Extended ε34 Phage TSP renatures after urea-acid unfolding

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Abstract: In antimicrobial-peptide/protein engineering, understanding the peptide/protein's adaptability to harsh environmental conditions such as urea, proteases, fluctuating temperatures, high salts provide enormous insight into the pharmacokinetics and pharmacodynamics of the engineered peptide/protein and its ability to survive the harsh internal environment of the human body such as the gut or the harsh external environment to which they are applied. A previous work in our laboratory demonstrated that our cloned Eε34 TSP showed potent antimicrobial activity against *Salmonella newington*, and more so, could prevent biofilm formation on decellularized tissue. In this work, the effects of urea-acid on the Eε34 stability is studied, and the results demonstrates that at lower pHs of 3 and 4 with urea the protein was denatured into monomeric species. However, the protein withstood urea denaturation above pH of 5 and thus remained as trimeric protein. The mechanism of denaturation of Eε34 TSP seems to show that urea denatures proteins by depleting hydrophobic core of the protein by directly binding to the amide units via hydrogen bonds. The results of our *in-silico* investigation determined that urea binds with Eε34 TSP with relative free energies range of -3.4 to -2.9 kcal/mol at the putative globular head binding domain of the protein. The urea molecules interacts with with the protein's predicted hydrophobic core, thus, disrupting and exposing the shielded hydrophobic moieties of Eε34 TSP to the solvent. We further showed that after the unfolding of Eε34 TSP via urea-acid, renaturation of the protein to its native conformation was possible within few hours. This unique characteristic of refolding of Eε34 TSP which is similar to that of the P22 phage tailspike protein is of special interest to protein scientists and can also be exploited in antimicrobial-protein engineering.

Keywords: Renaturation · Denaturation · Phage. Fusion protein, Podoviruses

1. Introduction

The epsilon 34 (ε34) phage is a dsDNA tailed-bacteriophage, carrying a 43.016Kbp in its capsid that expresses the coat protein, the tail machinery (which includes the tailspike proteins) that hydrolyzes the LPSs of its host, alongside several other structural and regulatory products. The entire sequence and the structural components of the phage have briefly been studied (**Uetake et al., 1958; Ayariga et al., 2021; Ayariga et al., 2021)**. Most of these studies have been limited to sequence similarities between the P22-like phages to which ε34 belongs (**Uetake et al., 1958**), lysogeny and lysogenic conversion abilities of ε34 and its phylogenetic relative known as ε15 on their host *S. newington* (**Villafane et al., 2008**; **Uetake et al., 1955**). Belonging to the temperate phages, ε34 is known to often alter its host cell surface antigens upon lysogenization in a process called "lysogenic conversion (**Uetake et**

al., 1958; Uetake et al., 1955; Uetake, 1959; Uetake and Hagiwara, 1961). This process is known to block super-infection of the host by the same phage specie (**Wright, 1971; Wright and Barzilai, 1971**). Modification of surface antigens has been demonstrated to be an important factor in bacterial virulence. For instance, Allison and Verma showed that serotype-converting bacteriophages and O-antigen modification in *Shigella flexneri* is an important virulence determinant that is conferred by temperate bacteriophages (**Allison and Verma, 2000**). Ɛ34 phage can both undergo lytic and lysogenic pathways (**Uetake et al., 1958**). Exploiting phage or phage components as antimicrobial agents has received resurgence in recent times (**Ayariga et al., 2021; Moradpour and Ghasemian, 2011; Fenton et al., 2011; Thandar et al., 2016**). For instance, phage lysin has been employed as an antimicrobial against multidrug-resistant *Acinetobacter baumannii* (**Thandar et al., 2016)** *Streptococcus pneumoniae* (**Resch et al., 2011**), biofilms of s*treptococcus suis* **(**Meng **et al., 2011)**. The tailspike of some phages too have been explored as antimicrobial agents (**Olszak et al., 2017; Waseh et al., 2010; Lee et al., 2020**). In our previous communication, we demonstrated that an engineered tailspike protein from Ɛ34 phage termed as EƐ34 TSP showed potent antimicrobial activity against *Salmonella newington*, and more so, could prevent biofilm formation on decellularized tissue (**Ayariga, et al., 2021; Ayariga et al., 2021**). In this work, we present the unique characteristics of this protein under urea-acid treatment, a condition that recapitulates the gastrointestinal conditions as well as the excretory system (**Miller and Wolin, 1981; Lu et al., 2018**) *in vitro*. Whiles there exist huge information on the destabilization power of urea on most protein, and while inference has been drawn based on the ability of aqueous urea to interact with both polar and nonpolar components of proteins, no data exist on the effect of urea, or urea-acid on Ɛ34 TSP. Hence, this work seeks to unravel the protein unfolding pathway via urea-acid. We further investigate the ability of Ɛ34 TSP to renature into its native state after complete denaturation via urea-acid. Similar works by Seckler and his colleagues in 1989 demonstrated that the trimeric *Salmonellae* phage tailspike protein, known as P22 TSP, can reconstitute *in vitro* after the protein had been unfolded via urea-acid (**Seckler et al., 1989)** . Finally, we demonstrate via computational analysis the interaction between urea molecules and Ɛ34 TSP. Thus in summary, in this study, we investigated *in vitro*; 1) the unfolding pathway of Eε34 TSP under the influence of urea tinkered with acid and 2) the ability of the Eε34 TSP to refold back to biologically active trimeric state after it had been unfolded completely via urea-acid denaturation, and 3) finally to we reconfirm via *in silico* analysis the interaction between urea molecules and the hydrophobic core of Eε34 TSP.

2. Materials and Methods

The cloning and validation of the clone of the Eε34 TSP has been carried out and published in a previous communication (**Ayariga et al., 2021**).

2.1. Urea-acid denaturation of Eε34 TSP

Samples of Eε34 tailspike protein in 50 mM Tris-HCl solution were titrated with urea-acid to a final concentration of 5 M urea and 2 mg/ml of the protein, and the pH adjusted to 3, 4, 5 or 6 via dropwise addition of 6N HCl. The reaction was allowed to run at room temperature and aliquots withdrawn at set time points; 0, 1, 2.5, 5, 10, 15, 20, 25, 30, and 60 minutes. Withdrawn aliquots were quickly mixed with stop buffer consisting of 50 mM Tris-HCl, 0.2% SDS, pH8 to a final concentration of 20 µg/ml. The large dilution acting as a buffer brought the acidic pH to neutrality. All samples were stored at -20 ˚C until all experimental samples from every set time point was taken. Samples from each time point were mixed with loading buffer consisting of 50 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.03% bromophenol blue, pH 7 and then electrophoresed in 10% polyacrylamide gel for 2 hours. Experiments were run in triplicates. Gels were stained with Coomassie blue and gel bands images acquired using Bio-Rad ChemiDoc XRS imaging system connected to PC operating Quantity One software. Densitometric values were acquired and plotted on graphs.

2.2. Renaturation of Eε34 TSP

2 mg/ml of Eε34 tsp samples was denatured in 5 M urea, 50 mM Tris (pH 3.0) for 30 minutes at 30 ˚C. Renaturation of the TSP was commenced by initial 15-minute dialysis of the denatured protein samples in excess buffer and followed with 20-fold dilution of the unfolded TSP using the renaturation buffer consisting of 50 mM Tris-HCl, 1mM EDTA, and pH 7.0 at 10 ± 4 ˚C to generate a final concentration of 100 µg/ml of TSP in solution. The refolding process was allowed to proceed at 10 ˚C with slight fluctuations in temperature at ±4 ˚C, and aliquots withdrawn at specific time points and refolding reaction stopped by the addition of loading buffer consisting of 0.2 mg/ml SDS, 0.16 M Tris/HCl, 250 mg/ml glycerol, 0. 1 µg/ml bromophenol blue, pH 7.0 then stored at -20 ˚C until all samples for all specified set time points were collected. All samples were then loaded in to 10% polyacrylamide gel and electrophoresed for two hours and 30 minutes at 100 volts. Protein bands were visualized by Coomassie blue staining. Gel images were acquired using Bio-Rad ChemiDoc XRS imaging system connected to PC operating Quantity One software. Densitometric values were acquired and plotted into graphs.

