Protein Refolding Guided by High-Throughput Differential Scanning Fluorimetry: a case study of an HtrA-Family Bacterial Protease

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Highlights

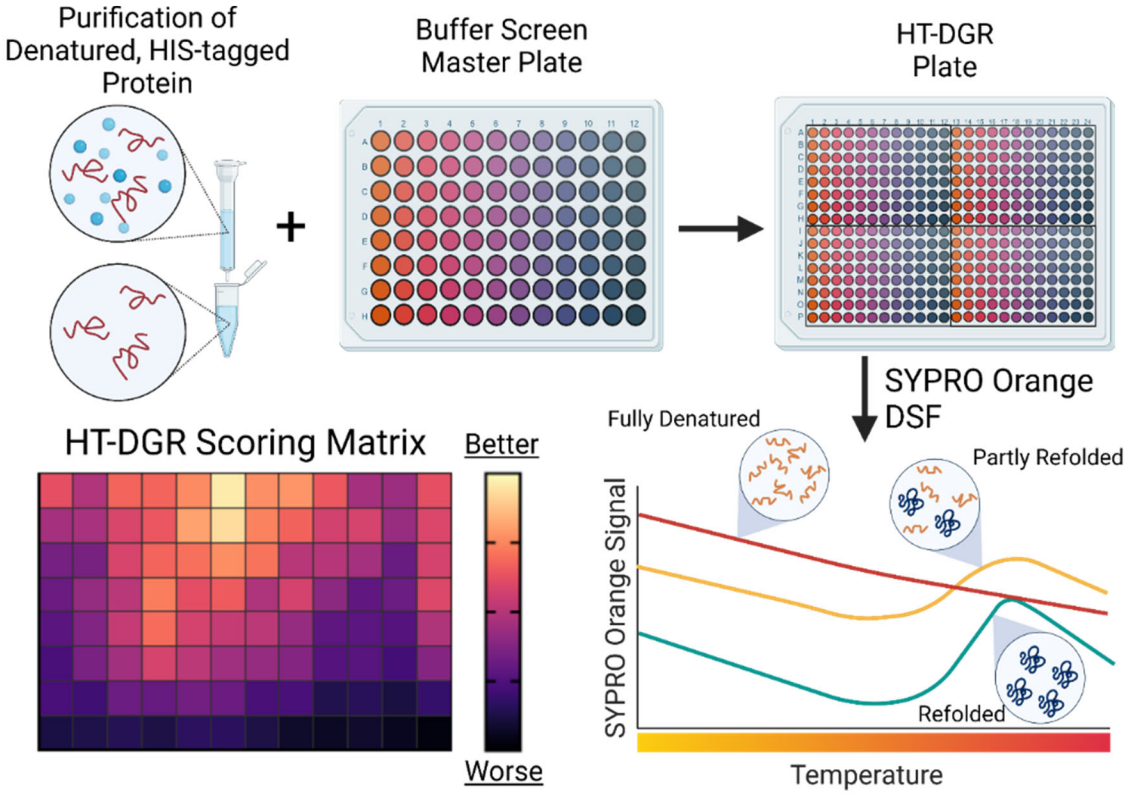
- Bacterial HtrA proteins represent an attractive target for next-generation antibiotics discovery.
- Natively purified HtrA proteins coelute with activating peptides and other contaminants, shifting the tertiary and quaternary structure and impacting activation of the protein.
- A denaturing purification of HtrA from inclusion bodies offers substantially improved yields while stripping protein of these contaminating species.
- Our extended screening method for optimal refolding conditions using a commercially available buffer screen and high-throughput differential scanning fluorimetry assay allowed us to generate HtrA protein with high purity and high yield.

Abstract

The high temperature requirement A (HtrA) serine protease family presents an attractive target class with recent interest for antibacterial development. These proteins contain numerous binding sites and regulatory loops and display diverse oligomerization patterns that depend on substrate occupancy and type. HtrA proteins that are purified natively often coelute with peptides and activating species, shifting oligomerization and protein structure to different activated populations. Here, a redesigned approach to HtrA production by purifying the protein from inclusion bodies under denaturing conditions, and screening for optimal refolding buffer composition using biophysical techniques that are function-agnostic and don't rely on any target-specific readouts or assays is described. This systematic workflow will translate to the production of HtrA-family proteins in higher quantities and of purer composition than the current literature standard.

