**Supplementary Data**

# Characterization of heat induced spherulites of lysozyme reveals new insight on amyloid initiation

**Pankaj Sharma, Neha Verma, Pradip Kumar Singh, Suresh Korpole and Ashish***
CSIR-INSTITUTE OF MICROBIAL TECHNOLOGY, CHANDIGARH, INDIA

**Address correspondence to:* Ashish, CSIR-Institute of Microbial Technology, Sector 39-A, Chandigarh INDIA 160036 Phone: +911726665472; Fax: +911722690685; Email: ashgang@imtech.res.in

**Table S1** Summary of protein concentrations used for studying amyloid formation by hen egg white lysozyme (and human lysozyme) using different biophysical methods.

| S.No | Paper | Year | Protein* | Protein conc. |
|---|---|---|---|---|
| 1 | Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis (Booth, Sunde *et al.* 1997). | 1997 | HL | [1]Cry.-10mg/ml<br>[2]IR- 5mg/ml<br>[3]CD-0.22mg/ml<br>[4]F- 0.2mg/ml<br>[5]MS-0.28µg/ml |
| 2 | Local cooperativity in the unfolding of an amyloidogenic variant of human lysozyme (Dhulesia, Cremades *et al.* 2010). | 2002 | HL | 8.69 mg/ml |
| 3 | Reduced Global Cooperativity is a Common Feature Underlying the Amyloidogenicity of Pathogenic Lysozyme Mutations (Dumoulin, Canet *et al.* 2005). | 2005 | HL | 0.1 mg/ml<br>[3]CD-0.2 mg/ml<br>[4]F-0.035mg/ml |
| 4 | Identification of the core structure of lysozyme amyloid fibrils by proteolysis (Frare, Mossuto *et al.* 2006). | 2006 | HL | 14mg/ml,<br>10mg/ml |
| 5 | Amyloid Protofibrils of Lysozyme Nucleate and Grow Via Oligomer Fusion (Hill, Robinson *et al.* 2009). | 2009 | HEWL | 17mg/ml |
| 6 | Structure of amyloid fibrils of hen egg white lysozyme studied by microbeam X-ray diffraction (Yagi, Ohta *et al.* 2009). | 2009 | HEWL | 0.01mg/ml |
| 7 | Characterization of Oligomeric Species on the Aggregation Pathway of Human Lysozyme (Frare, Mossuto *et al.* 2009). | 2009 | HL | [4]F-0.0336mg/ml<br>[3]CD-0.098mg/ml<br>[6]TEM-9mg/ml |
| 8 | Local Cooperativity in an Amyloidogenic State of Human Lysozyme Observed at Atomic Resolution (Dhulesia, Cremades *et al.* 2010). | 2010 | HL | [3]CD-0.28mg/ml,<br>[7]DSC-0.56mg/ml |
| 9 | Population of Nonnative States of Lysozyme Variants Drives Amyloid Fibril Formation (Buell, Dhulesia *et al.* 2011). | 2011 | HL | 0.98mg/ml |

| | | | | |
|---|---|---|---|---|
| 10 | Unfolding and aggregation of lysozyme: A thermodynamic and kinetic study by FTIR spectroscopy (Sassi, Giugliarelli *et al.* 2011). | 2011 | HEWL | 30mg/ml, 120mg/ml |
| 11 | Spatial Extent of Charge Repulsion Regulates Assembly Pathways for Lysozyme Amyloid Fibrils (Hill, Miti *et al.* 2011). | 2011 | HEWL | 0.42mg/ml |

* HL - Human lysozyme

HEWL- Hen egg white lysozyme

1. Cry. - Crystallization
2. IR - Infrared Spectroscopy
3. CD - Circular Dichroism
4. F - Fluorescence Spectroscopy
5. MS - Mass spectroscopy
6. TEM - *Transmission electron microscopy*
7. DSC - *Differential scanning calorimetry*

**Table S2**

Table of different states of lysozyme at different temperature and salt conditions are tabulated below.

| NaCl / Temperature | 0.9-1.2M | 1.2-1.3M | 1.3-1.4M | Above 1.4M |
|---|---|---|---|---|
| **35°C** | Tetragonal crystals | Tetragonal crystals | Tetragonal crystals | Aggregates |
| **40°C** | Tetragonal crystals | Tetragonal/ Orthorhombic crystals + Small Spherulites | Spherulites | Aggregates |
| **45°C** | Tetragonal crystals | Tetragonal/ Orthorhombic crystals + Spherulites | Spherulites + Aggregates | Aggregates |
| **50°C** | Spherulites + Aggregates | Aggregates | Aggregates | Aggregates |
| **55°C** | Aggregates | Aggregates | Aggregates | Aggregates |

**Table S3**

Table of residues involved in interactions between weakly associated lysozyme dimer and trimer. Among these the highlighted residues (bold and underlined) are also involved in interaction with arginine.

| **Lysozyme Dimer 3R0F** | | **Lysozyme Trimer 4CD4** | | | |
|---|---|---|---|---|---|
| Hydrogen bonding | Non-bonded contacts | Hydrogen bonding | | Non-bonded contacts | |
| Chain A–Chain B | Chain A-Chain B | Chain A-Chain B | Chain B-Chain C | Chain A-Chain B | Chain B-Chain C |
| Arg14-Asn37<br>Asp87-**Arg5** | Arg14 - Val2<br>Arg14 - Asn37<br>His15 - Val2<br>Pro79 - Arg128<br>Ser81 - Arg128<br>Asp87- Gly4<br>Asp87- **Arg5**<br>Thr89 - **Gly4**<br>Thr89 - Val2 | Asn77-Arg112<br>Ala82-Arg114<br>Asn93-Arg112<br>Asn93-**Lys116** | Arg73-Gly49<br>Arg73-Arg68<br>Gly102-Arg73 | His15-**Thr118**<br>Cys76-Arg112<br>Asn77-Arg112<br>Ile78-**Asn113**<br>Ala82-Arg114<br>Ala82-**Asn113**<br>Ser85-Arg114<br>Asp87-Arg114<br>Thr89-**Asn113**<br>Thr89-Arg114<br>Thr89-**Thr118**<br>Thr89-**Lys116**<br>Ala90-**Asn113**<br>Ala90-Arg114<br>Asn93-**Lys116**<br>Asn93-Arg112 | Trp62-Pro70<br>Trp62-Arg68<br>Trp62-Gly67<br>Trp63-Pro70<br>Arg73-Arg68<br>Arg73-Gly49<br>Lys97-Asp48<br>Asp101-Arg61<br>Asp101-Asp48<br>Gly102-Trp62<br>Gly102-Arg73<br>Asn103-Gly71 |