2.3. Homology modeling of ε34 TSP

2.3.1. Protein sequence extraction homology modeling of Ɛ34 TSP and urea 2D structure extraction

The protein sequences of Ɛ34 TSP (Gene ID: 7353089) was extracted from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/). The 3D structural modeling was performed by using Swiss-Modeler (http://swiss-model.expasy.org/) an online homology modeling and model evaluation program (and work published in a previous investigation of this protein (**Ayariga et al., 2021**). Subsequently the model's quality and validation of the model were assessed using structure assessment methods such as the QMEAN (**Benkert et al., 2011**) and Ramachandran plot analysis (http://mordred.bioc.cam.ac.uk). The 2D structure of urea (PubChem CID: 1176) was extracted from PubChem database (https://pubchem.ncbi.nlm.nih.gov/).

2.3.2. Homology modeling of Ɛ34 TSP

The wild type Ɛ34 TSP sequence was extracted from NCBI website and modeled following a similar process by Filiz et al., 2014 (**Filiz et al., 2014**). In short, the Ɛ34 TSP sequence was blasted against PDB to extract homologous templates for the modeling. The bifunctional P22 TSP, a homotrimer with PDB ID 2XC1.1.A (**Seul et al., 2014**) possessing 69.83% amino acid identities was used to model the HBD of the Ɛ34 TSP. The modeling of HBD of Ɛ34 TSP its 3D structure was carried out using the Swiss-Modeler (http://swiss-model.expasy.org/) program (**Arnold et al., 2006; Bordoli et al., 2009; Filiz et al., 2014**). The quality of the HBD model was evaluated to show a QMEAN of 0.66 ± 0.05 and a Ramachandran plot analysis as shown in Figure pred2B, the molProbity analysis showed a clash score of 3.09, a 96.83% Ramachandran favored structure, 0.00% Ramachandran outliers, 1.66% rotamer outliers and 0 bad bonds out of 2842 bonds. For the local quality estimate, the first 12 amino acids showed QMEAN of less than 0.3, whereas the rest of the structure showed amino acids having a local QMEAN greater than 0.4.

2.3.3. Molecular Docking analysis of Ɛ34 TSP- urea.

To investigate the interactions between the urea (PubChem CID: 1176) and Ɛ34 TSP, the 2D structure of urea was downloaded from the PubChem database and docked to Ɛ34 TSP using PyRx (an open-source software for performing virtual screening that combines AutoDock Vina, AutoDock 4.2, Mayavi, Open Babel etc). The modeled structure of Ɛ34 served as the receptor. The modeled Ɛ34 TSP was prepared using AutoDock Vina wizard,

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whereas the urea molecule was prepared by using the Open Babel tool. In summary, the 2D urea molecular structure was minimized and converted to a pdbqt format before uploading as a ligand. In the case of the receptor, the bond orders were assigned, and charged hydrogen atoms added to the protein. The receptor structure was also minimized using the AutoDock Vina wizard. Additionally, proteins were loaded into PyRx and converted to receptors, the receptor grid boxes were generated in PyRx using the build-in Vina Wizard module, the grid box was maximized to cover all active sites of the Ɛ34 TSP head binding domain (HBD) (Which demonstrate a globular structure and shows high hydrophobic regions). AutoDocking of urea to the HBD of Ɛ34 TSP was made by using the AutoDock wizard in-built in PyRx program to blind dock the urea molecule to the HBD of the Ɛ34 TSP with an exhaustiveness of 9. The free energies generated in docking results were saved and the models were generated were exported to Biovia Discovery Studio software (version; 21.1.0.278) for specific atomic-atomic interaction analysis between the ligand and the receptor.

2.4. Statistical analyses

For all statistical data, values were derived from multiple measurements (from replicates of 3 experiments) and averaged, the standard deviations were evaluated using P-values of Student's t-test (one-tailed, two samples of unequal variance, significance level $\alpha = 0.05$ was used unless otherwise stated).

3. Results

3.1 Urea denaturation profile

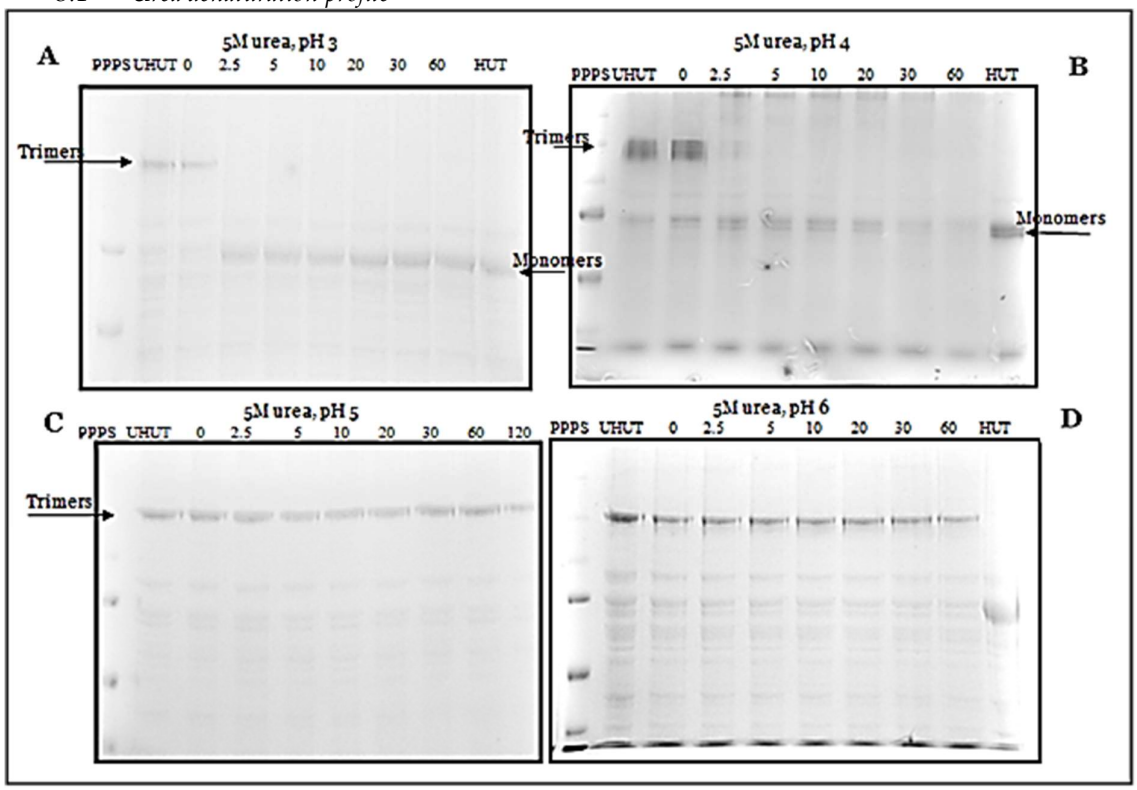

Figure 1. SDS PAGE analysis of denaturation kinetics of Eε34 TSP in urea-acid. The kinetics of denaturation of Eε34 tailspike protein in 5M urea, under varying pH were observed by SDS-PAGE (7.5% polyacrylamide gel). Protein samples were diluted with stock urea to urea set concentration of 5M, and the pH tinkered to 3, 4, 5 or 6 using 6NHCl. At the set time points, aliquots were withdrawn and quickly mixed with stop buffer consisting of 50 mM

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Tris-HCl, 0.2% SDS, pH8 to a final concentration of 20 µg/ml and stored temporarily at -20 ˚C until all sample reactions were quenched and collected. Samples were then electrophoresed via an SDS-polyacrylamide gel and imaged using the ChemiDoc XRS installed with Quantity one software.