Subject Category
Protein purification methods
Keywords
Protein refolding, protein purification, buffer screening, high-throughput screening, differential scanning fluorimetry, HtrA

Graphical Abstract



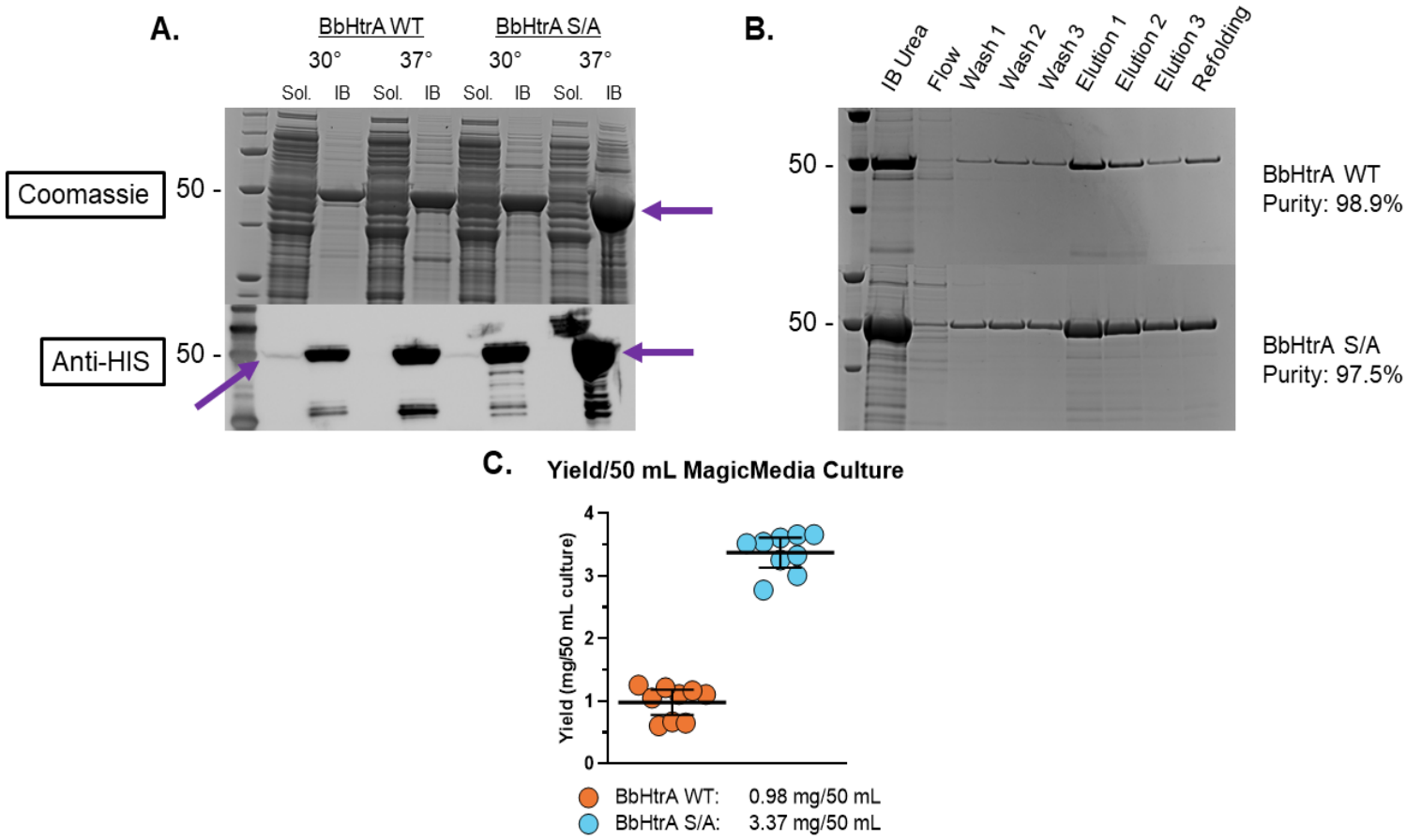


Figure 1 (A) Expression levels of BbHtrA protein in soluble vs. IB fractions of a 2 mL small-scale culture at different temperatures using the MagicMedia autoinduction system, tested by SDS-PAGE and immunoblotting. BbHtrA has an expected molecular weight of ~50 kDa (purple arrows). (B) Proof-of-concept workflow of a denaturing purification and refolding of BbHtrA proteins in PBS results in a highly pure protein. (C) The protein amount after purification and refolding was measured using the Bradford assay. Expression yields for BbHtrA WT and S/A reach 0.98 mg/50 mL (0.78 - 1.18 mg/50 mL 95% CI) and 3.37 mg/50 mL (3.13 - 3.61 mg/50 mL 95% CI) when testing 9 separate 2 mL small-scale cultures at 37 °C for 24 hours.