**Table S4**

Table of atoms of residues involved in hydrogen bonding between the dimeric and trimeric crystalline state of lysozyme are tabulated below.

| **Lysozyme Dimer PDB ID: 3R0F** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Atom 1** | | | | **Atom 2** | | | | |
| **S.No.** | **Atom Name** | **Residue Name** | **Residue Number** | **Chain** | **Atom name** | **Residue Name** | **Residue Number** | **Chain** | **Distance (Å)** |
| 1. | NH2 | ARG | 14 | A | O | ASN | 37 | B | 3.13 |
| 2. | OD2 | ASP | 87 | A | N | ARG | 5 | B | 3.22 |
| **Lysozyme Trimer PDB ID: 4CD4** | | | | | | | | | |
| 1. | O | ASN | 77 | A | NH1 | ARG | 112 | B | 3.19 |
| 2. | O | ALA | 82 | A | NH2 | ARG | 114 | B | 2.78 |
| 3. | ND2 | ASN | 93 | A | O | ARG | 112 | B | 3.29 |
| 4. | ND2 | ASN | 93 | A | O | LYS | 116 | B | 2.57 |
| 5. | NH1 | ARG | 73 | B | O | GLY | 49 | C | 3.27 |
| 6. | NH1 | ARG | 73 | B | O | ARG | 68 | C | 2.96 |
| 7. | O | GLY | 102 | B | NH1 | ARG | 73 | C | 3.29 |

**Table S5**

Comparison of previously resolved dimeric associations of lysozyme with our heat-induced dimeric and trimeric association (*cited references are below*).

| PDB Code | Space Group | Interchain H- Bonding | RMSD | Crystallization conditions | Summary |
|---|---|---|---|---|---|
| 1B2K | $P2_1$ Monoclinic | 119-87 | 4.04 | Lysozyme 50mg/ml, 0.14M NaI, pH 4.5, 291K. | Monoclinic crystals of lysozyme contain two protein molecules in an asymmetric unit. These molecules are independent and oriented parallel to each other. Ions $I^-$, $NO_3^-$ and $SCN^-$ are present between the two lysozyme molecules. These ions interact with both lysozyme molecules (19). |
| 1HF4 | $P2_1$ Monoclinic | 119-87 | 18.72 | Lysozyme 10mg/ml, 0-290mM $NaNO_3$, pH 5, 293K. | |
| 1LCN | $P2_1$ Monoclinic | 119-87 | 18.82 | Protein7.7 mg/ml, 50mM KOAc, pH 4.5, 190mM KSCN, 291K. | |
| 2D4K | $P2_1$ Monoclinic | 119-87 116-77 | 18.85 | 2% lys., 10% NaCl, 5% 1-propanol, pH 7.6, 313K | The mechanism of phase transition of protein crystals was elucidated by studying the structural changes in relation to change in solvation. Asymmetric unit contains two independent protein molecules (20). |
| 2D4I | $P2_1$ Monoclinic | 119-87 116-77 | 18.78 | 1% protein, 2% $NaNO_3$, pD 4.5, 298K. | |
| 1LJ3 | $P2_1$ Monoclinic | 119-87 116-77 | 18.73 | 0.1M NaOAc pH 4.6, 298K, 0.44M $NaNO_3$ | Monoclinic lysozyme crystals were grown as they contain two crystallographically independent molecules with slightly different packing environments. This helps in accessing the effect of normal and stabilizing additives (sucrose, trehalose, sorbitol and glycerol) on structure and hydration of lysozyme molecules (21). |
| 1LJ4 | $P2_1$ Monoclinic | 119-87 116-77 | 18.74 | 0.1M NaOAc pH 4.6, 293K, 0.44M $NaNO_3$ | |
| 1LJE | $P2_1$ Monoclinic | 119-87 | 18.77 | 0.1M NaOAc pH 4.6, 298K, 0.44M $NaNO_3$, 0.29M sucrose. | |
| 1LJF | $P2_1$ Monoclinic | 119-87 116-77 | 18.77 | 0.1M NaOAc pH 4.6, 293K, 0.44M $NaNO_3$, 0.29M sucrose. | |
| 1LJG | $P2_1$ Monoclinic | 119-87 116-77 | 18.74 | 0.1M NaOAc pH 4.6, 298K, 0.44M $NaNO_3$, 0.54M glycerol. | |
| 1LJH | $P2_1$ Monoclinic | 119-87 116-77 | 18.76 | 0.1M NaOAc pH 4.6, 298K, 0.44M $NaNO_3$, 0.54M glycerol. | |
| 1LJI | $P2_1$ Monoclinic | 119-87 116-77 | 18.73 | 0.1M NaOAc pH 4.6, 298K, 0.44M $NaNO_3$, 10% sorbitol. | |
| 1LJJ | $P2_1$ Monoclinic | 119-87 | 18.73 | 0.1M NaOAc pH 4.6, 298K, 0.44M $NaNO_3$, 0.29M trehalose. | |
| 1LJK | $P2_1$ Monoclinic | 119-87 116-77 | 18.79 | 0.1M NaOAc pH 4.6, 298K, 0.44M $NaNO_3$, 0.44M trehalose. | |
| 1LKR | $P2_1$ Monoclinic | 119-87 | 18.81 | 1% protein, 200 mM acetate buffer PH 8, 5%NaI. | Two lysozyme molecules are present in an asymmetric unit. $I^-$ ions are present between protein molecules, it links both molecules thus stabilizes the monoclinic form. Lysozyme molecules are oriented parallel to |