As can be observed Figure 1A, and Figure 1B, denaturation of Eε34 TSP occurred very quickly, with protein species existing predominantly in monomeric forms in the 5th minute onwards. A faint band can however be observed in the 2.5-minute mark for the 5 M, pH 4 (Figure 1B), but absent in the 5 M, pH 3, an indication of an incomplete unfolding of all species at the 5 M, pH4 in this time point, but a complete denaturation of all samples at the 5 M, pH3 at the same time point. The higher pH 5 and pH 6 as depicted by Figure 1C and Figure 1D show all samples in their trimeric states, indicative of resistance to denaturation at these conditions for all time points.

To understand in detail, the denaturation characteristics of Eε34 TSP under these set conditions, we repeated this experiment three times and band densities were quantitatively analyzed.

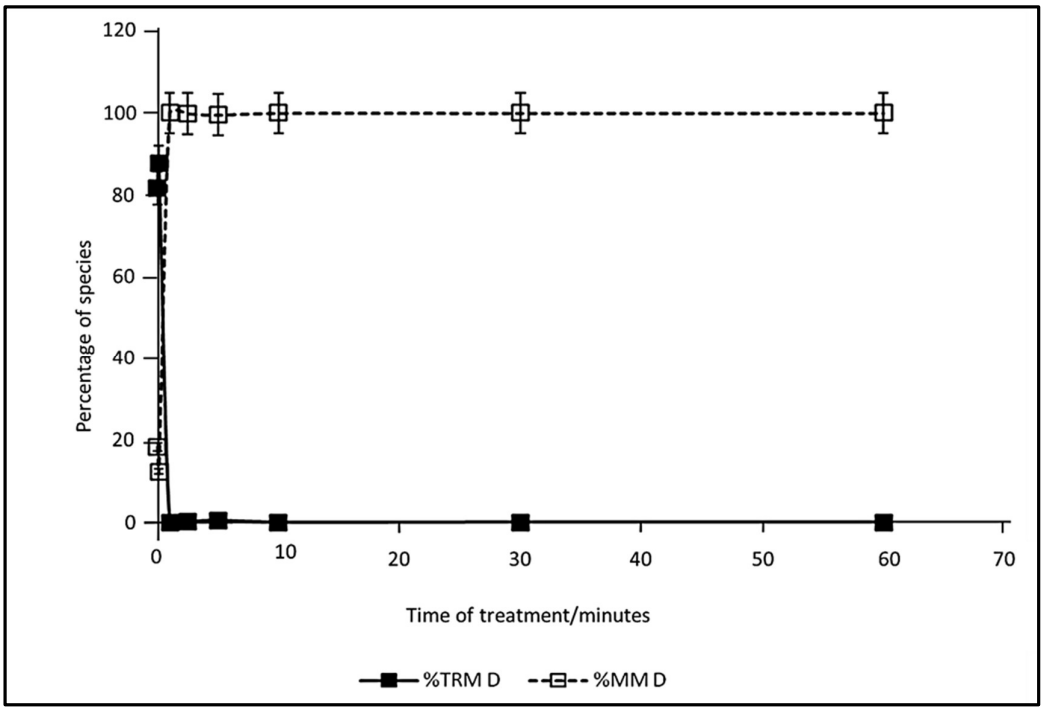

Figure 2. Chart following the kinetics of denaturation of Eε34 TSP in 5 M urea, pH 3. Chart following the kinetics of denaturation of Eε34 TSP in 5M urea, pH 3 compiled from the densitometric values obtained from bands of triplicate experiments. %TRM D = percentage trimeric Eε34 TSP species under denaturation condition of urea-acid, %MM D= percentage Eε34 TSP monomers under denaturation condition of urea-acid. p-value = 0.05, n= 3.

At pH 3, in 5 M urea (Figure 2), the quickest and sharpest drop of TSP species from trimeric forms to monomers is recorded. At an initial high of over 80% trimers, 60 seconds of treatment under this condition pulled the trimeric species curve down to almost 0%. No record of trimeric forms is seen with passing time afterward. While a quick drop in trimeric forms occurred in a space of a minute, this same space of time gave a sharp rise in the monomers, registering an average of 100% for monomers in only 1 minute of treatment. This percentage is maintained for monomers for all other time points extended from the 1-minute mark. The time point at which the two curves; monomeric species curve and trimeric species curve, intersected was recorded to be 30 seconds and registered a 50% split for both species type. This is indicative of complete denaturation at the highest speed for our TSP compared to all other conditions tested against the protein in this study.

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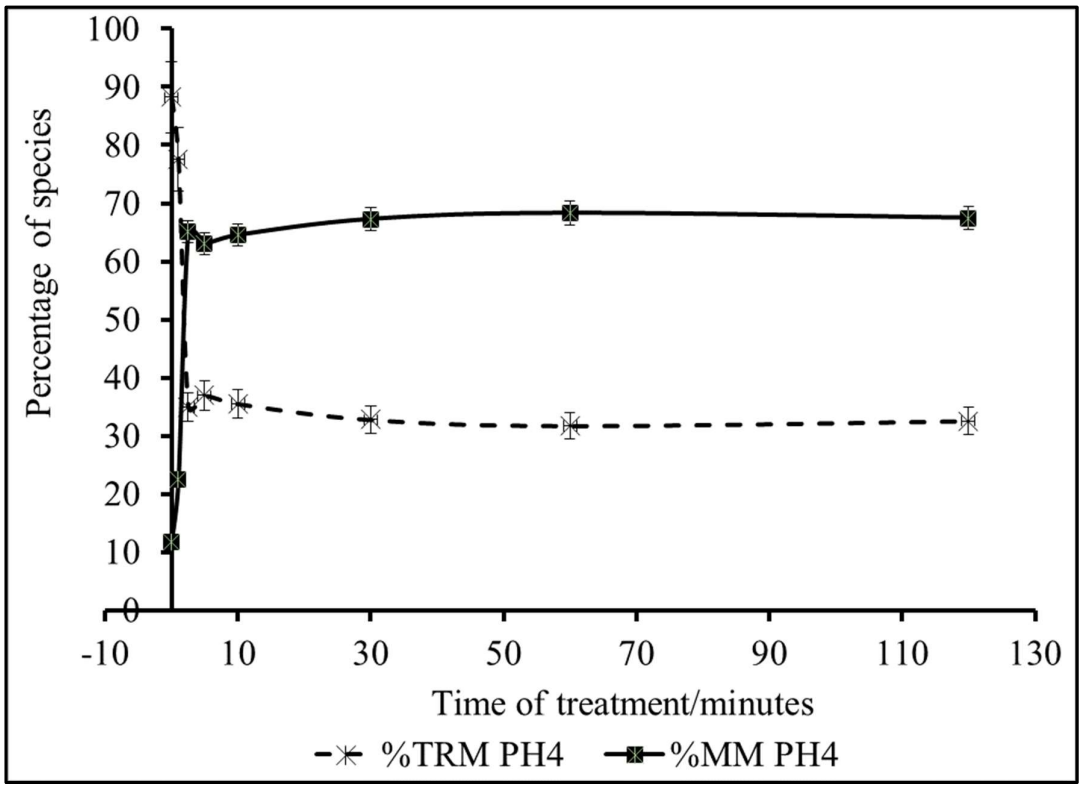

Figure 3. Chart following the kinetics of denaturation of Eε34 TSP in 5M urea, pH 4, results compiled from the densitometric values obtained from the bands of triplicate experiments. %TRM D = percentage trimeric Eε34 TSP species under denaturation condition of urea-acid, %MM D = percentage Eε34 TSP monomers under denaturation condition of urea-acid. p-value = 0.05, n = 3.

