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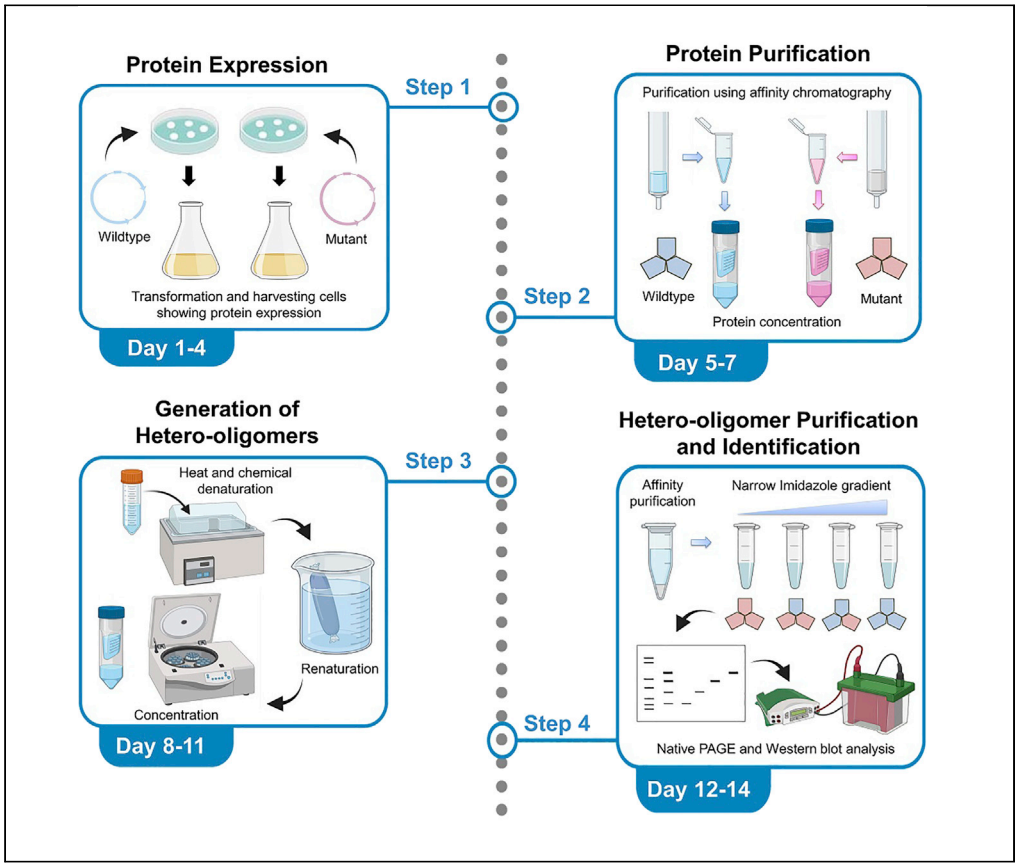
Purification of hetero-oligomeric protein variants using a modified tandem affinity purification approach



Here, we describe a protocol for artificially generating hetero-oligomeric protein complexes from the homo-oligomers using a sequential denaturation-renaturation strategy, followed by a modified affinity chromatography protocol used for their purification. This protocol enables one to obtain a homogenous population of hetero-oligomers and understand the contribution of each protomer through further biochemical and/or biophysical characterization.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Expression and purification of homo-oligomeric protein variants

Affinity purification of individual hetero-oligomer population from the mixture

Confirmation of hetero-oligomer identity through Native PAGE and Western blot analysis

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Purification of hetero-oligomeric protein variants using a modified tandem affinity purification approach

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SUMMARY

Here, we describe a protocol for artificially generating hetero-oligomeric protein complexes from the homo-oligomers using a sequential denaturation-renaturation strategy, followed by a modified affinity chromatography protocol used for their purification. This protocol enables one to obtain a homogenous population of hetero-oligomers and understand the contribution of each protomer through further biochemical and/or biophysical characterization.

For complete details on the use and execution of this protocol, please refer to Parui et al. (2022).[1]

BEFORE YOU BEGIN

This protocol generates hetero-oligomeric protein variants in *E. coli* by artificially reconstituting the protomers obtained from homo-oligomeric proteins, in order to elucidate the contributions of each protomer and/or their domains in governing the activity of the homo-oligomeric ensemble.[2–5] This method has been utilized to generate hetero-trimeric variants of a proapoptotic serine protease HtrA2.[1] We dissected its trimeric structure primarily by generating different heterotrimeric variants of HtrA2 differing in the number of a C-terminal protein-protein interaction domain - PDZ or active-site mutations.

The protocol primarily relies on the use of different affinity tags, at the N- or C-terminal ends of the two homo-oligomers, for efficiently separating the newly generated hetero-oligomers using a sequential denaturation-renaturation strategy.[2–6] In our case, wild-type HtrA2 protein (WWW) or its inactive mutant (S306A) was cloned in the bacterial expression vector pET-20b (+) containing a C-terminal His_6 histidine tag, while the PDZ-lacking HtrA2 variant (ΔΔΔ) was subcloned in a modified pMAL-c5E vector with a C-terminal His_3 tag and an N-terminal maltose binding protein (MBP) tag. A cleavage site for Tobacco Etch Virus (TEV) protease and an additional FLAG octapeptide (DYKDDDDK) tag were introduced after the MBP tag for efficient removal of MBP and to modify the overall pI (isoelectric point) of ΔΔΔ respectively. Difference in the property of the His-tags at the C-terminal end of the homo-oligomers helped in the subsequent purification of the hetero-oligomers. The FLAG tag insertion and single amino-acid substitutions were made via site-directed mutagenesis and verified by DNA sequencing facility.