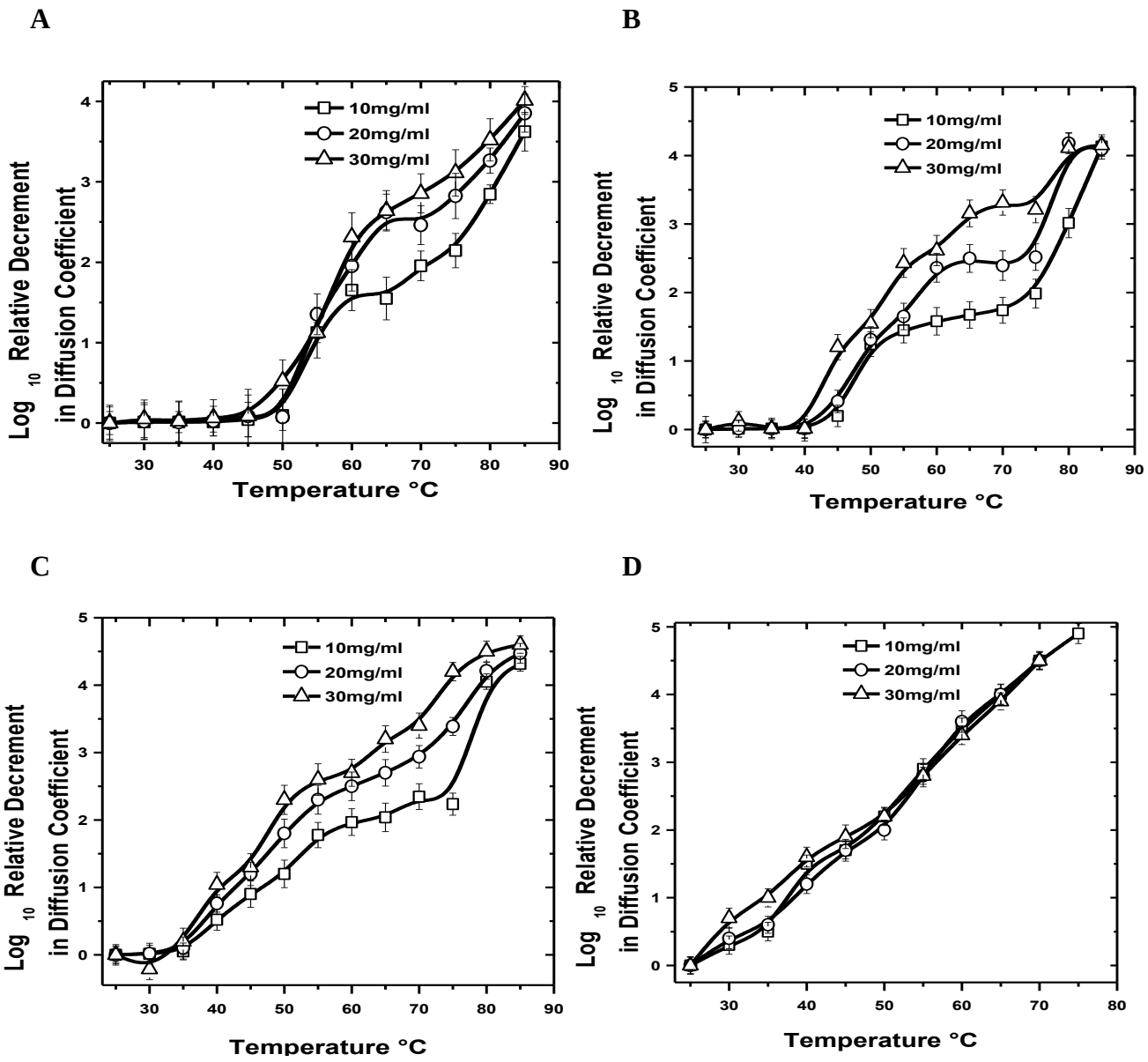
| | | | | | |
|---|---|---|---|---|---|
| | | | | | each other (22). |
| 1LYS | $P2_1$ Monoclinic | 113-81 | 18.82 | 10% NaCl, pH 7.6, 5% 1-propanol, 313K | The two molecules are parallel to each other (23). |
| 1UCO | $P2_1$ Monoclinic | 119-87<br>116-77 | 18.74 | Data not available | Lysozyme crystals were transformed from native to low humidity forms to investigate the effect of humidity on hydration, mobility and enzyme action of lysozyme (24). |
| 1V7T | $P2_1$ Monoclinic | 37-73 | 14.49 | 1%protein, 2% $NaNO_3$, 10mM NaOAc, 313K, pD 4.5. | A triclinic crystal was transformed into a new triclinic crystal. Transformed crystal contains two independent molecules of lysozyme oriented parallel to each other (25). |
| 1VDP | $P2_1$ Monoclinic | 119-87<br>116-77<br>112-74 | 18.78 | Data not available | Data not available |
| 3LYT | $P2_1$ Monoclinic | ----- | 18.70 | Data not available | Data not available |
| 4LYT | $P2_1$ Monoclinic | 119-87 | 18.75 | Data not available | Data not available |
| 5LYM | $P2_1$ Monoclinic | 119-87 | 18.79 | Data not available | Data not available |
| 1RCM | $P2_12_12_1$ Orthorhimbic | 66-39<br>81-41<br>65-41<br>41-81<br>41-65<br>39-66<br>43-68<br>68-43 | 14.73 | Protein 9mg/ml, 7% NaCl, 50mM NaOAc. | Reduction of disulfide bond between cyt. 6 and cyt. 127 displace the C-terminal segment from its conformation in the native protein, this make lysozyme a ubiquitination target. Asymmetric unit contains two lysozyme molecules oriented antiparallel to each other (26). |
| 3VFX Dimer | $P22_12_1$ Orthorhimbic | 87-5<br>14-37 | | 50mM sodium acetate, 1-1.5M NaCl, pH 3.8, temperature 318K | **Our Study** |
| 4DC4 Trimer | $P2_12_12_1$ Orthorhimbic | A-B<br>B-C<br>77-112<br>73-49<br>93-112<br>73-68<br>93-116<br>102-73<br>82-114 | | 50mM sodium acetate, 1-1.5M NaCl, pH 3.8, temperature 318K | **Our Study** |