As depicted in Figure 3, at room temperature Eε34 TSP trimeric form existed in equilibrium with their monomeric counterparts, holding a huge share of approximately 90% while 10% represented monomers. However, in less than a minute, treatment of Eε34 TSP to 5 M urea, pH 4 produced a drop in the percentage trimers to approximately 77.5%, at 2.5 minutes less than 35% TSP exist in trimeric state. The rest of the time points maintained an average of approximately 34% trimeric state TSP species. Monomeric species however stood at 12.5% at 0-time point, increased steeply to 65.1% in only 2.5 minutes and stayed approximately the same for the entire experimental period of 120 minutes. The inverse relationship between the monomeric forms and the trimers are best seen at the early seconds of the treatment, with these two curves intersecting at the 89th second to record a percentage of 50% trimers and 50% monomers.

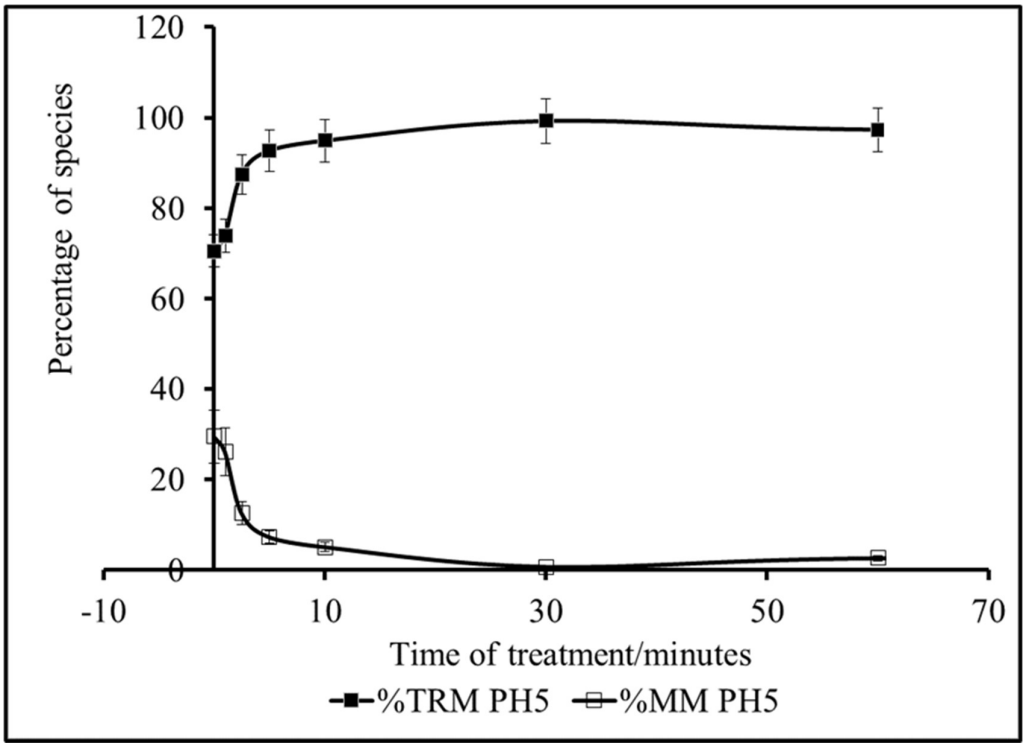

Figure 4. Chart following the kinetics of denaturation of Eε34 TSP in 5M urea, pH5, results compiled from the densitometric values obtained from the bands of triplicate experiments. %TRM D = percentage trimeric Eε34 TSP species under denaturation condition of urea-acid, %MM D= percentage Eε34 TSP monomers under denaturation condition of urea-acid. p-value = 0.05, n = 3.

Treatment of Eε34 TSP to 5M urea, pH 5 (Figure 4) produced a simple almost linear curve with nearly a zero slope. In this study, at 0-time point, the Eε34 TSP samples used had 70.5% trimeric and 29.5% monomeric species, then within half a minute, treatment of Eε34 TSP with 5M urea, pH 5 resulted in a slight increase in percentage of trimeric TSP population, then a 2.5-minute treatment showed an increased to about 87.5% trimeric TSP, 5 minutes gave an average percentage of 92.3% and a 94.9% at 10 minutes. Over an average of 99.3% trimers were recorded at time points exceeding the 10-minute mark.

The monomeric forms however, decreased with time, starting slightly over 29.4% and dropping continuously to a low of 5% at 10 minutes, and 0.7% at the 30th minute. It maintained an average low of 2.6% at the 60th minute mark.

This seems to indicate that at pH 5, in 5M urea, Eε34 TSP is resistant to denaturation, although it also seems to show a pattern in which some species of monomers are forced into association to form trimers.

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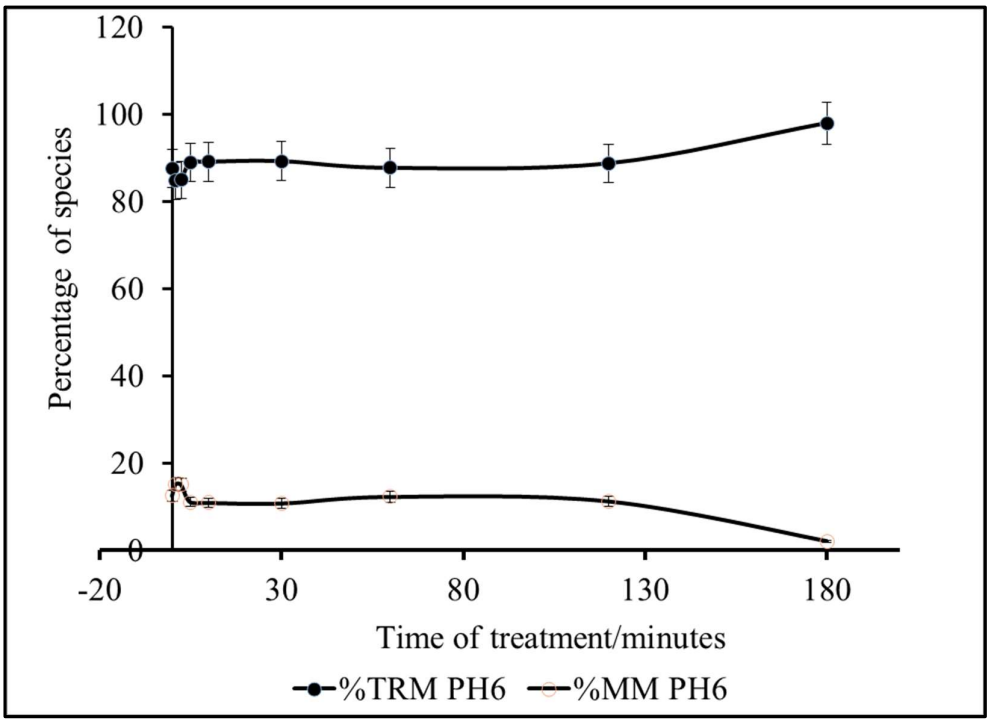

Figure 5. Chart following the kinetics of denaturation of Eε34 TSP in 5M urea, pH6. Results compiled from the densitometric values obtained from the bands of triplicate experiments. %TRM D = percentage trimeric Eε34 TSP species under denaturation condition of urea-acid, %MM D= percentage Eε34 TSP monomers under denaturation condition of urea-acid. P-value = 0.05, n = 3.

As shown in Figure 5, the trimeric species indicated 87.5% abundance at 0-time point, an incremental drop to an average of 84.8% was recorded at just 10 seconds and at 2.5 minutes later 84.9% abundance of the trimers was registered. A slight gain in percentage to 88.9% came at the 5th minute, and average abundance for the rest of the time points stayed at an average of 88% beyond the 5th minute mark.