This protocol is specifically beneficial for the purification of those hetero-oligomeric protein variants that have minimal differences in their biochemical properties such as molecular weight or pI from their wild-type counterparts, and are difficult to purify using conventional size-exclusion or ion-exchange chromatography. Therefore, before you begin, clone the homo-oligomers in appropriate expression vectors of suitable *E. coli* strains for protein expression and production.[7]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-FLAG	Sigma-Aldrich, India	Cat# F3165
IgG HRPO (goat antimouse)	Thermo Scientific	Cat# 31430
Bacterial and virus strains		
E. coli DH5α	New England Biolabs (NEB, Ipswich, USA)	N/A
E. coli BL21 (DE3)	New England Biolabs (NEB, Ipswich, USA)	N/A
E. coli Rosetta (DE3) pLysS	Novagen, Billerica, MA, USA	N/A
Chemicals, peptides, and recombinant proteins		
Ampicillin sodium salt	Sigma-Aldrich, India	Cat# A-9518
Chloramphenicol	MP Biomedical, India	CAS Number 56-75-7
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Sigma-Aldrich, India	Cat# I5502
Luria Bertani (broth and agar)	HiMedia Laboratories, India	Cat# RM 301
Superdex-200 10/300 HR column (120 mL)	GE Healthcare, Sweden	Cat# GE28-9893-35
Superdex-200 Increase 10/300 HR column (24 mL)	GE Healthcare, Sweden	Cat# GE28-9909-44
ReSource 15S cation-exchange column (1 mL)	GE Healthcare, Sweden	Cat# GE17-1180-01
Ni-NTA	Nucleo-pore, Genetix Biotech Asia Pvt. Ltd	Cat# NP-40211
Amylose	New England Biolabs (NEB, USA)	Cat# E8021S
2-Mercaptoethanol (β-ME)	Sigma-Aldrich, India	Cat# M7522
Triton-X 100	Sigma-Aldrich, India	CAS number 60-24-2
Imidazole	Sigma-Aldrich, India	Cat# I5513
Bradford reagent	Sigma-Aldrich, India	B6916
Sodium dodecyl sulfate	Sigma-Aldrich, India	Cat# L3771
Puregene Unstained Protein Ladder	Genetix Biotech Asia Pvt. Ltd.	N/A
3 Color Prestained Protein Ladder (10–250kDa)	Genetix Biotech Asia Pvt. Ltd.	Cat# PG-PMT2922
Coomassie Brilliant Blue G250	MP Bio	CAS# 6104-58-1
Recombinant DNA		
pET-20b(+) HtrA2 WWW	Parui et al.[1]	N/A
pMAL-c5E HtrA2 ΔΔΔ (modified with TEV protease cleavage site)	Parui et al.[1]	N/A
Software and algorithms		
Image Lab™ - version 6.0.0 build 25 (2017)	Bio-Rad laboratories, Inc.	N/A
Other		
GenElute™ Plasmid Miniprep Kit	Sigma Aldrich	SKU# PLN70
Quick change site directed mutagenesis Kit	Stratagene, USA	Cat# 200530
Pierce™ ECL Western Blotting Substrate Kit	Thermo Fisher Scientific, USA	Cat# 32106
Amicon® Ultra-15 Centrifugal Filters Amicon® Ultra-4 Centrifugal Filter Unit Amicon Ultra-0.5 Centrifugal Filter Unit (30kDa and 10 kDa MWCO)	Merck Millipore	Cat# Z71785
SnakeSkin™ Dialysis Tubing (10K MWCO, 22 mm)	Thermo Fischer Scientific, USA	Cat# 68100
Nitrocellulose membrane	Millipore, Italy	N/A
UV-visible absorption spectrophotometer	Thermo Fischer Scientific, USA	N/A
NanoDrop, Model-ND 1000	Thermo Fischer Scientific, USA	N/A
ÄKTApurifier UPC 100	GE Healthcare, United Kingdom	N/A
NGC Medium-Pressure Liquid Chromatography Systems (NGC Quest 10 Plus and NGC Discover 100 pro)	Bio-Rad Laboratories, Inc.	N/A
UVP Bioimaging System	Analytik Jena US LLC, USA	N/A
Invitrogen wet transfer mini blot module	Thermo Fisher Scientific, USA	N/A
ChemiDoc™ MP Imaging System	Bio-Rad laboratories, Inc.	N/A

MATERIALS AND EQUIPMENT

Isopropyl-β-D-thiogalactopyranoside (IPTG)		
Reagent	Final concentration	Amount
IPTG	1 M	1.19 *g*
Autoclaved Milli Q	N/A	Up to 5 mL
Total	**N/A**	**5 mL**
Filter-sterilized using 0.22 μm filter and autoclaved.		
Store the stocks at -20°C in dark conditions. Do not refreeze after thawing.		

10X Phosphate buffer, pH 8.0		
Reagent	Final concentration	Amount
di-Sodium hydrogen phosphate (Na_2HPO_4) (1 M)	200 mM	200 mL
Sodium dihydrogen phosphate (NaH_2PO_4)(1 M)	N/A	Volume to adjust pH up to 8.0
NaCl	1 M	58.44 *g*
Autoclaved Milli Q	N/A	Up to 1 L
Total	**N/A**	**1 L**
Filter-sterilized using 0.45 μm filter and autoclave. Store at 4°C.		

Lysis Buffer, pH 8.0		
Reagent	Final concentration	Amount
Phosphate buffer (10X)	1X	1 mL
Triton X-100	0.1%	10 μL
Glycerol	3%	300 μL
Imidazole (1 M)	1 M	100 μL
Autoclaved Milli Q	N/A	Up to 10 mL
Total	**N/A**	**10 mL**
Filter-sterilized using 0.45 μm filter and use freshly prepared buffer.		

Equilibration Buffer (Buffer A), pH 8.0		
Reagent	Final concentration	Amount
Phosphate buffer (10X)	1X	10 mL
Glycerol	3%	3 mL
Autoclaved Milli Q	N/A	Up to 100 mL
Total	**N/A**	**100 mL**
Freshly prepare the buffer and store at 4°C.		

Elution Buffer (For His-tag protein purification), pH 8.0		
Reagent	Final concentration	Amount
Equilibration buffer	N/A	50 mL
Imidazole (1 M)	10 mM–500 mM	Depending upon the gradient selected for elution
Freshly prepare the buffer and store at 4°C.		

Elution Buffer (For MBP-tag protein purification), pH 8.0		
Reagent	Final concentration	Amount
Equilibration buffer	N/A	50 mL
Maltose (1M)	10 mM–20 mM	Depending upon the gradient selected for elution
Freshly prepare the buffer and store at 4°C.		

