ of jacalin. This is in accordance with previous studies wherein osmolytes have been reported to decrease the $T_m$ of certain proteins [233]. Thus, it can be inferred that a given protein shows increased stability only in some osmolytes but not in others. Table 3.6 shows the effects of various additives on the thermal unfolding transition temperature ($T_m$) of jacalin.
Figure 3.10 Effects of different additives on thermal unfolding of jacalin. 5 µM jacalin was incubated with 0 M, 0.5 M, 1 M and 2 M (i) Glycine, (ii) Sarcosine, (iii) Sucrose (iv) Trehalose, (v) NaCl, (vi) Ammonium sulfate (AS), (vii) Sorbitol, (viii) Tween 20, (ix) betaine and (x) glycerol and incubated for about 18 h at RT. The thermal denaturation of the samples were monitored by increasing the temperature from 20 to 85°C at 1°C/min. Jasco V730 UV–Vis spectrophotometer was used to record the absorbance at 276 nm.
Table 3.6  Effects of additives (stabilizers) on thermal unfolding transition temperature ($T_m$) of jacalin

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration (M)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0.5</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>64.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>66.7</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>0.5</td>
<td>62.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>63.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>66.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.5</td>
<td>63.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>66.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>71.5</td>
</tr>
<tr>
<td>Trehalose</td>
<td>5</td>
<td>65.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>66.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>72.4</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5</td>
<td>65.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>68.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>74.3</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>0.5</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>72.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>75.6</td>
</tr>
<tr>
<td>Betaine</td>
<td>0.5</td>
<td>61.3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>62.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>63.5</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.5</td>
<td>63.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>64.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>65.7</td>
</tr>
</tbody>
</table>
### Additive Concentration % $T_m$ (°C)

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration (%)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbitol</td>
<td>5</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>66.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>68.4</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>60.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>68.6</td>
</tr>
</tbody>
</table>

#### 3.3.10 Protective effects of SDS

#### 3.3.10.1 Effects of SDS on thermal-induced unfolding of jacalin

It was observed that jacalin showed remarkable thermal stability in the presence of 1 and 3 mM (figure 3.11). 3 mM SDS was shown to completely prevent thermal denaturation of jacalin, while, 1 mM SDS significantly increased the $T_m$ of jacalin.
Figure 3.11  Effects of SDS on thermal-induced unfolding of jacalin. 5 µM jacalin was incubated with 0 mM, 1 mM and 3 mM SDS and incubated for about 18 h at RT. The thermal denaturation of the samples were monitored by increasing the temperature from 20 to 85°C at 1°C/min. Jasco V730 UV–Vis spectrophotometer was used to record the absorbance at 276 nm.

3.3.10.2 Effects of SDS on thermal aggregation of jacalin

When heat induced aggregation of jacalin was assessed in the presence of different concentrations of SDS, as shown in figure 3.12, there was no relation between the critical micelle concentration (CMC) of SDS and its effects on preventing aggregation of jacalin. SDS at concentrations of 3 mM and higher was shown to effectively prevent the aggregate formation. Significantly, even 1 mM SDS was shown to exhibit remarkable effects in preventing the formation of aggregates [figure 3.12 (i)].

The ability of post-micellar concentrations of SDS to effectively obstruct the formation of protein aggregates have been reported earlier [234,235]. While, low SDS at low concentrations are known to favor protein aggregation [236-239]. Yet, in the present study, when jacalin was subjected to thermal
denaturation in the presence of low concentrations of SDS (0-1 mM), no significant increase in absorbance was noticed, thereby, indicating that low concentrations of SDS did not induce thermal aggregation of jacalin [figure 3.12(ii)].