Proteases from the high temperature requirement A family are widely expressed across the animal kingdom and essential to the survival and infectivity of bacteria and other microbes of concern to public health, making them particularly tractable as next-generation antibiotic targets for drug discovery campaigns [1, 2]. HtrA homologs display a wide range of quaternary structures that vary in oligomerization and shape, and the majority of HtrA protein structures remain unstudied [3-9]. All known bacterial HtrA-family proteins form homo-oligomers that shift to higher-order oligomeric structures by different signals, most often by allosteric regulations through the PDZ domains [7, 10-14]. When a substrate binds to the regulatory PDZ domain, it causes conformational changes in the regulatory loops of the protease domain, removing blocks from the oxyanion hole and the S1 specificity pocket and allowing for proteolytic or holdase-like chaperone activity [4]. Previous groups have reported the presence of contaminating peptides that co-purify with and influence the oligomerization of HtrA [4]. Following up, Bai et al. directly show copurified DegQ substrates by mass spectrometry after native purification from *E. coli* culture [15]. Purifying HtrA proteins under denaturing conditions would provide a means of removing any potential HtrA-bound ligands. The *B. burgdorferi* HtrA, BbHtrA, is a therapeutic target shown to contribute to borrelial dissemination in the host and the pathogenesis of Lyme disease [16-19]. This manuscript presents a roadmap and protocols for an improved overexpression and denaturing purification of BbHtrA followed by function-agnostic biophysical screening for the optimal refolding conditions.

Cloning and expression of the HtrA protein

The BbHtrA amino acid sequence was parsed using the SignalP 3.0 signal peptide detection program with hidden Markov model (HMM) and neural network modes. Interestingly, the HMM model highlights a signal peptide cleavage site between position 28 and 29, while the neural network mirrors prior literature in detecting a peptide cleavage site between positions 37 and 38 and was chosen moving forward [16]. The BbHtrA gene strand (AAC66500, 1326 bp) was cloned into the pET28a(+)-TEV vector using the NdeI/BamHI restriction sites, retaining the N-terminal 6XHIS tag and TEV protease site for downstream tag cleavage. These constructs were verified at the T7 promoter for correct insertion, after which BbHtrA WT and the inactive S226A point mutant (S/A) were transformed into BL21 Star (DE3) chemically-competent *E. coli* which contain a rne131 mutation that leads to higher mRNA stability and thus higher-level protein expression over

standard BL21 strains. For the transformation reaction, 10 ng of plasmid in 5 µL ddH_2O was mixed gently with one vial of BL21 Star cells and transformed following the manufacturer's standard protocol. 80 µL of the reaction was streaked onto kanamycin agar plates and incubated at 37 °C overnight, after which the protein expression of three separate colonies was checked with a small-scale culture according to the manufacturer's protocol (2 mL culture of Terrific Broth at 37 °C overnight, 50 µg/mL kanamycin, 0.5 mM IPTG). The culture was spun down (2,000 x g, 10 min, 4 °C) and lysed using 250 µL BugBuster Master Mix for 30 min at room temperature (RT). After spinning down the crude lysates (20,000 x g, 30 min, 4 °C) and collecting the suspension, 5 µL of each lysate (1:50th of the original culture) was separated using SDS-PAGE and analyzed by immunoblotting for the histidine tag (1:2500 α-HIS). Clones with the highest expression of protein for each mutant were selected to move forward (Fig S1A).

The biomass and BbHtrA yield was further improved by using a high-density autoinduction media, an expression system that is intervention-free after inoculation and improves the yields of target proteins under T7 promoter by using differentially-metabolized carbon sources as compared to traditional multistep LB-IPTG induction [20, 21]. BbHtrA WT and S/A were overexpressed in 2 mL MagicMedia autoinduction media at both 30 and 37 °C to monitor protein yield and whether the protein is expressed solubly or not. As observed in prior testing with BbHtrA and other HtrA homologs in-house, HtrA proteins tend to express most in inclusion bodies (IB) and shift expression towards the soluble fraction when the temperature is lowered at the expense of total yield (Fig 1A). The expression of the S/A mutant was also higher relative to the wildtype protein, though the protein almost entirely appears in the inclusion body fraction (Fig 1A). A pilot denaturing purification was then performed to assess the purity using 0.2 mL Ni-NTA spin-columns according to manufacturer's protocol and subsequent refolding in PBS (1X, pH 7.4). The resulting preparations of BbHtrA WT and S/A had a purity of 98.9% and 97.5%, respectively, when analyzed by SDS-PAGE and Coomassie staining (Fig 1B). Testing the target protein yield of 9 separate small-scale cultures (performed in parallel at 37 °C for 24 hours) with the Bradford assay gave a final yield for BbHtrA WT and S/A at 0.98 mg/50 mL MagicMedia (0.78 - 1.18 mg/50 mL 95% CI) and 3.37 mg/50 mL MagicMedia (3.13 - 3.61 mg/50 mL 95% CI) (Fig 1C), respectively.