STEP-BY-STEP METHOD DETAILS

Recombinant protein expression using bacterial cells

Timing: 4 days

The recombinant protein constructs are expressed in bacterial cells such as *E. coli* BL21 (DE3) and Rosetta (DE3) pLysS strains. Single isolated colonies of the transformed cells are further picked up and grown ~16–18 h in Luria Bertani (LB) broth (containing the appropriate antibiotic depending upon the expression vector used) at 37°C. These cultured cells serve as the primary source for plasmid isolation (by miniprep method, as per manufacturer's instructions) or as the primary culture for protein expression.

1. For protein expression, inoculate about 1–2% (v/v) of the ~16–18 h grown primary culture to each liter of LB broth containing the appropriate antibiotic.
   a. Grow the cells further at 37°C (180 rpm) until the optical density (OD) at 600 nm reaches up to ~0.6 AU.

   Note: The antibiotic used in the media should correspond to the antibiotic resistance gene present in the plasmid as well as in the competent cells used for protein expression.

2. Once the required OD is achieved, induce protein expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM to the cultured cells and incubate at 18°C for 18 h at 180 rpm.
3. Collect 10 μL samples of the pre- and post-induction cultures for analysis of protein expression by SDS-PAGE.
4. Harvest the induced cells for protein production by centrifuging at 4°C using Beckman Coulter centrifuge (Rotor ID JA-10) or equivalent for 30–45 min at - ~3000 × *g* and store at −80°C until protein purification.

Protein purification by affinity chromatography

Timing: 10–12 h

All the steps for cell lysis, solubilization, and protein purification are to be carried out on ice, with all the buffers pre-chilled at 4°C.

Cell lysis and protein solubilization

5. For every gram of the harvested cell pellet, add about 5 mL of lysis buffer (Ni-NTA lysis buffer/ MBP protein purification lysis buffer).

   ⚠ CRITICAL: Resuspend the pellet in the solution using a glass rod or by gentle vortexing, with intermittent incubation on ice, to avoid protein degradation by heat generated during homogenization.

6. Transfer the solubilization mixture to a clean pre-chilled sonicator tube and sonicate the homogeneous cell suspension using a cell sonicator, with the following parameters:
   a. Sonication Pulse: 1 min - On, 2 min - Off.
   b. Amplitude: 50%.
   c. Number of cycles: 5–6 (depending on the lysate volume and clarity of suspension).

⚠ CRITICAL: Perform this step on ice or at 4°C to prevent protein degradation. The number of sonication cycles and the amplitude of sonication can be standardized depending on the stability of the protein.

7. Centrifuge the sonicated mixture at ~17,000–31,000 × *g* (Beckman Coulter JA-25.50 rotor or equivalent) for 50–60 min at 4°C depending on the volume of cell lysate.

   Note: The particulates (unlysed cells and cellular debris) in the lysate pellet settle at the bottom of the sonication tube, while the soluble protein is present in the supernatant.

8. After centrifugation, ensure not to disturb the pellet and transfer the supernatant carefully to a clean, chilled 50-mL Falcon tube.
   a. To check the efficiency of protein solubilization, prepare 20 μL sample of the supernatant and a sample of pellet (small amount of pellet homogenized in SDS sample loading buffer buffer) for analysis by SDS-PAGE.
9. Filter the supernatant once more using a sterile 0.45 μm syringe filter to remove any particulates or remnant debris and transfer the filtered supernatant in clean pre-chilled falcon tube.

Protein purification

For this part of the protocol, the homo-oligomeric protein variants are purified using affinity chromatography based on the type of affinity tag present in each variant. In our case, WWW with a C-terminal histidine tag was purified using Ni-NTA resin (Nucleo-pore, Genetix Biotech Asia Pvt. Ltd), while ΔΔΔ with N-terminal maltose binding protein (MBP) fusion tag was purified using amylose resin (New England Biolabs). These protein variants were purified using the equilibration buffer (20 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0) containing 100 mM NaCl and 3% (v/v) glycerol) and the composition of buffers used for protein elution varied with the type of affinity tag present in each variant. All the buffers used for this process are to be pre-chilled at 4°C before starting with the purification process.

10. Load the filtrate obtained in step 9 onto fresh affinity purification resin (Ni-NTA Agarose for Histidine-tagged proteins or Amylose resin for MBP-tagged proteins) taken in a glass/plastic column used for manual purification (or pre-packed resin columns used with FPLC instrument) and is well equilibrated with the equilibration buffer.
11. Gently mix the solution at a constant slow speed by placing the purification column on the rocker for 45–60 min at 4°C to promote efficient protein binding to the resin.
12. After incubation, allow the resin beads to settle and collect the flow-through containing the non-specific cellular proteins.
    a. As the flow-through should not contain the protein of interest, prepare a small volume of sample (~20 μL) for SDS-PAGE analysis to get an estimate of the amount of unbound target protein and save the flow-through until the analysis at 4°C is done.
13. Wash the column with the equilibration buffer until the coloration in Bradford's test (used for protein content estimation, performed in micro-titer plates as per the manufacturer's instructions) becomes negligible or the UV absorbance at 280 nm for the eluant (for purifications performed using FPLC instrument) is nearly to the baseline.
14. For the elution of the bound target protein, increase the concentration of imidazole (for Ni-NTA resin) or maltose (for amylose resin) in the elution buffer in a stepwise manner.
15. For purification using Ni-NTA resin, wash with ~30 mL of 10 mM, 50 mM, 100 mM, and 250 mM imidazole-containing elution buffer, while for MBP-tagged protein purification, wash with ~30 mL of 10 mM and 20 mM maltose-containing elution buffer. His-tag proteins (with ~6 histidine residues) generally elute in 250–300 mM imidazole-containing buffer while MBP-tagged proteins elute in 10 mM maltose-containing elution buffer.

Note: The concentration gradients selected for elution of the target protein of interest need to be standardized depending on the type of impurities present in the lysate supernatant. If there are more impurities, increase the gradient range and the number of washes for each gradient buffer.