The monomers at the other hand registered an average of 12.5% at the 0-time point at room temperature and peaked at 15.1% at 2.5 minutes then dropped down to 11.1% at the 5-minute and continue to exist in the lower percentages below 10% average for all readings beyond the 5-minute mark.

This demonstrates that EƐ34 TSP is very stable in 5M urea, pH 6. Repeating the same experimental conditions with an extended period (from 60 minutes to 180 minutes) was carried out to gain insight to the nature of TSP at 5M urea, pH 6 for extended time points (Data not shown). After 180 minutes, at least 97.9% of TSP existed in their trimeric form in all samples while less than 2.1% remained as monomers.

To appreciate the kinetics and gain greater insight of the characteristics of the protein under the varying conditions for the study, a graph focusing in the first five minutes of the kinetics are made for comparison, thus providing for a holistic view comparing the kinetics of the four denaturation curves (Figure 6).

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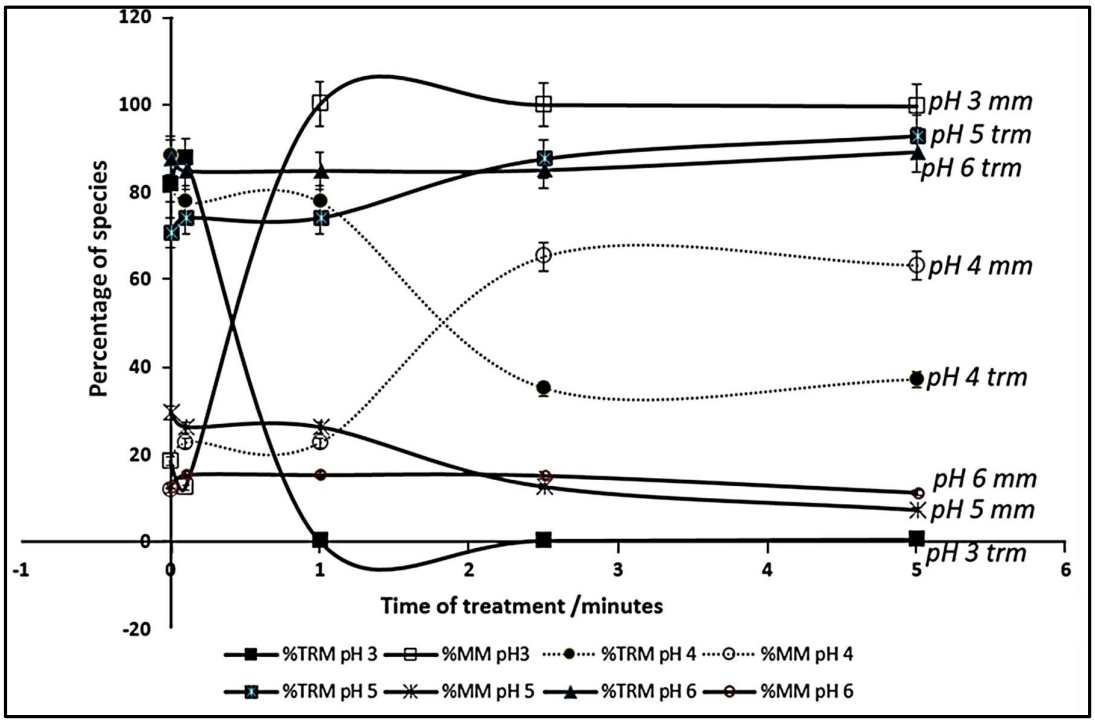

Figure 6. The first 5-minute time course of *in vitro* denaturation of Eε34 TSP via urea acid set at varying pH

As shown in Figure 6, 5 M Urea, pH 3 gave the sharpest gradient in monomeric species production followed by 5 M Urea, pH4. 5 M Urea at pH 5 and 6 respectively produced less than 20% monomeric species of Eε34 TSP on the average. An inverse relationship existing between monomeric species and the trimers saw the sharpest fall of trimers in the 5 M Urea, pH3, followed by 5 M Urea, pH4. The 5 M Urea, pH5 and pH6 maintained an almost flat curve, registering a high of over 80% trimers in average.

3.2 Renaturation kinetics of Eε34 TSP

Protein folding, unfolding, tagging, proteolysis, aggregation and reconstitution are simultaneous events occurring in the cytosolic milieu of cells (**Seckler et al., 1989; Frydman, 2001; Clark and King, 2001**). For instance, while aggregation could arise due to hydrophobic interactions between "wrongly ex- posed" protein surface areas produced by temperature and ionic strength [**van der Kamp and Daggett, 2010**) *in vivo,* the cell employs several biochemical processes to rescue protein from aggregating (**Soto and Pritzkow, 2018**), more so, *in vitro* folding experiments have demonstrated that cytosolic globular proteins possess small folding equilibrium constants and do unfold and refold many times during their life cycle (**Seckler et al., 1989**). The reconstitution of the P22 tailspike protein from denatured chains *in vitro* has been documented (**Seckler et al., 1989**).

To investigate the refolding pathway of extended ε34 TSP, this protein samples were subjected to complete denaturation via 5M urea, pH 3 for half an hour and subjected to dialysis in excess buffer consisting of 50 mM Tris, 1 mM EDTA, pH 7.0 at room temperature for a brief period of 15 minutes, afterward; samples were 20-fold diluted by the renaturing buffer (50 mM Tris, 1 mM EDTA, and pH 7.0 pre-chilled to 10 ˚C) to commence renaturation. Reconstitution reaction was incubated at 10 ±4 ˚C. Renaturation was stopped at the following designated time points by the addition of a quenching solution consisting of 0.2 mg/ml of SDS, 50 mM Tris/HCl, 250 mg/mL glycerol, 0.1 µg/ml bromophenol blue, pH 7; The set time points were 0-minute, 10 minutes, 30 minutes, 60 minutes, and 3 hours, 4 hours, 5 hours, and 9 hours and results of each of these time points shown in lane 2 to lane 10 respectively. Lane 1 is the standard protein ladder (Colored Prestained Protein Standard (CPPS), New England Biolabs). The samples were separated by electrophoresis in 10% polyacrylamide gel and visualized by Coomassie blue staining.

A similar method has been employed by Seckler and co. in their renaturation study of P22 (**Seckler et al., 1989)**, the exception however in this case was the additional use of dialysis method to precede the commencement of the renaturation process instead of direct initial 50-fold dilution employed by the said investigators. Dialysis tubes served three major advantages; 1) There is no loss of protein concentration during the dialysis with the concomitant quick reduction in the denaturant (urea) concentration. 2) Protein samples were incubated in an excess of 5M urea, pH 3 which served to quickly denature the protein within half an hour, the high acidity of the solution was quickly buffered to neutrality during the brief period of dialysis. Previous denaturation study showed that protein samples were completely denatured in this process within the first 2 minutes; 3) Simple protein detection/visualization method such as Coomassie blue staining can be employed instead of the costlier silver staining process. The renatured samples were run in 10% polyacrylamide gel. The untreated control as in lane 2 Figure 6 migrated to the position characteristic of the Eε34 TSP at its trimeric state.