Screening for optimal refolding conditions

Purifying functional protein from inclusion bodies presents unique challenges around the proper solubilization and refolding of denatured proteins to their native conformations while producing acceptable yields. Biter et al. established a differential scanning fluorimetry (DSF) -guided workflow to screen buffers for the optimal refolding conditions by diluting purified, denatured protein into a matrix of buffer agents and salt concentrations and then monitoring for the presence and magnitude of thermal melting transitions [22]. This method is especially powerful because the primary readout is agnostic to the target protein's function, providing information on refolding conditions without the requirement for functional testing of your target protein. Here, the utilization of previous DSF-guided refolding workflow is greatly improved by compression into a 384-well format to increase sample throughput and the introduction of a new scoring parameter to better analyze thermal unfolding curves.

To perform the high-throughput DSF-guided refolding (HT-DGR) screen, IBs containing BbHtrA S/A were resuspended in Denaturing/Binding buffer (20 mM phosphate buffer, pH 7.5, 5 mM KCl, 500 mM NaCl, 8M urea, 20 mM imidazole) at a concentration of 100 mg IB/mL, shaken at RT for 1 hour, and centrifuged at 20,000 x g for 10 min at RT. The denatured protein was then purified as described earlier using 1.0 mL Ni-NTA columns. The Hampton Research Solubility and Stability Screen 2 was used as the primary buffer screen because it has a wide-range pH, buffering agent and NaCl concentration matrix in 96-well format and was used internally with good results in other buffer screening projects (Fig 2A). The recommended dilution of 4X from the master plate was used to construct the HT-DGR screen (buffering agent final concentration: 50 mM, NaCl concentration: 0-1000 mM). The 96-well plate was compressed into the four quadrants of a 384-well plate using an Apricot PP-384 pipettor by dispensing 10 µL of each well from the master plate into 28 µL of water, making a 4X dilution in a final volume of 40 µL (after the subsequent protein dispense). Purified BbHtrA S/A was diluted to 5 mg/mL in denaturing buffer and 2 µL of protein was dispensed into each well of the 384-well plate for a final protein concentration of 0.25 mg/mL (5 µM). The plates were sealed with a foil and placed on a shaker at 100 rpm at 4 °C. Samples taken at 30- and 90-minute incubation periods gave low and noisy signal, which prompted an extension to an 18-hour incubation to allow for a more complete refolding. A greater refolding signal is expected from shorter incubation times at higher incubation temperatures as reflected in the Biter et. al protocol, however, higher yields of desired protein refolding are anticipated at lower temperatures due to less protein precipitating through aggregation [22-24].

Finally, the DSF assay plate was constructed by dry-spotting 5 nL of SYPRO Orange with an acoustic dispenser (Echo 555, Labcyte) into a 384-well PCR plate. The plate was spun down at 200 x g for 1 min before dispensing 5 µL of the refolding reaction per well and mixing using an Apricot PP-384 pipettor. The plate was sealed, spun down, and immediately run on a Roche LightCycler 480 II using standard DSF conditions (20-95 °C, 0.13 °C/second, 4 acquisitions per °C). The resulting thermal shift data set was then analyzed using Roche Thermal Shift Analysis software to derive target unfolding parameters, namely the first derivative curves, T_{agg} values, slope of the derivative peak, and initial signal before heating, using standard program parameters.