16. The washing with a particular elution buffer should be carried out until the coloration for a particular gradient in Bradford's test becomes negligible or the UV absorbance at 280 nm for the eluant fraction nearly reaches the baseline.
17. During standardization, collect ~20 μL sample for each elution gradient and prepare SDS-PAGE samples to check the amount of impurities that get eliminated in each step of buffer wash. The flow-through from the lower gradient steps should be stored at 4°C until the analysis.
18. The elution step for the gradient at which the target protein elutes should be performed under cold conditions by placing the collection tubes on ice and the fractions have to be stored immediately at 4°C to avoid protein degradation.
19. Collect samples to check the purity of the protein of interest in the collected fractions through SDS-PAGE analysis. Store the pure protein fractions (>95% pure, as estimated by SDS-PAGE) at 4°C for immediate use or flash freeze and stored at −80°C for long-term use depending on the stability of the protein.

Removal of affinity tag

Timing: 1–2 day

For enzymatic assays where the affinity tag of the target protein can interfere with the readout of the assay, removal of the tag becomes essential. For this purpose, a protease cleavage site is generally introduced between the affinity tag and the target protein gene during the stages of cloning. In our case, the N-terminal MBP tag in the ΔΔΔ protein variant was interfering with the recombination of the heterotrimer during the renaturation process. Therefore, we preferred to cleave the tag with TEV protease before proceeding to the next steps of hetero-oligomer generation.

20. Pull together the elution fractions of the purified protein variant and add the recommended units of TEV protease in a protease to target protein ratio as recommended by the manufacturer or as per the amount standardized for laboratory-purified TEV protease.

Note: A typical protease to target protein ratio (w/w) of 1:50 to 1:200 generally works out effectively for most of the target proteins.

21. Incubate the mixture for 12–18 h at 4°C–22°C, depending upon the activity previously determined for the TEV protease. Furthermore, incubate a small aliquot of the uncleaved target protein at similar conditions, which serves as a negative control for SDS-PAGE analysis.
22. After the incubation period, prepare 20 μL samples (control and test) for SDS-PAGE analysis to determine the successful cleavage of the affinity tag from the target protein.

Note: If the amount of uncleaved protein is still visible in the SDS-PAGE sample, increase the amount of TEV protease in the cleavage process.

23. After SDS-PAGE analysis, load the cleaved protein onto an appropriate resin (affinity or size-exclusion chromatography) or use a protein concentrator with an appropriate molecular-weight cut-off for separating the cleaved tag. In our case, since the ΔΔΔ variant had a C-terminal histidine tag, we preferred to incubate the MBP cleaved protein fraction onto Ni-NTA resin and repeated the steps of purification, as suggested in steps 13–19. The cleaved MBP tag elutes in the flow-through, while the target protein now binds to the Ni-NTA resin through the C-terminal His tag.

24. Perform the washing with the gradient buffers as mentioned in steps 15–16 for Ni-NTA purification. Depending upon the amount of impurity imparted by the presence of the affinity tag, standardize the volume of gradient buffer used in each washing step.
25. Elute the target protein using 10–250 mM imidazole containing elution buffer depending on the number of histidine residues present in the C-terminal tag. In our case, since the ΔΔΔ variant had three histidine residues in the C-terminal tag, the target protein was eluted in 50 mM Imidazole. Save the flow-through and each elution fraction until the analysis at 4°C.
26. Take 10 μL samples during each step of gradient wash and elution to elucidate the pattern of purification of cleaved target protein through SDS-PAGE analysis.
27. For analysis of tag separation performed using protein concentrator or size-exclusion chromatography, prepare SDS-PAGE samples for each step of separation (filtrate and concentrated samples) to estimate the amount of pure target protein obtained after tag cleavage.

Sample preparation

Timing: 5–10 h

Before proceeding with the step of hetero-oligomer generation using sequential denaturation-renaturation strategy, we need to start with a suitable concentration of the homomeric protein variants. To achieve this, the purified elution fractions for a particular variant are pooled together and protein concentration is carried out until a particular concentration is reached. The concentration of both the parent proteins should be such that there is no chance of aggregation of the protein molecules during the process of denaturation and renaturation.

28. For carrying out protein concentration, use either an Amicon tangential-flow spin filter or an Amicon stirred cell concentrator with an appropriate molecular-weight cut-off for the target protein.
29. Prepare the membrane of these concentrators by extensively rinsing first with autoclaved deionized water (two-three times) to remove any debris and then equilibrate it with the respective equilibration buffer ~16–18 h at 4°C.
30. Pool the eluted pure fractions for a particular protein obtained from the affinity chromatography purification, load it onto the equilibrated membrane concentrator and perform the concentration process at 4°C, until the desired concentration and volume is reached. Discard the filtrate at each regular interval to maintain the reservoir load on the concentrator membrane.

Note: For spin filters, centrifuge the concentrators at a speed recommended in the manufacturer's guidelines to prevent the rupturing of the membrane.

31. Run the concentrated sample on SDS-PAGE to determine the purity of the protein and identify the presence of any impurity (cleaved tag or uncleaved protein).

Note: Concentration of the target protein can also result in amplifying the amount of the impurities that may have been previously present in negligible amounts and is now visible on SDS-PAGE. If any impurity is present, run the concentrated sample through the size-exclusion column and separate the pure protein fraction.

32. Estimate the concentration of the final protein sample for the two homomeric parent variants by performing Bradford measurements (as per manufacturer's guidelines) or by measuring the absorption of the sample at 280 nm using the UV-visible spectrophotometer.

Generation of hetero-oligomeric protein variants using the protomer mixing strategy

Timing: 1–4 days

For generating the hetero-oligomeric variants, one needs to perform a protomer mixing strategy where the two asymmetric homomeric parent variants are reconstituted together at a particular concentration, and subjected to a series of sequential denaturation-renaturation process. However, before starting with the protomer mixing strategy, one should be aware of the stability of individual parent homomers towards the denaturation scheme. This can be achieved by performing thermal and/or chemical denaturation studies using fluorescence spectroscopy, circular dichroism spectroscopy, or nano-DSF (Nano differential scanning fluorimetry). If both the homomer variants have melting temperatures within a suitable range, perform heat denaturation of the reconstituted mixture of parent homomers at a temperature that is at the lower end of the two melting temperatures to prevent the denaturation of the lesser stable variant. However, if the parent proteins are less thermostable, then one may opt for chemical denaturation as an alternative, using reagents such as guanidium hydrochloride and urea.[2–5] The optimal concentration of the denaturant to be used for the final process can be standardized by performing folding studies of each parent homomer at different concentrations of the chemical denaturant using circular dichroism spectroscopy. This study will provide a detailed understanding of the unfolding-refolding dynamics of the parent homomeric proteins and help in deciding the final denaturant concentration. The standardized denaturant concentration or melting temperature finally chosen for the purpose of denaturation of the reconstituted mixture of parent protein should be such that the condition destabilizes the quaternary structure of each variant and is enough to dissociate the parent oligomer into its individual folded protomers. In our case, since both the parent oligomers were found to be highly thermostable, the use of simultaneous heat and chemical denaturation process was employed.