3.2 SDS-PAGE ANALYSIS OF RENATURATION

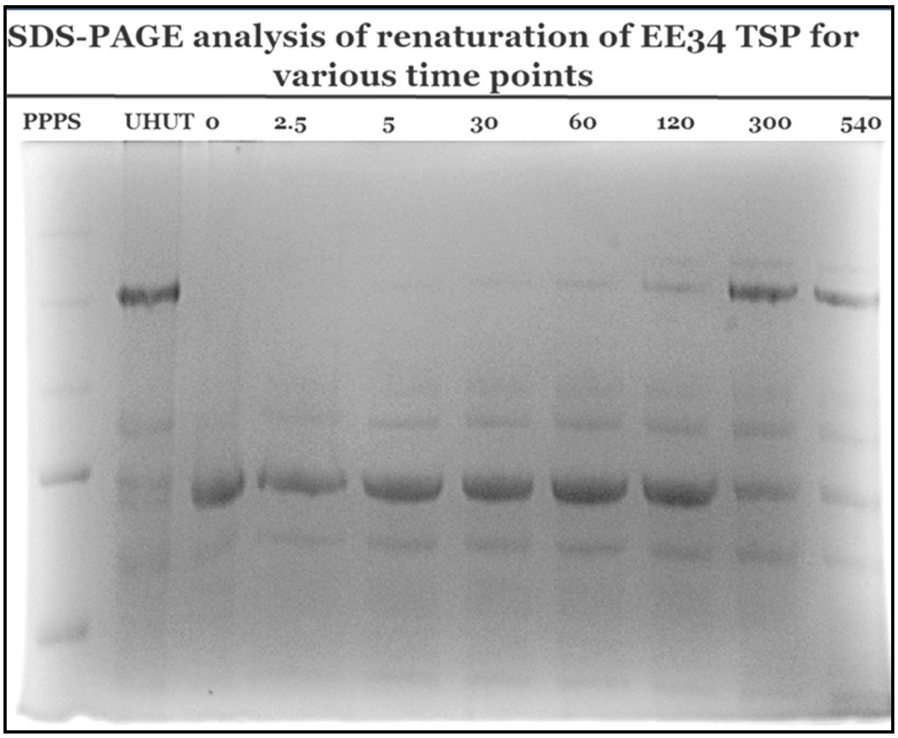

Figure 7. The renaturation kinetics of ε34 TSP into its trimeric state as observed in a 10% SDS-PAGE from fully denatured samples. Samples of EƐ34 TSP were denatured in 5M urea, pH 3 for half an hour, the renaturation process was commenced via first 15 minutes' dialysis and followed with 20-fold dilution of Eε34 TSP samples using renaturation buffer consisting of 50 mM Tris, 1 mM EDTA, and pH 8.0 at 10 ±4 ˚C. Samples were withdrawn from the renaturation reaction at set time points and the reaction quenched by the addition of quenching solution composed of 50 mg/ml SDS, 50 mM Tris-HCl, 250 mg/ml glycerol, 0.1 mg/ml bromophenol blue, pH 7.0. The set time points were 0 minute, 2.5 minutes, 5 minutes, 30minutes, and 1 hour, 2 hours, 5 hours, and 9 hours as shown in lane 3 , lane 4, lane 5, lane 6, lane 7, lane 8, lane 9 and lane 10 respectively. Lane 1 represented Prestained Perfect Protein Standard (PPPS) (Novagen), lane 2, is unheated and untreated control sample. Samples were run in 10% SDS-PAGE, visualized by Coomassie blue R-250 staining. Gels were scanned using Chemi-Doc fitted with Quantity One software.

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3.3 *Quantitative analysis of renaturation of Eε34 TSP after samples*

To quantitatively analyze the renaturation of EƐ34 TSP after the protein samples had been subjected to an initial 5 M urea, pH 3 denaturation processes for half an hour we followed EƐ34 TSP refolded species using SDS-PAGE and quantitated the densitometric values of the arising bands. As shown in Figure 8, the renaturation characteristic is indicative of hysteresis; a quicker denaturation process that occurred in less than 60 seconds in 5M urea, pH 3, took over 300 minutes to produce 80.6% trimers again, and about 275 minutes of renaturation time to produce 50% native conformers. While in less than 30 seconds, Eε34 TSP recorded 50% trimers in the denaturation process. It was also observed that 100% trimeric forms were not possible within the reach of our experimental time frame even after we allowed renaturation to continue for an extended period. Upward inflection of our trimeric species curve started at the 180-minute mark, recording a slight increase in trimeric species from approximately 0.2% to 1.3%, and then rose steadily to 26.2% average at the close of the 240th minute. The 300-minute mark recorded 80.6% trimeric species. The downward trajectory of the monomeric species also started at the 180th minute mark, registering a lowered percentage of 98.6%, a drop from 99.8%. Then 73.8% monomers were recorded at the close of the 240th minute and a lowest of 19.4% at the end of the 300-minute time point.

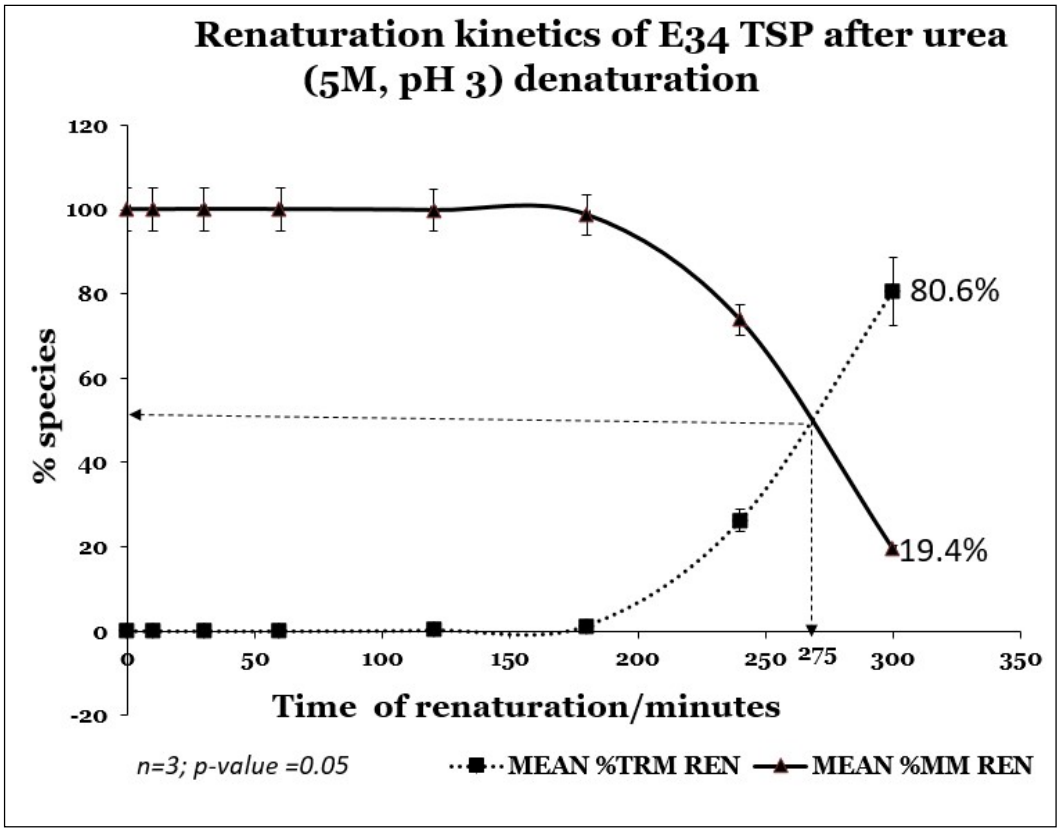

Figure 8. Renaturation kinetics of Eε34 TSP after complete urea-acid denaturation. Renaturation profile for Eε34 TSP after samples had been subjected to an initial 5 M urea, pH 3 denaturation processes for half an hour. Renaturation process was commenced via 20-fold dilution of Eε34 TSP samples using renaturation buffer consisting of 50 mM Tris, 1 mM EDTA, and pH 8.0 at 10 ±4 ˚C. Samples were withdrawn from the renaturation reaction at set time points and the reaction quenched by the addition of quenching solution composed of 50 mg/ml SDS, 50 mM Tris-HCl, 250 mg/ml glycerol, 0.1 mg/ml bromophenol blue, pH 7.0. The set time points were 0 minute, 10 minutes, 30 minutes, 60 minutes, and 3 hours, 4 hours, 5hours. Data were obtained via averaging 3 replicates of densitometric values of electrophoretic bands after SDS-PAGE electrophoresis of renaturation samples arising from three separate experiments.