Figure 3.12 Effects of different concentrations of SDS on thermal induced aggregation of jacalin. Jacalin was subjected to 70°C for 1 h, in the presence of (i) high (1-8 mM) and (ii) low (0-1 mM) concentrations of SDS. Jasco V730 UV–Vis spectrophotometer was used to record the absorbance at 276 nm after overnight incubation at RT. (iii) Representative images illustrate the heat induced aggregation of jacalin in the presence of SDS.
3.3.10.3 Effects of SDS on concentration-dependent aggregation of jacalin

When the protective effects of SDS on different concentrations of jacalin was analyzed, 3 mM SDS was found to confer protection to 5 and 10 µM jacalin, against aggregation. As shown in figure 3.13, when 5 and 10 µM jacalin were incubated at 70°C for 1 h, difference in amount of aggregates formed was observed. However, 3 mM SDS was found to be sufficient in preventing aggregation of both the concentrations studied.

Figure 3.13 Effects of SDS on concentration-dependent aggregation of jacalin. 5 and 10 µM jacalin at neutral pH, with and without 3 mM SDS were subjected to 70°C for 1 h. Representative images taken after tubes were incubated overnight at RT show the presence of aggregates when 5 and 10 µM jacalin were heated without SDS (-) while the samples remain turbid free when heated with 3mM SDS (+).
3.3.10.4 Effects of SDS on temperature-dependent aggregation of jacalin

When the effects of SDS on temperature-dependent changes in turbidity of jacalin was analyzed, 3 mM SDS was found to dramatically prevent heat induced aggregation of 3 and 5 µM jacalin [figure 3.14 (i), (ii) & (iii)].
Figure 3.14 Effects of SDS on temperature-dependent aggregation of jacalin. 3 µM and 5 µM jacalin at neutral pH was subjected to thermal denaturation at 350 nm “with” and “without” 3 mM SDS. The samples were mixed and left overnight at RT after which the samples were subjected to temperature ranging from 20°C to 85°C at 1°C/min. Graph showing difference in absorbance at 350 nm of (i) 3 µM jacalin and (ii) 5 µM jacalin. (iii) Representative images of 3 µM and 5 µM jacalin post thermal denaturation, showing varying degrees of turbidity. The samples remained clear when the protein was thermally denatured with 3 mM SDS, denoting absence of aggregates.

3.3.10.5 Effects of SDS on time-dependent aggregation of jacalin

When jacalin was subjected to heating at 70°C for 30 mins along with 3 mM SDS, no significant increase in absorbance was detected at 350 nm [figure 3.15 (i) & (ii)]. This further demonstrates the strong potential of SDS to inhibit thermal aggregation of jacalin.
Figure 3.15  Effects of SDS on time-dependent aggregation of jacalin. (i) 5 μM and (ii) 10 μM jacalin were thermally denatured, with and without 3 mM SDS, at 55°C, 60°C and 70°C for 30 mins and changes in absorbance was continuously recorded at 350 nm.
3.3.10.6 Rayleigh light scattering measurements

To further confirm the protective effects of SDS, the presence of aggregates was monitored by light scattering intensity at 350 nm. As shown in figure 3.16, the amount of light scattered was insignificant when jacalin was incubated at 25°C for 1 h. For samples incubated at 70°C for 1 h; remarkable increase in light scattering intensity was observed which denotes formation of large amounts of aggregates. Further, the amount of light scattered was found to be high in case of 10 µM jacalin as compared to the light scattered when 5 µM jacalin is heated. When jacalin was heated with 3 mM SDS, a drastic decrease in the amount of light scattered was observed at both the concentrations. Upon heating 10 µM jacalin with 3 mM SDS, a slight increase in light scattering that was observed may be due to the formation of negligible quantities of protein aggregates that are traceable only by the light scattering assay which can be attributed to the sensitivity of the technique.