Analyzing the Thermal Shift Dataset

Most thermal shift analysis reports unfolding data by determining the midpoint of the sigmoidal fit of thermal unfolding curves as the primary readout for target thermal stability, known as the T_{agg}. Recent work on DGR assays have

also compared protein stability by reporting the multiplication of the T_{agg} by the height of the first derivative peak, correlating peak height as a measure of the protein refolding yield [22, 24]. In this work, the 'DGR score' is synthesized to describe the refolding stability and yield as represented by the multiplication of the T_{agg} by the maximum slope of the first derivative peak, and then dividing this result by the initial SYPRO Orange signal before the thermal ramp has been applied. The initial signal of a DSF curve using SYPRO Orange is a convenient indicator of the unfolding status of a sample at baseline; because the dye emits fluorescence energy only after binding to hydrophobic regions in unfolded and aggregated protein species, the initial signal may be assumed to be directly correlated with the amount of unfolded, aggregated, or disordered material present in a refolding reaction.

The results of the BbHtrA S/A HT-DGR screen show reproducible T_{agg} values across the replicate refolding reactions on our plate, but there is only a small ~1 °C difference in T_{agg} among the majority of buffer conditions tested (Fig S1B). The analysis improves after integrating the initial signal and slope of the derivative peak into a "DGR" score, giving the best refolding signals with MES, BisTris, Imidazole, and HEPES buffering agent at pH range 6.0 to 7.5 and NaCl concentrations below 100 mM (Fig 2B). Indeed, the BbHtrA protein quaternary structure is known to be sensitive to the concentration of salt in buffer, presenting substrate-independent shifts in oligomeric assemblies at different concentrations of NaCl, which fits well with the apparent sensitivity of the refolding process to salt levels [16]. The first derivative of the thermal unfolding curves of the top four selected buffers show single, sharp peaks, unlike less folded or unfolded populations in suboptimal buffer conditions (Fig 2C, green curves are optimal conditons, yellow are suboptimal). The thermal stability of BbHtrA S/A in top refolding conditions was further analyzed using nanoDSF, an orthogonal differential scanning fluorimetry method that monitors shifts in both amino-acid autofluorescence and in backscattering aggregation signals during a thermal ramp [25, 26]. Following standard nanoDSF experimental design, 5 µL of refolded protein was loaded into standard capillaries and onto the capillary tray of a Prometheus NT.48 instrument (Nanotemper) and run using a 5.0 °C/min thermal ramp at 50% LED excitation. Each condition produced strong peaks at ~65 °C, in agreement with T_m and T_{agg} measurements, with the MES buffer system chosen to move forward as it produced the sharpest, highest peak in the backscattering aggregation curve (Fig S1C). Notably, an analysis of the HT-DGR screen using only T_{agg} as the endpoint parameter would advance sodium citrate tribasic [pH 5.0, NaCl 1000 mM] (Fig S1B, green square) as a top candidate, though the shallow slope and high initial signal in the DSF thermal unfolding curves indicate an unfavorable refolding buffer (Fig 2C, S1D).

The amino acid L-arginine is often included as an additive in refolding buffers to deter aggregation of target proteins by reducing disordered protein-protein interactions along the refolding process [24, 27, 28]. Attempts to integrate L-arginine into the primary buffer screening at high-millimolar concentrations typically used in protein refolding appears to interfere with the SYPRO Orange thermal shift readout, so the alternative nanoDSF technique was applied to samples including L-arginine as described before (Fig S1E). As expected, there was no signal in the negative control water condition. The inclusion of 500 mM arginine in water produced a significant signal, while only minimal signal was apparent when only NaCl was present (Fig 2D). The MES buffer condition was markedly improved by the addition of 500 mM arginine, which induced a significant shift in the signal span in both intrinsic amino acid fluorescence at 330 nm (from 32.0 to 55.5) and the backscattering sensor for sample aggregation (from 9.25 to 20.79) (Fig 2D). Notably, while the increase in signal span indicates more properly folded protein contributing to unfolding signals, the presence of arginine did not significantly shift the T_{agg} or T_{onset}, suggesting that arginine enhances refolding through a mechanism that does not affect thermal stability of the protein and in agreement with previous work on the role of arginine in protein refolding [27].