3.4. Computation analysis of urea- ε34 TSP interaction

Calorimetric titration analysis of the interaction of urea with proteins has been studied and demonstrates that urea binds to proteins leading to their unfolding. It has been revealed that the binding of urea by protein follows a drop in enthalpy and entropy (**Makhatadze and Privalov, 1992**). Analysis of the binding sites of urea to the studied proteins showed that urea binds by forming hydrogen bonding (**Makhatadze and Privalov, 1992**) which significantly restricts the conformational freedom within the polypeptide chain. In a molecular dynamics simulations of chymotrypsin inhibitor in 8 M urea at 60 °C the protein unfolded; however it retained its native structure in water at the same temperature (**Bennion and Daggett, 2003**). They also observed that the overall process of unfolding in urea resembles the protein's unfolding process in thermal denaturation simulations above the protein's Tm of 75 °C (**Bennion and Daggett, 2003**). Notably, they demonstrated that the initial unfolding step was the expansion of the hydrophobic core (**Bennion and Daggett, 2003**) which led to the solvation of the core by water and urea.

In this work, as shown in Figures 10 and 11, urea binds to the hydrophobic core of the HBD of ε34 TSP via the amino acid residues as depicted in Table 1. It is clear from Table 1, that urea is engaged in hydrogen bonding with the protein. The in vitro characterization of the denaturation of Eε34 TSP by urea alone showed that urea could not denature Eε34 TSP at room temperature, even at higher concentrations up to 7 M (data not shown). However, at lower pH conditions, urea at 5 M was able to denature Eε34 TSP (Figures 1A, 1B, 2 and 3). Proving that urea might require an initial expansion of the hydrophobic core of Eε34 TSP similar to what has been described by Bennion and Daggett, 2003, in order to access and bind to the shielded hydrophobic core.

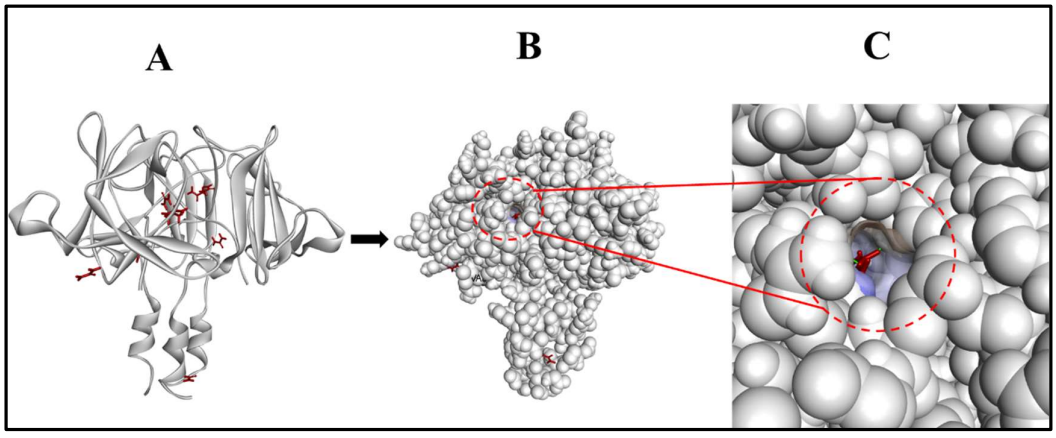

Figure 9. Putative head binding domain (HBD) of Ɛ34 TSP (grey) docked to urea molecules (red). (A) Cartoon structure of the HBD of putative Ɛ34 TSP interacting with urea in red. (B) Space filled structure of the HBD of Ɛ34 TSP showing urea interacting in the hydrophobic core of the protein. (C) An enlarged view of the urea enclosed by the hydrophobic interacting atoms of HBD of Ɛ34 TSP.

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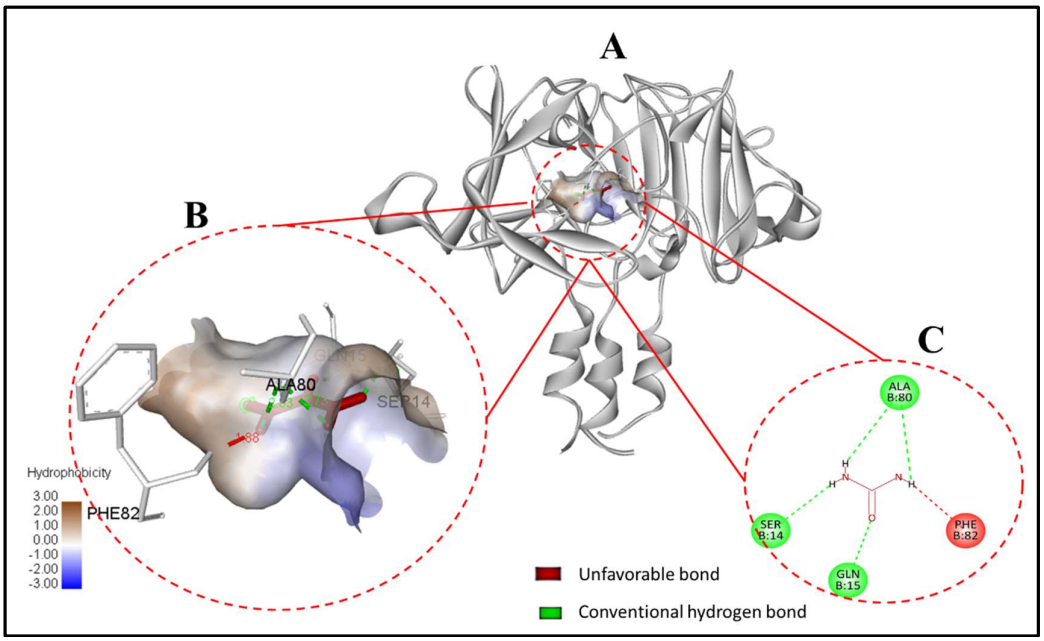

Figure 10. Putative head binding domain (HBD) of Ɛ34 TSP (grey) docked to urea molecules (red). (A) Cartoon structure of the HBD of putative Ɛ34 TSP interacting with urea in red enclosed in a hydrophobic binding site. (B) The interaction of urea with the specific interaction atoms of the HBD of Ɛ34 TSP, also depicting the degree of hydrophobicity of the binding cavity. (C) 2D depiction of urea molecule binding to four amino acids in the highly hydrophobic binding pocket of HBD of Ɛ34 TSP. The free relative energy recorded was -3.4 kcal/mol. Specific interactions are shown in Table 1.