33. Mix 500–1000 μL of each concentrated parent homomeric protein variant in equimolar concentrations (50–60 μM) and dilute the reconstituted mixture up to 15-fold in the equilibration buffer that additionally contains 6 M guanidinium chloride (pH 8.0).

    Note: The volume and the concentration of homomeric proteins to be used in this step needs to be standardized depending on the stability of the concentrated protein samples.

34. Incubate the mixture at 50°C for 1–2 h (depending upon the protein stability) under slow rocking conditions for efficient denaturation into individual protomers.
35. To recombine the protomers into hetero-oligomeric variants, transfer the denatured protein mixture to a dialysis apparatus having the membrane with an appropriate molecular-weight cut-off (10 kDa) and is well pre-equilibrated with buffer A.
36. Seal the dialysis apparatus at both ends and place it in a beaker containing 1 L–3 L (approximately 200–500 times greater than the sample volume) of renaturation buffer (the dialysate) pre-chilled at 4°C.

    Note: The renaturation buffer is similar to buffer A (pH 8.0) that additionally contains 300 mM arginine and varies in the glycerol content (starting from 15%–5%). For the first buffer exchange, use renaturation buffer with 15% glycerol.

37. Place a magnetic stir bar inside the beaker and incubate this assembly under constant gentle stirring for 3–4 h at 4°C.
38. After incubation, carefully replace half the volume of the used dialysate renaturation buffer in the beaker with fresh renaturation buffer containing 10% glycerol. Incubate under constant gentle stirring conditions for 3–4 h at 4°C.

    Note: This step dilutes the concentration of the contaminants diffused in the dialysate and gradually reduces the total glycerol content of the dialysate to 12.5%, thus preventing the protein sample from undergoing instant shock due to fresh buffer exchange.

39. Replace the equilibrated renaturation buffer entirely with fresh renaturation buffer containing 10% glycerol and continue the incubation for 3–4 h at 4°C.
40. Repeat steps 38 and 39 by gradually decreasing the glycerol content until the final dialysis is performed in renaturation buffer containing 5% glycerol. At this step, the protein variants are expected to regain the quaternary structure by reassociation of the protomers.
41. For removal of arginine from the reconstituted protein mixture, perform two-step dialysis with a fresh buffer A containing only 5% glycerol in a similar way as described for steps 38–39 and incubate at 4°C for 3–4 h at each step.
42. After complete dialysis, carefully transfer the renatured protein mixture from the dialysis apparatus to pre-chilled falcon tubes.
43. Filter the solution using a sterile 0.22 μm syringe filter to remove any aggregated protein or particulate matter formed during dialysis or particulate matter.
44. Load the filtrate onto fresh pre-equilibrated membrane concentrator and concentrate the renatured protein mixture (as described in steps 29–30) back to the original volume used before diluting with the denaturant.
45. Transfer the concentrated sample to a clean micro-centrifuge tube and spin it for 10 min at ~13,500 × *g* and 4°C for removal of any aggregated protein.
46. Carefully separate the supernatant and store it in a fresh micro-centrifuge tube at 4°C, until use for the further steps of purification.
47. For analysis of the hetero-oligomer generation, prepare 20 μL samples of the concentrated renatured mixture for SDS-PAGE and Native PAGE.

Note: SDS-PAGE analysis will help to determine the purity of the total renatured mixture and most importantly, help in identifying the presence of any degradation that may have occurred during the denaturation-renaturation process. Through Native PAGE, one can get a correct estimate of the success of the renaturation process. For a protein with 'N' number of protomers, one should observe 'N+1' number of bands in Native PAGE, where two bands will be of the homomeric parent protein variants while the rest indicate the hetero-oligomeric variants. Depending on the native molecular weight of each variant ensemble present in the renatured mixture, calculate the percentage of Native PAGE gel to be prepared.

48. Run two sets of identical Native PAGE gels for all the samples at a constant voltage of 50 V for 5 h at 4°C to avoid any degradation of the protein. Use one gel for Coomassie staining and set aside the other unstained Native PAGE gel for western blot analysis (described below) to confirm the identity of the hetero-oligomers formed in the protomer mixing strategy.

Separation of the hetero-oligomeric protein variants

Timing: 10–12 h

For further separation of the different homo- and hetero-oligomeric variants from the renatured protein mixture, one can employ size-exclusion chromatography or ion-exchange chromatography[6] if the variants have a significant difference in their molecular weights or pI respectively. However, if there are no significant differences in the biochemical properties of the variants then one can opt for the tandem affinity tag-based purification protocol for separation, as described below.

49. For separation based on differences in the histidine tag residues, load the concentrated renatured protein mixture onto fresh Ni-NTA resin pre-equilibrated with buffer A. Take about 1–2 mL of the resin for about 500–700 μL of the concentrated protein mixture in a pre-chilled Eppendorf tube depending on the binding capacity of the resin.
50. After incubation, allow the resin beads to settle by giving a fast spin in a micro-centrifuge (~5000–6000 × *g*) at 4°C for 1 min.

51. Collect the flow-through carefully and prepare a small volume of sample (~20 μL) for SDS-PAGE analysis to get an estimate of the amount of unbound target protein or the presence of any degraded proteins. Save the flow-through at 4°C until the analysis through SDS-PAGE.
52. Wash the column with the equilibration buffer till the coloration in Bradford's test (used for protein content estimation, performed in micro-titer plates) becomes negligible or the 280 nm signal for the eluant (using FPLC instrument) is near the baseline.
53. For the elution of the bound target protein variants, increase the concentration of imidazole in the elution buffer in a stepwise manner.