As a final test of the MES buffer system with arginine, the refolding reaction was scaled up to 1 mg of total purified BbHtrA S/A diluted to the same concentration as the buffer screen (0.25 mg/mL) and then dialyzed overnight into the final refolding buffer (50 mM MES, pH 6.5, 50 mM NaCl, 500 mM L-arginine). In parallel, native BbHtrA S/A was produced using cell-free protein expression systems following manufacturer's suggested concentrations and protocol with 500 ng plasmid DNA in 50 µL reaction volume. Following buffer exchange of the two preparations to PBS, the protein was analyzed using nanoDSF as described earlier. There was no significant difference in the T_{agg} between cellfree native BbHtrA and the scaled-up refolded protein, interpreted as final confirmation of an optimal refolding of the BbHtrA protein as a result of HT-DGR-guided buffer design (Fig 2E). Further confirmation of correct BbHtrA refolding was given by the proteolytic cleavage of fluorescent fragments from a casein-BODIPY substrate by wildtype BbHtrA, produced using the above described protocol. Briefly, 50 nM of BbHtrA WT or S/A was mixed with 10 µg/mL casein-BODIPY in a 50 µL reaction and immediately read on a Tecan Infinite M1000 in kinetic mode for 20 minutes, where the increase in fluorescence when incubated with BbHtrA WT, but not catalytically-inactive BbHtrA S/A, indicates the proteolytic liberation of fluorescent peptide fragments from the casein-BODIPY substrate (Fig S1F).

Overall, the HT-DGR assay is a widely-accessible method to rapidly screen buffer conditions for optimal refolding of a target protein without dependence on any functional assay. Additionally, by testing conditions from the primary buffer screen with a complementary thermal shift approach, more data is revealed on favorable refolding conditions that would otherwise not be detectable with the SYPRO Orange readout. The flexibility of the HT-DGR format allows researchers to finetune the buffer conditions and concentrations tested with target-dependent changes without interfering with assay design. Finally, a protocol is established for significantly improving the purity and yield of an HtrA-family homolog that will enable more accurate biophysical characterization of these proteins.