**Table S6**

Table of residues involved in interaction with arginine and benzyl alcohol bound to lysozyme and resolved in PDB IDs 4EOF (w arginine) and 4II8 (w benzyl alcohol).

| **Ligand** | **Residues involved in interaction with ligand (PDB ID: 4EOF)** | **Residues involved in hydrogen bonding with ligand (PDB ID: 4II8)** |
|---|---|---|
| Arginine 1 | Asn113,Lys116,Gly117 and Thr118 | Asn113 and Gly117 |
| Arginine 2 | Gly4,Arg5 and Cys6 | Cys6 |
| Benzyl alcohol | Glu57,Asn59,Ala105 andGlu35 | Glu57 |

**Figure S1**




Tracking increase in particle size of lysozyme as a function of temperature, protein and salt concentration in solution. **(A)** Relative change in diffusion coefficient at 10 mg/ml (-□-), 20 mg/ml (-○-) and 30 mg/ml (-Δ-) protein concentrations on heating lysozyme from 20 to 85°C in 150 mM, **(B)** 300 mM, **(C)** 450 mM and **(D)** 600 mM NaCl at pH 3.8 is presented here. (*Lines are spline curves plotted to guide the eye on the trend*).

**Figure S2**

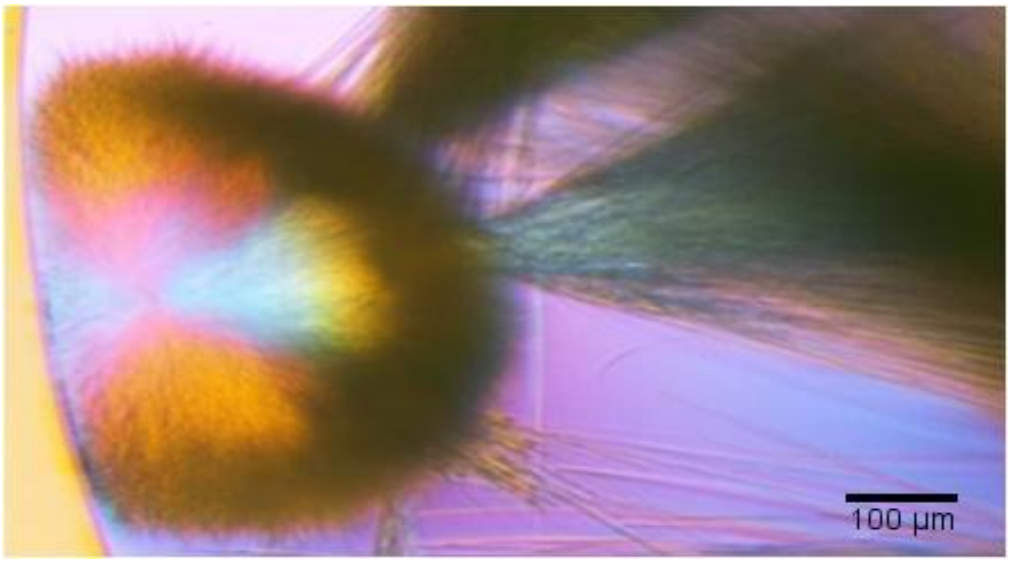
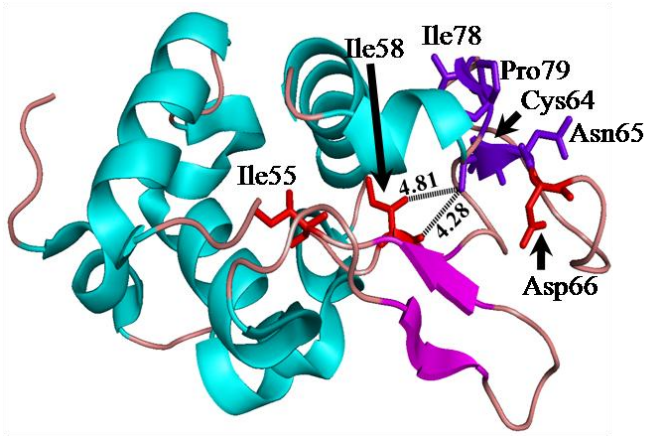


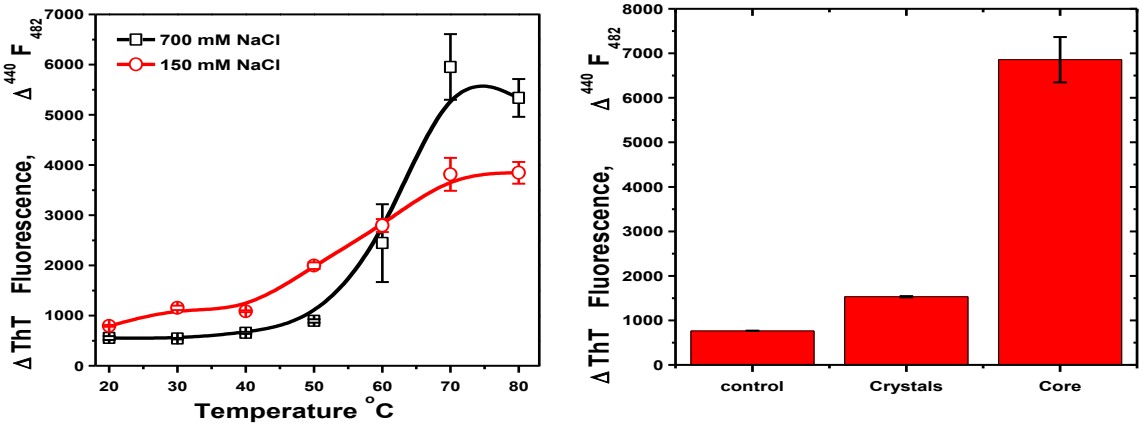

**Image of HEWL spherulite:** This image displays a clear Maltase-cross extinction pattern when observed between crossed polarizers. This observation shows that core of spherulites is composed of outwardly oriented fibrils.