Note: Since the protein variants differ in the total number of histidine residues present in the terminal tags, a very shallow gradient of imidazole (ranging from 10–250 mM, with a 10 mM interval) in the elution buffers should be used for their separation.

54. For the elution of a particular protein variant, incubate about 200 μL of each buffer gradient to avoid unnecessary dilution of the variants. The impurities are eluted in the flow-through fraction (up to 30 mM imidazole buffer washes), followed by elution of the first homomeric parent protein with the lowest number of histidine residues between 40–50 mM imidazole buffer washes.

Note: The elution step for each gradient should be performed under cold conditions by placing the collection tubes on ice.

55. The hetero-oligomers then elute between 50–200mM imidazole buffer washes in the increasing order of the histidine residues present in the tag.
56. This is followed by the elution of the second homomeric parent protein with the highest number of histidine residues.

Note: In our case, ΔΔΔ eluted in 50 mM imidazole, while WΔΔ and WWΔ eluted in 80–130 mM imidazole and WWW in 250 mM imidazole containing elution buffers. Depending on the elution profile, the interval between the gradients and the number of washes for each gradient buffer can be standardized.

57. Elute the protein at each step by micro-centrifuging the beads (at ~5000–6000 × *g*) and then carefully collecting the supernatant. Store each fraction immediately at 4°C to avoid protein degradation.
58. During standardization, collect ~20 μL sample of every elution gradient wash to check the amount of protein variant through SDS-PAGE as well as Native PAGE analysis.
59. Run the native PAGE gels in a set of two – use one gel for Coomassie staining and set aside one unstained gel for western blot analysis (described below) to confirm the purity of the separated hetero-oligomers in comparison to the homomers present in the renatured mixture.
60. After PAGE analysis, store the fractions containing a single protein variant (>95% pure, as estimated by Native PAGE) at 4°C for immediate use or flash freeze and stored at −80°C for long-term use depending on the stability of the protein.

Confirmation of hetero-oligomer formation using Western blot

Timing: 1–2 days

61. For analysis of the previously run Native PAGE gels (steps 48 and 59), transfer the gel slice containing the separated protein bands from the unstained native PAGE gel onto the nitrocellulose membrane using Invitrogen wet transfer mini blot module and 1X transfer buffer, at a constant voltage of 15 V for 60–90 min.

62. Once the transfer is over, remove the membrane and block it with 5% bovine serum albumin (BSA) prepared in 1X TBST (Tris-buffered saline with Tween-20) buffer for 1 h. Stain the gel with Coomassie Blue to check the transfer efficiency.
63. After the incubation for blocking, wash the membrane three-six times with 1X TBST buffer for 5–10 min intervals each.
64. Depending on the type of tag present in the protein variants, probe the protein bands in the blot using primary antibody against the tag present at the C-terminal or N-terminal end of the variant.

Note: In our case, since all the homo- and hetero-oligomers also differed in the number of FLAG tag at the N-terminal end (originally introduced in ΔΔΔ protein construct), the western blot was performed using monoclonal anti-FLAG antibody.

65. Incubate the membrane ~16–18 h with the recommended/standardized dilution of the primary antibody (1:2000 dilution for anti-FLAG antibody) under constant rocking conditions at 4°C. The dilution of the antibody used can be standardized depending on the manufacturer's guidelines.
66. On the following day, remove the membrane from the antibody solution and wash the membrane three-six times with 1X TBST buffer for 5–10 min intervals each.
67. Incubate the membrane further at ~22°C–24°C for 1 h with the recommended/standardized dilution of the secondary antibody (1:5000 dilution of goat anti-mouse IgG HRPO secondary antibody).

Note: Prepare the antibody dilutions in 1X TBST and add 1% BSA to prevent non-specific interactions.

68. Wash the membrane three-six times with 1X TBST buffer for 5–10 min intervals each and store in 1X TBST buffer at 4°C until probing the blot.
69. Develop the blot using an enhanced chemiluminescence kit (as per manufacturer's instructions) in the ChemiDoc™ MP Imaging System with optimum exposure.

EXPECTED OUTCOMES

Protomer mixing strategy is a very reliable technique that helps to reconstitute hetero-oligomeric protein variants from their homo-oligomeric counterparts and preserve the native properties of the variants. Following this detailed protocol, we were able to purify four different combinations of the HtrA2 trimeric variants (Figure 1A). The homo-oligomeric protein variants were first purified up to >95% purity (Figure 2A and 2B). The presence of newly formed heterotrimers (WWΔ and WΔΔ) in the concentrated renatured protein mixture was confirmed by Native PAGE. Since all the variants differ in their pI (due to the presence of FLAG tag that was strategically introduced in ΔΔΔ) and molecular weight, four distinct bands (two bands of parental homotrimers and two bands of recombined heterotrimers) were obtained with good resolution and in the order of decreasing pI values (Figure 3A). The heterotrimeric HtrA2 variants were further confirmed by probing with anti-FLAG antibody, as one of the subunits (Δ) contained a FLAG tag and the variants would therefore differ in the number of FLAG epitopes (Figure 3B). The separation of these closely-related HtrA2 variants (having a minimal difference in pI and molecular weight) from the renatured protein mixture was performed by employing a modified version of affinity chromatography using a very narrow gradient of imidazole (Figure 3C). After separation, the identity of these separated HtrA2 variants (homo- and hetero-trimers) was confirmed by Native-PAGE. The hetero-trimers so obtained were further used for biochemical and biophysical characterization,[1] which enhanced our understanding of the influence of the PDZ domain on the total residual activity and specificity of the HtrA2 protease.

LIMITATIONS

The described protocol is not suitable for protein variants that are highly unstable in nature, as they may not be able to withstand the conditions prescribed for the denaturation-renaturation process.