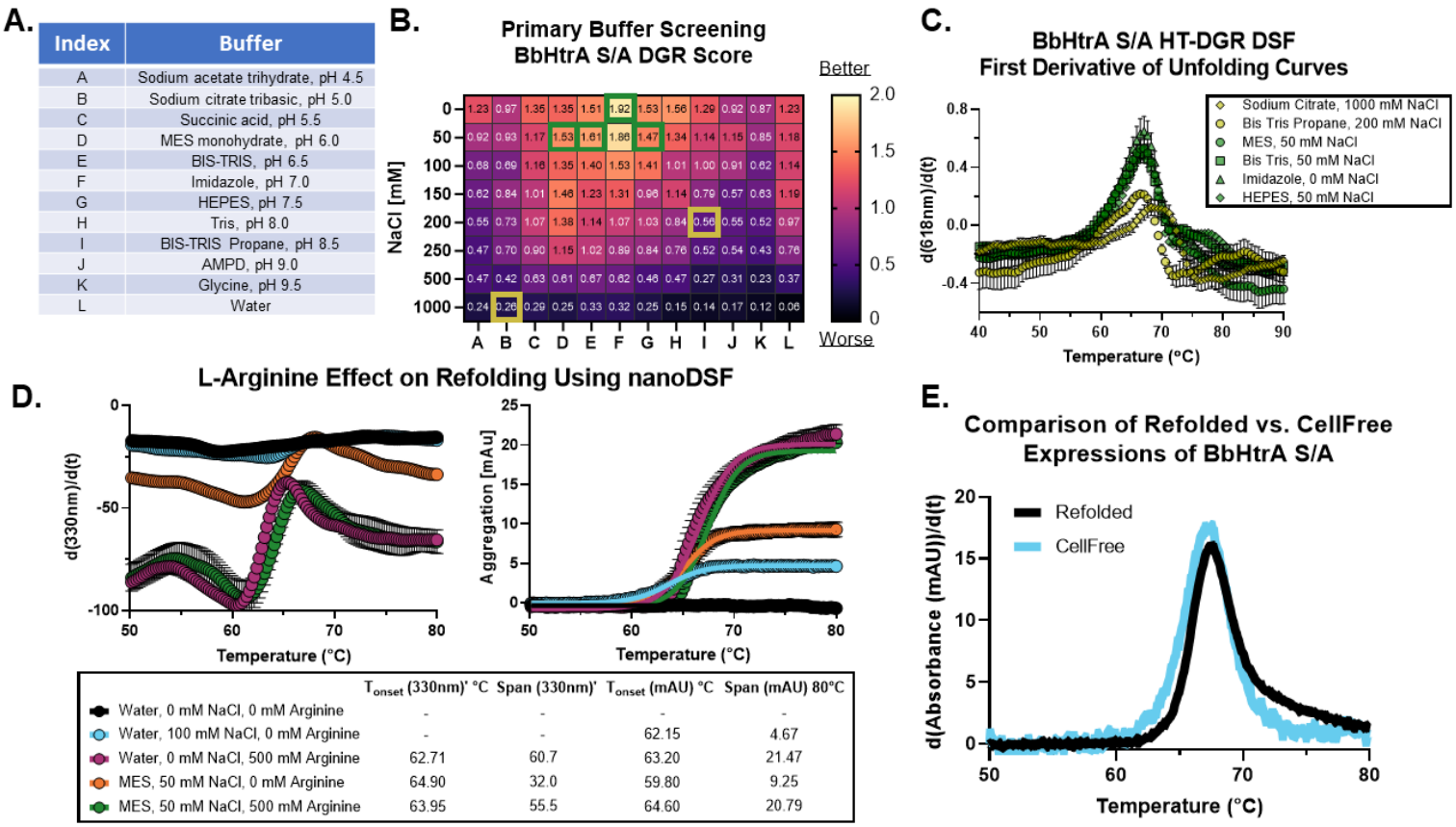


Figure 2 (A) Buffer compositions tested in our HT-DGR screen using the Hampton Research Solubility and Stability Screen 2. These buffers were diluted to a final concentration of 50 mM, and paneled in a matrix with 8 concentrations of NaCl (final 0 – 1000 mM). (B) DGR scores for BbHtrA S/A show a more optimal folding reaction at lower NaCl concentrations around 0 - 100 mM and buffer pH between 6.0 – 7.5. The top four conditions chosen for additive screening are shown with a green box, and representative suboptimal conditions are shown with a yellow box. (C) DSF first derivative unfolding curves for high and low DGR scoring conditions show clear differences in peak height and slope. Note the initial values between different buffering systems indicating different degrees of total unfolded population: Sodium citrate: 55.79 ± 8.19, BisTris Propane: 45.90 ± 7.75, MES: 31.58 ± 6.65, BisTris: 31.58 ± 6.73, Imidazole: 20.87 ± 8.67, HEPES: 33.41 ± 5.75. (D) Comparison of the effects of L-arginine on the refolding efficiency of BbHtrA S/A using nanoDSF. The T_{onset} and span of the 330nm fluorescence first derivative and aggregation readouts are given, showing the improvement in refolding yield in the presence of arginine. (E) Comparison of the thermal unfolding profiles of BbHtrA S/A expressed using cellfree native conditions and optimized refolding conditions show similar thermal unfolding profiles.

Author Contribution Statement

MR: Conceptualization, Methodology, Experimentation, Validation, Analysis, Writing, Review & Editing BB: Conceptualization, Methodology, Review & Editing UP: Conceptualization, Supervision AS: Conceptualization, Methodology, Supervision, Review & Editing