![Rayleigh light scattering](image)

**Figure 3.16 Rayleigh light scattering.** Light scattering intensity of jacalin at 25 and 70°C, with and without SDS.
3.3.10.7 Circular dichroism measurements (far UV-CD spectra)

A far UV CD spectra can be used to detect modifications that occur in the secondary structural contents of a protein. Here, the changes in the secondary structure of jacalin that was subjected to heating with and without SDS, was monitored by far UV-CD spectra. At 25°C, as expected for a typical β-sheet protein, a single minimum was detected at 218 nm. This was in complete agreement with an earlier report [43]. When the temperature was raised over 55°C, the disappearance of negative peak at 218 nm signified loss of regular secondary structure [figure 3.17 (i)]. When jacalin along with SDS were subjected to a temperature of 25°C, a significant difference in the negative minimum at 218 nm was detected [figure 3.17 (ii)]. The observed effect may be attributed to the ability of SDS to induce the formation of secondary structural contents. Interestingly, when jacalin along with 3 mM SDS were heated to above 55°C, a dramatic shift in spectra was noticed as a single peak at 208 nm was detected. This shift in spectra could be attributed to the ability of SDS to increase the α-helical content of the protein.

The observed change in far UV CD spectra of jacalin that is subjected to heating with SDS may be due to induction of intrachain interaction between the hydrophobic residues. Usually, the hydrophobic residues of a protein will be exposed upon unfolding and the interchain interactions between them will eventually lead to protein aggregation. Here, the helical propensity of jacalin seems to be enhanced by SDS. The high content of α-helical conformers that are present in the denatured state of protein reduces considerably the chances of aggregate formation [240]. Such transition from α to β or vice versa are known to commonly occur when the proteins are treated with certain additives and also upon subjecting the proteins to thermal denaturation. For instance, when heated with high concentration of CTAB which is a cationic surfactant, the “all β” lectin Con A transformed to form an α helical structure [241]. Likewise, α helical to β sheet conversion was found to be responsible for the formation of amyloid fibrils in prion protein, which consist of high proportion of helical content [242].
Figure 3.17  Far UV-CD spectra of jacalin with and without SDS. The far UV-CD spectra of 5 μM jacalin that was subjected to different temperatures (i) without and (ii) with 3 mM SDS in the wavelength range of 190-250 nm.
3.3.10.8 Biological activity of jacalin that was thermally denatured in the presence of SDS

Haemagglutination assay was used to ascertain if jacalin that was subjected to heat denaturation along with SDS was able to retain its biological activity. SDS is known to lyse the erythrocytes. Hence, the mixture of jacalin and SDS was diluted to 1:16 factor so as to reduce the concentration of SDS so that the cells are not lysed. As shown in figure 3.18, jacalin that was subjected to thermal denaturation along with SDS was found to partially retain its ability to cause agglutination of erythrocytes.

Figure 3.18 Biological activity of jacalin that was thermally denatured in the presence of SDS. The biological activity of jacalin that was subjected to thermal denaturation with and without 3 mM SDS was ascertained by haemagglutination assay. The RBCs were added after the samples were appropriately diluted.

First panel : native jacalin.
Second pane : native jacalin with SDS.
Third panel : jacalin that was heat denatured
Last panel : jacalin that was heat denatured with SDS.
3.4 CONCLUSION

It is well known that the post-micellar concentration of SDS are effective in preventing the formation of aggregates [234,243,244]. Surfactants like SDS alter the aggregation behavior of proteins, thereby, preventing aggregation. However, these detergents are incapable of stabilizing the native state of the protein and in many proteins do not restore the function [224,225]. However, in the present study, jacalin was able to partially retain its biological activity after having subjected to heat denaturation along with SDS. Interestingly, at high temperatures, when jacalin was heated along with SDS, the single minimum at 218 nm converted into a negative peak at 208 nm. This might be due to increase in α-helical content when jacalin is heat denatured along with SDS. It is proposed that upon heating with SDS, jacalin turns into structure. This might be due to ability of SDS to induce intrachain hydrogen bonding among the exposed hydrophobic residues and not the interchain binding which will favor protein aggregation.