**Figure S3**




One chain of trimeric lysozyme (PDB ID 4DC4) showing residues homologous to human lysozyme responsible for amyloid formation (red) and residues involved in formation of additional β-strands in our structure (purple).

**Figure S4**

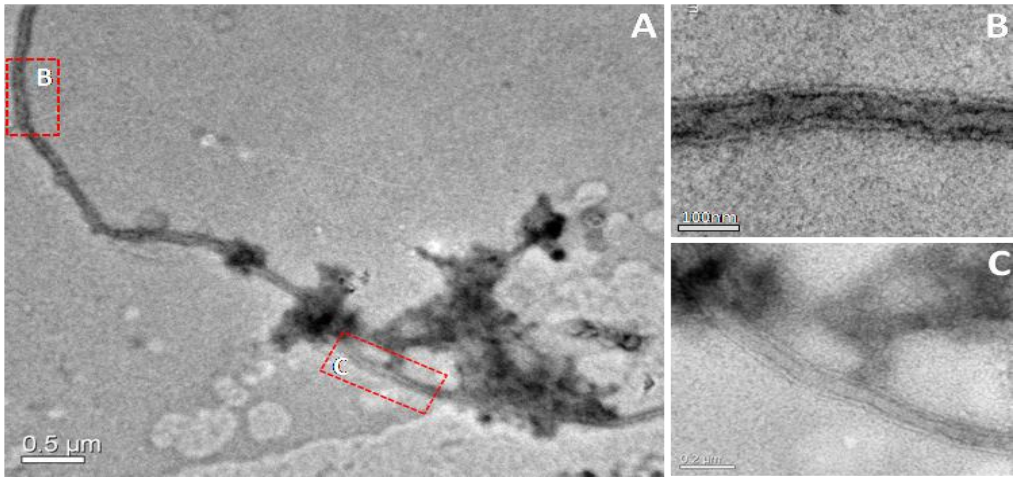



**ThT staining of lysozyme samples:** Lysozyme samples (20 mg/ml) were incubated for 3 days at different temperatures (20 to 80°C).

**(A)** A portion of the samples were stained with ThT dye, known to bind and thus stain amyloid specific structural features. Significant increase in the fluorescence with increment in temperature in the presence of 150 and 700 mM NaCl in buffer confirmed that amyloid fibrils were formed above 50°C.

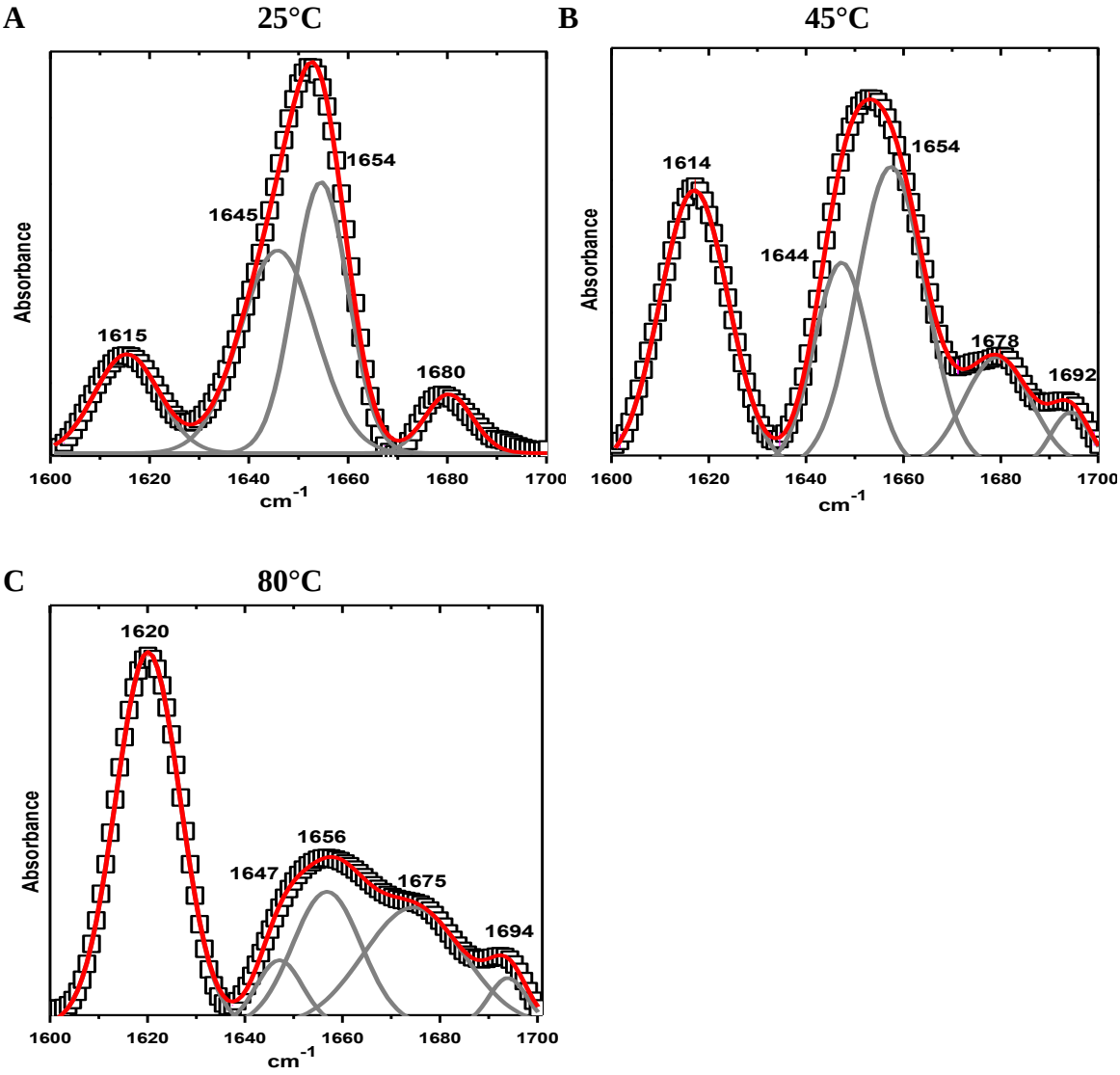
**(B)** Also, crystalline outgrowths and core of spherulites were stained with ThT. Significant difference in the fluorescence value of the core compared to crystalline outgrowths reconfirmed that core is composed of amyloid fibrils.