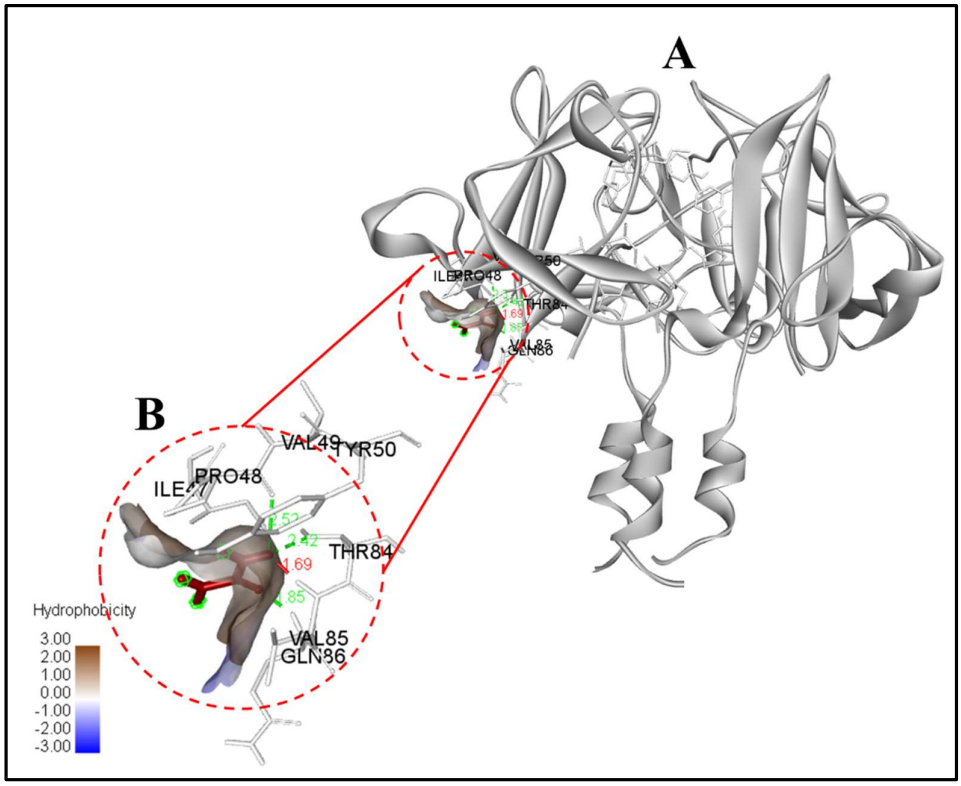

Figure 11. Putative head binding domain (HBD) of Ɛ34 TSP (grey) docked to urea molecules (red). (A) Cartoon structure of the HBD of putative Ɛ34 TSP interacting with urea in red at a slightly less hydrophobic region compared to Figure 10. (B) The interaction of urea with the specific interaction atoms of the HBD of Ɛ34 TSP, also depicting the degree of hydrophobicity of the binding cavity. The free relative energy recorded was -2.9 kcal/mol. Specific interactions are shown in Table 1.

Model	Residue	Bond distance/Å	Bond type	ΔG/kcal/mol
1	GLN15	1.98	Hydrogen Bond	-3.4
1	ALA80	2.83	Hydrogen Bond	
1	SER14	2.19	Hydrogen Bond	
1	ALA80	2.73	Hydrogen Bond	
1	PHE82	1.88	Unfavorable bond	
6	GLN86	1.85	Hydrogen Bond	-2.9
6	PRO48	2.52	Hydrogen Bond	
6	THR84	2.42	Hydrogen Bond	
6	VAL85	1.69	Unfavorable bond	

Table 1. The site-specific interaction of urea with the HDB of Ɛ34 TSP, depicting the interacting amino acids, the bond distances, the bond types recorded as well as the relative free energies predicted to be released upon interaction of urea and Ɛ34 TSP.

4.0 Discussion

The analysis of the urea-acid denaturation process of Eε34 TSP have helped identify two major aspects of the protein; 1) first it proofs that, the protein have no intermediate since no intermediary band was noticeable, however two forms of the protein were recorded, thus the monomeric and the trimeric states of the protein. 2) It showed that the unfolding and reconstitution follows separate unique pathways. The renaturation studies demonstrated that Eε34 TSP can be renatured with a substantial good yield under refolding conditions *in vitro*. While it took 48 hours for P22 TSP to achieve 80% renaturation (**Seckler et al., 1989**), 5 hours of renaturation of Eε34 TSP produced similar results. In the case of P22 TSP renaturation began slowly at 3-hour time point, and progressively increased with time to achieve complete refolding at 96 hours (**Seckler et al., 1989**), Eε34 TSP however did not achieve 100% renaturation even after extended time points, however, began similarly at the 3-hour time mark, and progressed quickly to achieve 80.6% trimeric forms at 5-hour time point. In the limits of experimental errors, and the slight difference in experimental conditions for which our protein was subjected to e.g. temperature fluctuation within the limits of 10 ±4 ˚C and a lower dilution of 20-fold, whereas the study by Seckler and his colleagues on P22 TSP received a constant temperature of 10 ˚C and with higher dilution of 50-fold, it seems plausible to assume that Eε34 TSP refolds quicker than P22 TSP. An argument that our lower dilutions (20-fold instead of the 50-fold dilution used by Chen and King) might have resulted in higher concentration of our polypeptide chains, which might present higher frequency of collision and association into fully matured trimeric species, whereas the higher dilution of P22 TSP decreased the polypeptide collision frequency and hence their frequency of association and oligomerization is feasible. Nonetheless, it is interesting to observe that both studies recorded an initial reconstitution starting at the 3-hour time point. The reconstituted products

of EƐ34 TSP after the 300-minute mark and the 540-mark showed native-like characteristics since these species were resistant to SDS under room temperature and hence migrated at a trimeric level as demonstrated via the SDS-PAGE result (Figure 7).

This *in vitro* renaturation system would enable a deeper understanding of the folding and association pathway of the EƐ34 TSP. Analytic comparison of the *in vitro* renaturation of both P22 TSP and EƐ34 TSP further elucidates the functional and structural similarities these two proteins share. A major drawback of this study is the fact that this is conducted in *an in vitro* condition, thus will substantially differ from *an in vivo* folding and chain association processes since in *in vivo* conditions, nascent polypeptides are assisted to fold at unfavorable conditions by molecular chaperone (**Eustace and Jay, 2004**). The obvious question that lingers here is, to what extend will such information regarding *in vitro* refolding process guide and inform our understanding of the complex *in vivo* folding process in the ε34-gp19 translational process in the life cycle of the ε34 phage? Since there exist a complex and dynamic cellular environment of the host cell compared to the test tubes, and the presence of molecular chaperones *in vivo* to assist unfolded TSPs or nascent polypeptides fold into their proper conformations but absent *in vitro* further compounds issues. The chances of post proteolytic processing of the TSPs if administer *in vivo* are not captured in these *in vitro* assays, and these can possibly add up to confound and or limit our full grasp of the true nature and property of folding, unfolding, and refolding of our TSP *in vivo* when administered orally or intravenously as an antimicrobial. However, it can be glimpsed from this study that even within the limits of *in vitro* experimentation, reconstitution of Eε34 TSP is possible.

The *in-silico* studies (Figures 9, 10 and 11) further support established knowledge that urea binds to solvent inaccessible hydrophobic regions, thus destabilizing the protein's core and subsequently leading to the protein's denaturation. The EƐ34 TSP however showed high resistance to urea denaturation at higher pHs (Figures 1, 4 and 5).

5.0 Conclusion

In conclusion, this work unraveled the Eε34 TSP unfolding, and renaturation pathways, demonstrating the high stability of the protein against urea denaturation. Furthermore, this work demonstrated the ability of Ɛ34 TSP to renature into its native SDS resistant state after complete denaturation via urea-acid. Finally, we demonstrated via computational analysis the interaction between urea molecules and Ɛ34 TSP. Thus in summary, in this study, we investigated *in vitro*; 1) the unfolding pathway of Eε34 TSP under the influence of urea tinkered with acid and 2) the ability of the Eε34 TSP to refold back to biologically active trimeric state after it had been unfolded completely via urea-acid denaturation, and 3) finally to we reconfirm via *in silico* analysis the interaction between urea molecules and the hydrophobic core of Eε34 TSP. These discoveries taken together shows that Eε34 TSP is a stable protein and shows similar characteristics as the well-studied P22 TSP. Thus, providing evidence that the use of this protein as an antimicrobial agent against *S. newington* in an *in vivo* system could be feasible.

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Conflict of interest: All authors declare that they have no conflicts of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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