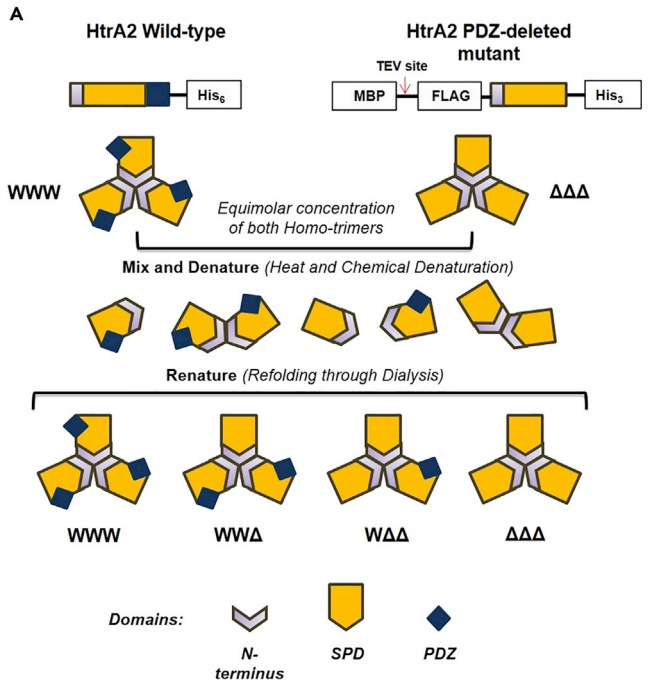

Figure 1. Protomer-mixing strategy for hetero-oligomer generation

(A) Schematic model depicting the denaturation-renaturation strategy used to generate heterotrimeric variants of HtrA2 serine protease (WWΔ and WΔΔ).

Moreover, proteins that are prone to undergo aggregation during the concentration process might also not serve as good starting material for this protocol. Therefore, one needs to have a prior understanding of the protein's stability (wild-type and mutant) through various biophysical studies before successfully employing this protocol.

TROUBLESHOOTING

Problem 1

Purified homo-oligomeric protein solution becomes turbid or degrades during concentration (steps 30 and 31).

Potential solution

When the volume of the concentrated sample is reduced down to a certain volume (from 5 mL original volume to 1 mL concentrated volume), stop the concentration process at definite intervals and mix the sample by gentle pipetting to prevent the accumulation and subsequent aggregation of the concentrated protein on the filter membrane.

Equilibrate the membrane with buffer containing 3%–5% glycerol to prevent the sticking of the protein molecules to the filter membrane.

Problem 2

The renatured protein mixture solution becomes turbid or shows the presence of particulates (steps 42 and 43).

Potential solution

Reduce the concentration of the homo-oligomers used for setting up the denaturation step.

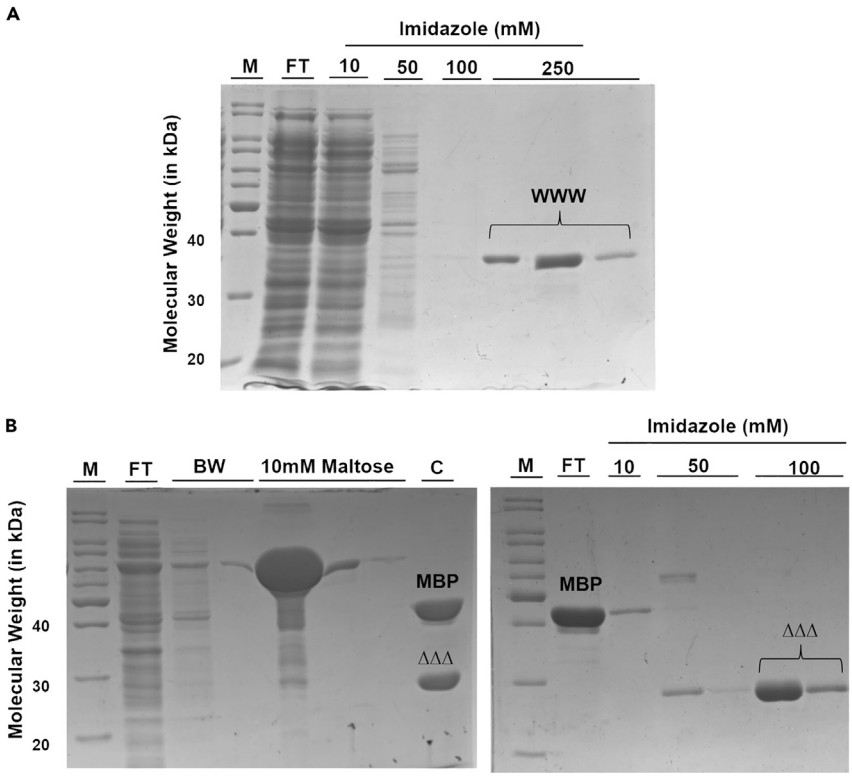

Figure 2. Purification profiles for homo-oligomers

(A) Purification of HtrA2 wild-type homotrimeric variant (WWW: ~36 kDa, with a C-terminal His_6 tag) using Ni-affinity chromatography.

(B) Purification of HtrA2 PDZ-deleted homotrimeric variant (ΔΔΔ: ~23 kDa, with an N-terminal MBP tag & C-terminal His_3 tag). The MBP-ΔΔΔ fusion protein was purified using amylose resin, followed by TEV protease cleavage to remove MBP and a second round of purification using Ni-NTA resin. In (A) and (B), M: Protein marker, FT: Flow-through, BW: Buffer wash, C: MBP-cleaved protein fraction.

Increase the amount of glycerol in the elution buffers used for the purification of the homo-oligomers.

Standardize the denaturing conditions (reduce temperature or molarity of denaturant).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and/or reagents should be directed to and will be fulfilled by the lead contact, Dr. Kakoli Bose (kbose@actrec.gov.in).

Materials availability

This study did not generate any unique reagent/s. The commercially available reagents used in this study are listed in the key resources table.

Data and code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. All the other relevant data procured by the usage of this protocol are present in the paper and the supplemental information (Parui et al., 2022).