**Figure S5**



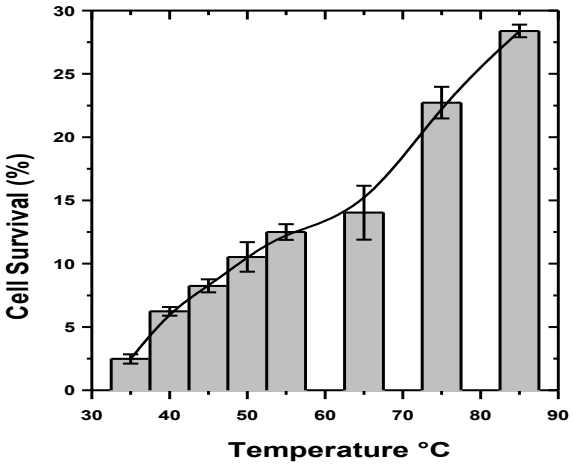

**(A)** TEM images of thin spikes emerging out of spherulites.
**(B and C)** Images show detailed architecture of fibrils on 100 and 200 nm scale, respectively.

**Figure S6**




FTIR spectra for lysozyme (20mg/ml, 700 mM NaCl and pH 3.8) collected at **(A)** 25, **(B)** 45 and **(C)** 80°C. Experimental data (□), post buffer subtraction and baseline correction is shown. Curve fitting was done considering the experimental profile to be composed of multiple Gaussian peaks close to 1620, 1645, 1655, 1675 and 1690 $cm^{-1}$. The search and fitting process yielded peaks closer to these numbers (shown in grey lines). The addition of the curves in grey lines is shown as red line, and in all cases the summation represented the experimental data (as can be seen by the overlap). Area under the computed peaks (grey lines) for β-sheet(s) and α-helical content were used to estimate secondary structural content in the HEWL sample at the temperature studied.

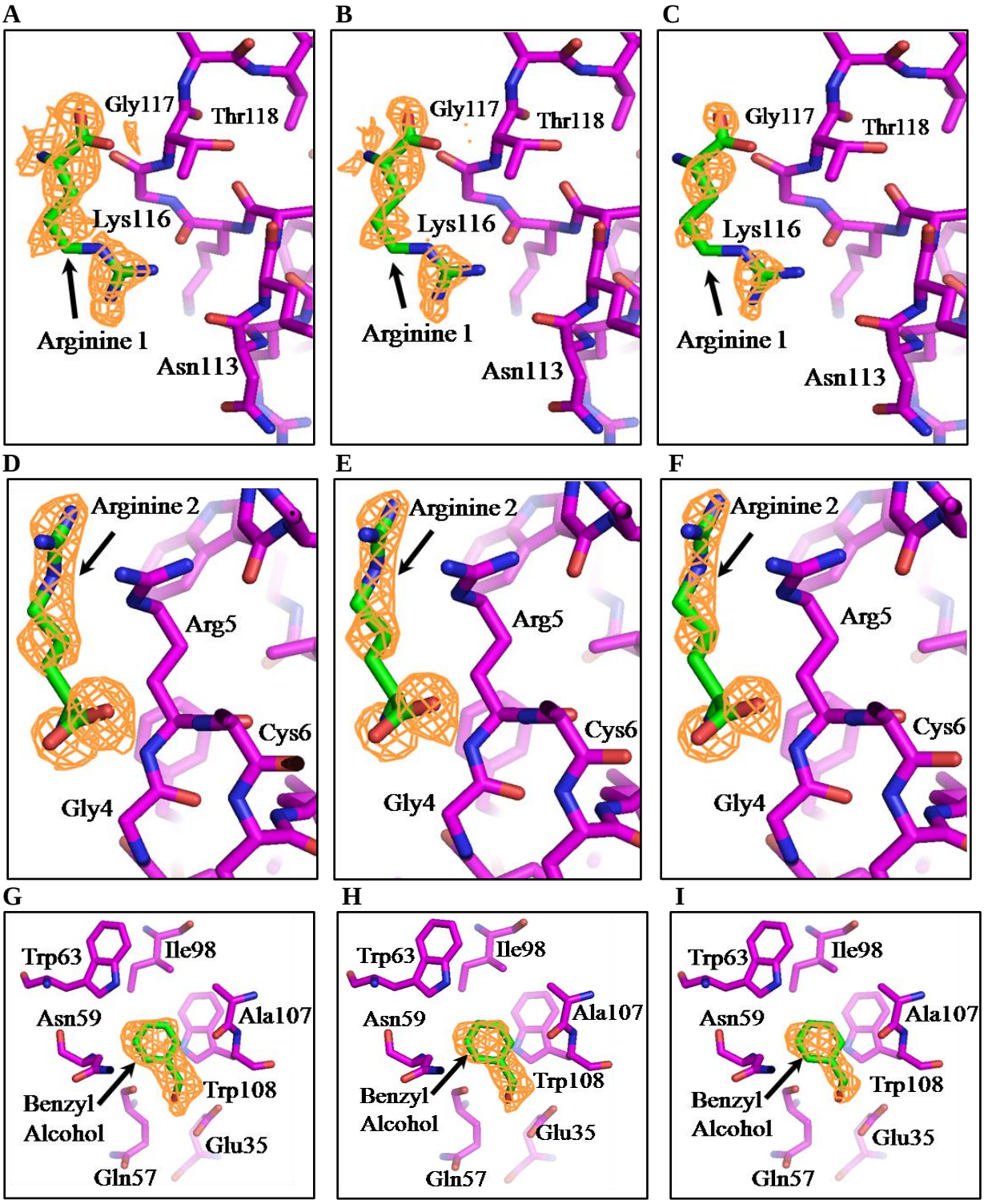
**Figure S7**




**Lysozyme Activity by Lytic Assay:** Purified lysozyme (~20 mg/ml) was dialyzed against filter sterilized buffer containing 500 mM NaCl and 50 mM NaOAc at pH 3.8. The dissolved lysozyme was incubated for one hour at different temperatures by gradually increasing the temperature from 35, 45, 50, 55, 65, 75 to 85°C. Protein samples were collected at each temperature for microbe lysis ability assay. *Micrococcus luteus* cells were grown at 30°C in 10 ml nutrient broth and the culture was harvested at mid log phase ($A_{600}$≈0.6). The cells were resuspended in 1 ml of lysozyme samples, collected at above mentioned temperatures. The resultant suspension was further incubated for 30 minutes at 35°C and the lysozyme activity was measured by colony forming unit (CFU) count after overnight incubation of plates at 30°C.