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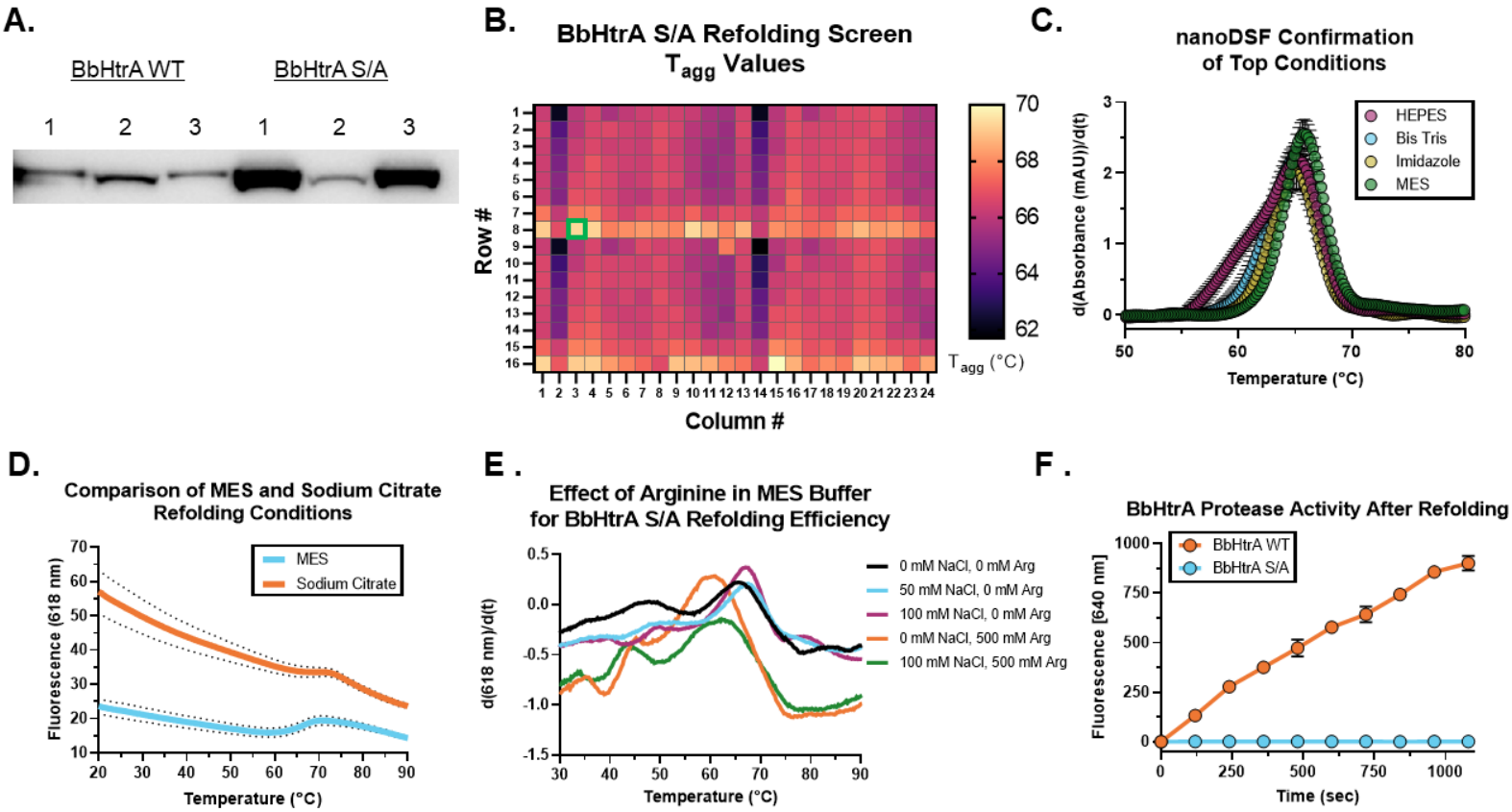
Supplementary



Supporting Figure 1 (A) Immunoblot using anti-HIS antibody against three transformation reactions of BbHtrA WT and BbHtrA S/A pET28a constructs. (B) Heatmap of T_{agg} values from an HT-DGR screen for the refolding of BbHtrA S/A. The green box is around the sodium citrate tribasic [pH 5.0, NaCl 1000 mM] condition. (C) The first derivatives of the backscattering curves for the top four conditions using nanoDSF. Data shown is the mean of three replicate biological reactions with error bars showing standard deviations. (D) The first derivatives of the backscattering curves for the top condition from the HT-DGR screen (MES) and the top condition when analyzed using T_{agg} value only (sodium citrate). Data shown is the mean of three replicate biological reactions with error bars showing standard deviations. (E) The effect of arginine on the refolding of BbHtrA S/A is analyzed using the first derivatives of the DSF thermal unfolding curves for the top refolding buffer condition. Data shown is the mean of three separate biological reactions. (F) The protease activity of BbHtrA WT and S/A after refolding was tested using a casein-BODIPY substrate. Data shown are three independent biological replicates, with error bars showing standard deviations.

Materials

- OneShot BL21 Star (DE3) Chemically Competent Bacteria (Invitrogen #C601003)
- Terrific Broth (ThermoFisher #A1374301)
- BugBuster Master Mix (Millepore Sigma #71456)
- MagicMedia (ThermoFisher # K6803)
- Kanamycin (Millepore Sigma #K1637-1G)
- IPTG (Millepore Sigma #5810-5GM)
- PBS 10X (Millepore Sigma #6505-OP)
- L-Arginine (Millepore Sigma #A5006)
- NaCl (Millepore Sigma #S9888)
- Ni-NTA spin columns (ThermoFisher 0.2 mL:#88224 1.0 mL:#88225)
- Pierce Coomassie Plus (Bradford) Assay Reagent (ThermoFisher #23238)
- Imperial Protein Stain (ThermoFisher #24615)
- SYPRO Orange (5,000X stock in DMSO, ThermoFisher #S6650)
- Solubility and Stability Screen 2 (Hampton Research #HR2-413)
- 384-well white PCR plate (Roche #04729749001)
- 384-well nonbinding reaction plate (Corning #3965)

263 • α-HIS antibody (Cell Signaling Technology #2365)
264 • nanoDSF standard capillaries (Nanotemper #PR-C002)
265 • NEBExpress CellFree Protein Expression Kit (New England Biolabs #E5360L)
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