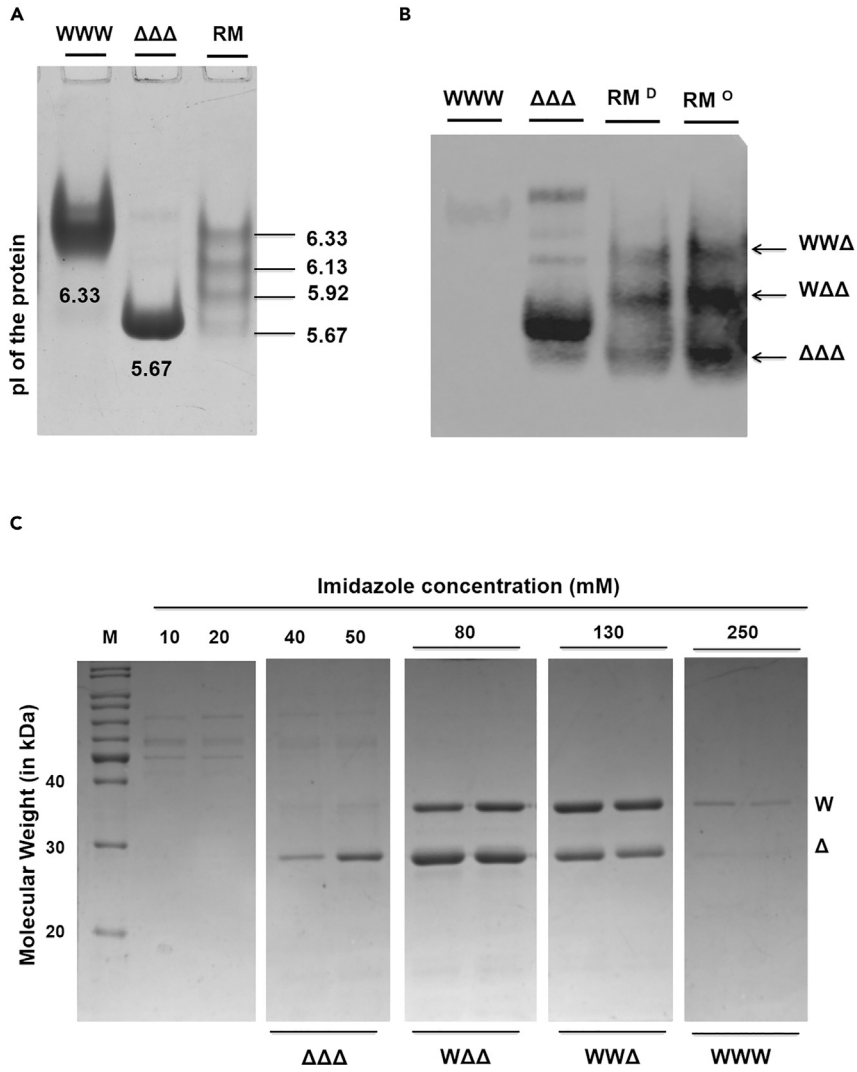

Figure 3. Hetero-oligomeric HtrA2 variants with one or two PDZ domains

(A) Native PAGE showing electrophoretic separation of the two additionally formed hetero-oligomeric variants in the renatured reaction mixture (RM), in comparison to the parent homotrimers – wild-type (WWW) and PDZ-deleted construct (ΔΔΔ). The concentration of homotrimers loaded in the respective lanes was same as that used at the start of the denaturation process, in order to check the presence of any higher order oligomer formation at this concentration.

(B) Western Blot analysis confirming the biochemical identity of the heterotrimers. Native PAGE gel, as described in (D), was used to transfer the protein variants and perform subsequent blotting analysis. Since ΔΔΔ consisted of an N-terminal FLAG tag, the variants containing Δ subunit (WΔΔ, WWΔ and ΔΔΔ) were readily probed in the reaction mixture using Anti-FLAG antibody, with a few non-specific bands observed for ΔΔΔ. The protein bands were probed in the renatured reaction mixture in diluted form (RM^D) as well as in the original concentration (RM^O), in order to confirm the identity of heterotrimers.

(C) SDS-PAGE showing the purity of the heterotrimeric HtrA2 variants. On the basis of number of histidine residues present in the C-terminal His tag of each trimer, the heterotrimeric variants were separated from the reaction mixture using a very narrow gradient of imidazole present in the elution buffer. Thus, WΔΔ eluted first in the elution buffer washes containing 80 mM imidazole, followed by WWΔ in 130 mM imidazole-containing buffer and WWW in 250 mM imidazole-containing buffer. M: Protein marker. Adapted from Ref.[1]

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AUTHOR CONTRIBUTIONS

A.L.P. and K.B. conceptualized and designed the project. A.L.P. performed the experiments and optimized the protocols described in this method article. A.L.P. and K.B. performed data analysis and conceived the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Parui, A.L., Mishra, V., Dutta, S., Bhaumik, P., and Bose, K. (2022). Inter-subunit crosstalk via PDZ synergistically governs allosteric activation of proapoptotic HtrA2. Structure *30*, 1307–1320.e5. https://doi.org/10.1016/j.str.2022.06.001.

2. Burns, D.L., and Schachman, H.K. (1982). Assembly of the catalytic trimers of aspartate transcarbamoylase from folded monomers. J. Biol. Chem. *257*, 8638–8647. https://doi.org/10.1016/S0021-9258(18)34176-0.

3. Coe, J.E., and Ross, M.J. (1987). Hamster female protein, a pentameric oligomer capable of reassociation and hybrid formation. Biochemistry *26*, 704–710. https://doi.org/10.1021/bi00377a007.

4. Deville-Bonne, D., Le Bras, G., Teschner, W., and Garel, J.R. (1989). Ordered disruption of subunit interfaces during the stepwise reversible dissociation of Escherichia coli phosphofructokinase with potassium thiocyanate. Biochemistry *28*, 1917–1922. https://doi.org/10.1021/bi00430a073.

5. Le Bras, G., Auzat, I., and Garel, J.-R. (1995). Tetramer-dimer equilibrium of phosphofructokinase and formation of hybrid tetramers. Biochemistry *34*, 13203–13210. https://doi.org/10.1021/bi00040a036.

6. Schneider, F., Hammarström, P., and Kelly, J.W. (2001). Transthyretin slowly exchanges subunits under physiological conditions: a convenient chromatographic method to study subunit exchange in oligomeric proteins. Protein Sci. *10*, 1606–1613. https://doi.org/10.1110/ps.8901.

7. Parui, A.L., Chaganti, L.K., Kulkarni, R., and Bose, K. (2022). Cloning and gene manipulation. In Textbook on Cloning, Expression and Purification of Recombinant Proteins, K. Bose, ed. (Springer Nature Singapore), pp. 13–56. https://doi.org/10.1007/978-981-16-4987-5_2.