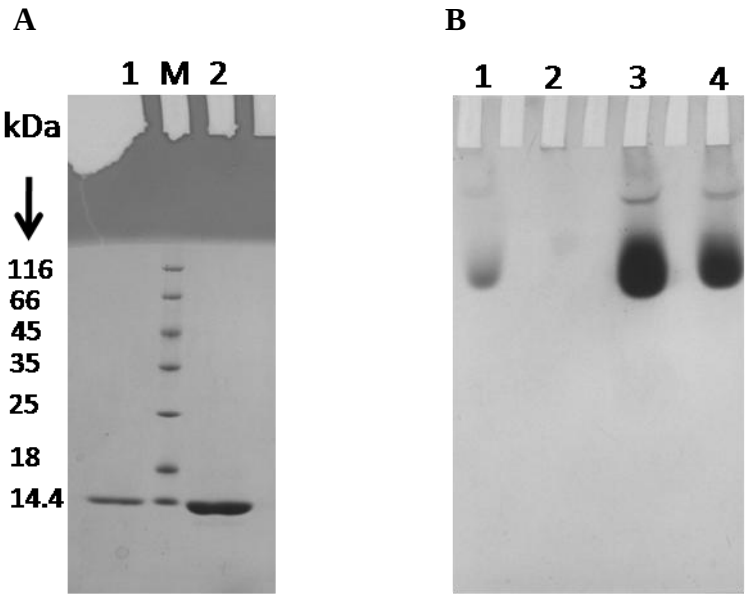
The above plot shows the cell survivability in % after treating with HEWL sample earlier heated at increased temperature.

**Figure S8**




(**A**,**B,C,D,E and F**) Electron density maps (orange mesh) for arginine and (**G,H** and **I**) benzyl alcohol with lysozyme residues involved in interaction. The *Fo-Fc* maps contoured at 2.0 σ (first pannel), 2.5 σ (second pannel) and 3 σ (third pannel) are shown as orange mesh.

**Figure S9**




**SDS-PAGE of crystalline needle shaped outgrowths and core of spherulites:**

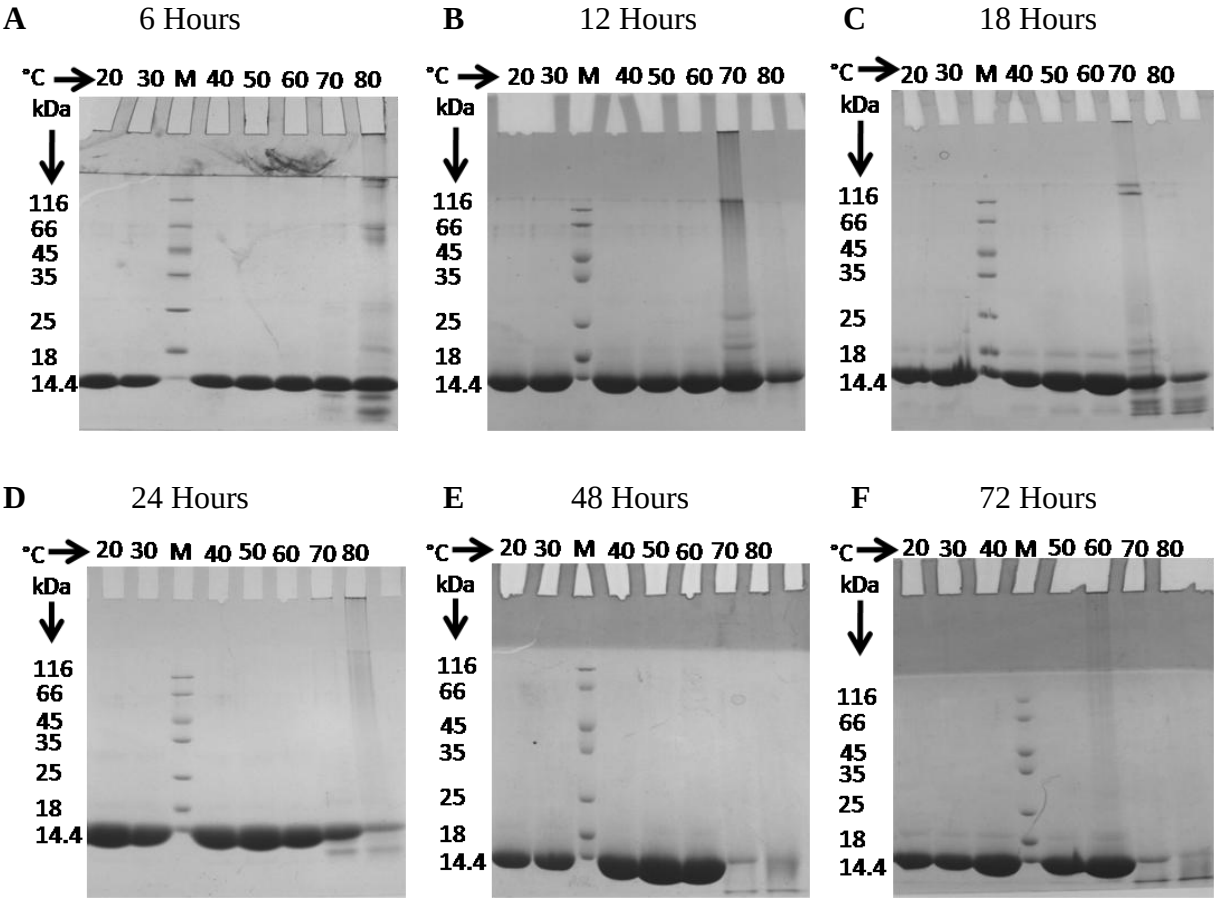
**(A)** SDS-PAGE of (**lane 1**) crystalline outgrowths and **(lane 2)** core of the spherulites formed during crystallization attempts of HEWL at 45°C were loaded on SDS-PAGE.

**(B)** Native- PAGE. Samples were: (lane 1) freshly prepared HEWL solution, (lane 2) core of the spherulites, (lane 3) tetragonal crystals and (lane 4) crystalline outgrowths from spherulites grown at 45°C.

Nothing could be seen in lane 2, probably because the association was too large to enter the gel.

These results confirmed that there was no degradation of HEWL protein during crystallization set-ups which led to spherulites described in this work.

**Figure S10**

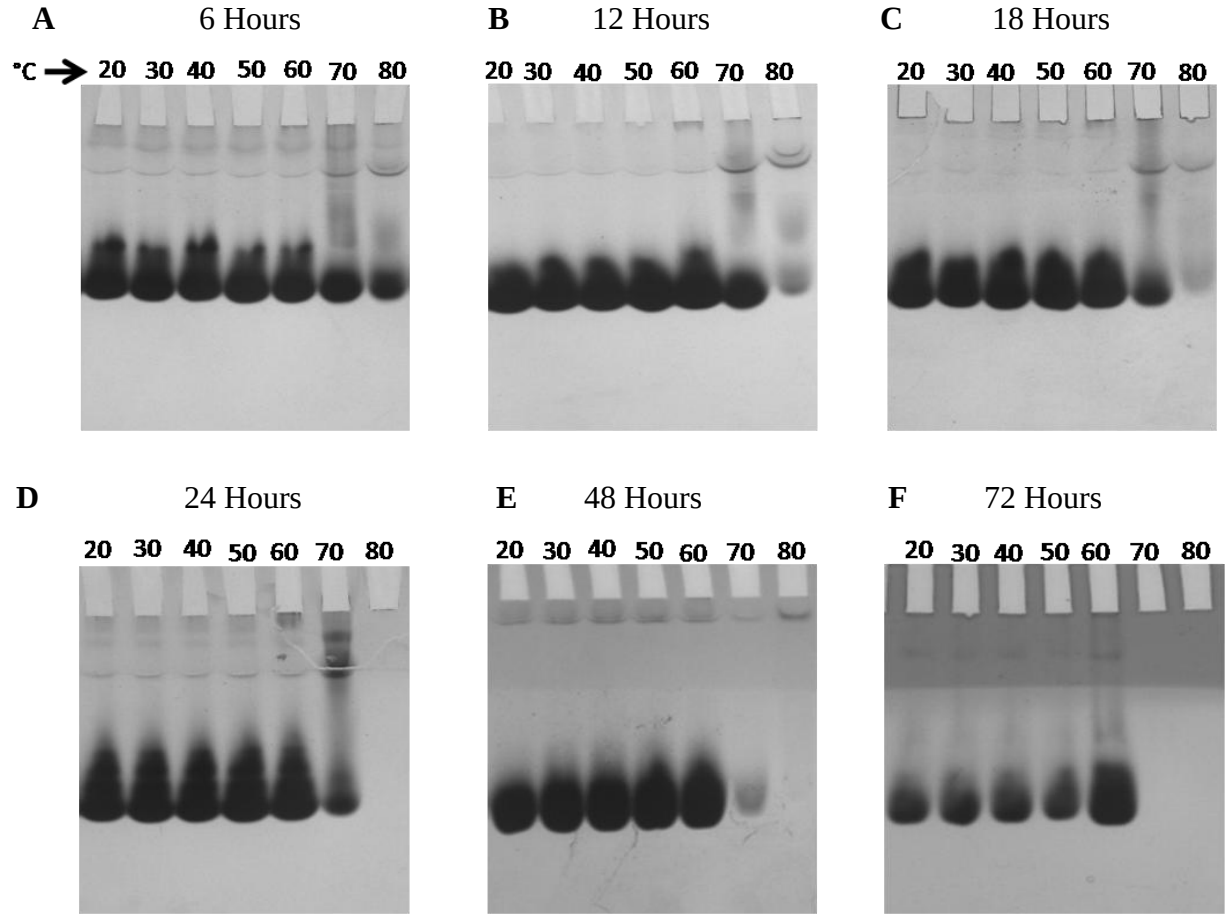



**Effect of temperature on HEWL as a function of time characterized by 15% SDS-PAGE is shown here.**

HEWL at 20 mg/ml in 700 mM NaCl was incubated 20 to 80°C, then 3µl of sample is loaded in each well. SDS-PAGE gels were run with samples heated for (A) 6, (B) 12, (C) 18, (D) 24, (E) 48 and (F) 72 hours.

Remarkable primary observation is that no HEWL protein degradation due to heating was observed below 60°C up to 72 hours.

**Figure S11**




**Effect of temperature on HEWL as a function of time as characterized by 10% Native-PAGE is shown here.**

The 10% native-PAGE was run using the protocol similar to that of SDS-PAGE (**shown in Figure S9**) except for the absence of SDS in the loading buffer and the gel.

HEWL at 20mg/ml in 700 mM NaCl was incubated 20°C to 80°C, 3µl of sample is loaded in each well. PAGE gels were run with samples heated for (A) 6, (B) 12, (C) 18, (D) 24, (E) 48 and (F) 72 hours.

Primary observation was the observation of higher order oligomers started after 6 hours above 60°C as depicted by reduction in band intensity. After 12 hours, bands corresponding to 70°C and 80°C start disappearing as more protein units were converted to higher order oligomeric state.

Importantly, under 60°C, most of the HEWL protein was present in native monomeric state up to 72 hours of incubation. (*The tailings of the bands do suggest some low order association, but we are refraining from commenting on those as the gels are deliberately overloaded since their M/Z values would be identical*).