Electronic Supplementary Material (ESI) for RSC Advances.

Thermodynamic, structural and functional properties of membrane protein inclusion bodies are analogous to purified counterparts: Case study from bacteria and humans

Ankit Gupta, Bharat Ramasubramanian Iyer, Deepti Chaturvedi,[#] Svetlana Rajkumar Maurya[#] and Radhakrishnan Mahalakshmi*

Molecular Biophysics Laboratory, Department of Biological Sciences, Indian Institute of Science Education and Research, Bhopal. India.

[#]These authors contributed equally.

*E-mail: maha@iiserb.ac.in.

SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

1. Reagents and chemicals

All ultrapure reagents for protein expression, inclusion body preparation, protein purification and protein folding, including lauryldimethylamine oxide (LDAO), were procured from Sigma-Aldrich Co. LLC. N-dodecylphosphocholine (DPC) and diphytanoylphosphatidylcholine (DiPhPC) were obtained from Avanti Polar Lipids, Inc. All enzymes and PCR reagents used for cloning and proteolysis (proteinase K) were procured from New England Biolabs Inc. Ion exchange columns for protein purification were obtained from GE Healthcare. Chloroform (HPLC grade) and methanol (>99% purity) were obtained from Merck-Millipore Ltd.; methanol was distilled before use.

2. Cloning and protein expression

All proteins that were chosen for this study are summarized in Table S1. Genes for all the proteins were cloned without the signal sequence in the IPTG-inducible pET-3a vector between NdeI and BamHI sites, and confirmed by DNA sequencing. Cloning of *ompX-om14* fusion,[1] *ail*,[2] *ompX*,[3] as well as *vdac-2* WT and *vdac-2* C0,[4] have been reported elsewhere. For all other genes (*rv1698c* from *M. tuberculosis* H37Rv, *pagN*, *pagC*, *pagP* from *S. typhimurium* LT2, *pagP* from *E. coli*), amplification was carried out directly from the respective genomic DNA using the designed primers listed in Table S2. Plasmids containing the desired gene were transformed in chemically competent *E. coli* BL21 (DE3), C41 or C43 cells (www.overexpress.com), and induced with 1.0 mM IPTG, when the culture attained an $OD_{600} \approx 0.8$. All the proteins were produced as inclusion bodies by induction for ~3 h, except hVDAC-2 WT and hVDAC-2 C0,

which were induced for ~7 h, before harvesting for inclusion body preparation (IBP). Selectively ^{15}N-labeled protein was obtained by growing the cultures in M9 media containing ^{15}N ammonium sulphate as the sole nitrogen source.[3]

3. Protein purification

All IB pellets were dissolved in the appropriate buffer (20 mM sodium acetate pH 5.0 for Ail, 20 mM Tris-HCl pH 8.5 for Rv19698c, OmpX, PagP and 20 mM Tris-HCl pH 9.5 for hVDAC-2 WT and hVDAC-2 C0) containing 8 M urea. All samples were centrifuged at 45000 x *g* for 1 h and filtered through a 0.22 μm filter. Samples were applied to a 20 ml HiPrep 16/10 Q FF (for OmpX, Rv1698c, hVDAC-2 and PagP) or a 20 ml HiPrep SP FF (for Ail) and eluted using an NaCl gradient.[3-5] Peaks containing the purified protein were pooled, dialyzed against water and lyophilized to obtain a white powder. Typical yields were between 30 mg – 100 mg/L culture.

4. Pulse proteolysis

Pulse proteolysis was carried out with proteinase K, using minor modifications of reported protocols.[3, 6] Briefly, proteinase K (PK) or proteinase K buffer (control) was added to the refolding reaction to achieve a final concentration of 2 μg/μl PK for Ail and OmpX, and 0.2 μg/μl PK for PagP. The refolding reaction contained ~0.5 μg/μl protein in 25 and 20 mM LDAO respectively (for Ail and OmpX) or ~0.25 μg/μl protein in 50 mM DPC (for PagP-Ec and PagP-St) prepared in 20 mM Tris-HCl pH 8.5 (for Ail) and pH 9.5 for OmpX, PagP-Ec and PagP-St. All samples were incubated at either 25 °C or 37 °C for 1-2 min,[3, 6] and analysed on 15% SDS-PAGE without arresting PK activity.

5. Development and optimization of a modified inclusion body preparation protocol applicable for several transmembrane proteins

5.1 Comparative analysis of protocols for effective bacterial cell lysis (step 1)

Several protocols are available for IBP. We first narrowed down the available protocols to distinct methods used for cell lysis (Fig. S1). For methodology development, we specifically chose the Met-less OmpX-Om14 fusion construct,[1] expressed as IBs in *E. coli* C41 cells, for two reasons : (i) Om14 is a yeast mitochondrial protein, with five cysteine residues that pose problems in protein handling due to non-specific disulphide formation under alkaline pH conditions; (ii) Om14, so far, has not been generated in quantities sufficient for biophysical studies using known expression systems, due to the inherently low stability of the over-expressed protein.[1, 7] Purity levels, therefore, are low, and this protein demands multiple purification steps to obtain sufficiently pure protein.

The various lysis protocols (Fig. S1) yielded protein purities in the range of 30 - 40% (Fig. S2A). Based on the densitometry quantification (Fig. S2B), we chose protocols 2, 4 and 6B and merged the components into a single protocol (described in section 5.3). It must be mentioned here that while the use of sucrose increased cell lysis efficiency due to osmotic effect in protocol 3, the associated solution viscosity posed difficulties in the subsequent sample processing and efficient recovery of the IB pellet and resulted in substantial loss of the desired protein in the lysate supernatant, and was therefore not pursued further. The three chosen protocols allowed us to combine major elements that resulted in the rapid and efficient cell lysis: (i) exposure of the cell pellet to liquid nitrogen facilitated easy re-suspension (time reduced from ~10 min to 1 - 2 min); (ii) treatment with lysozyme completed cell lysis; (iii) rigorous sonication

at 4 °C effectively sheared DNA and other membrane fractions, resulting in solubilization of these impurities.

5.2 Removal of host membrane proteins and other lipid impurities (step 2)

Host lipids from the cell lysate form one of the major contaminants in protein preparations. Triton-X-100 (TX100) and triton-X-110 are popularly used to disrupt membrane complexes and solubilize host membrane proteins for effective removal of the membrane fraction. The protocol involves the re-suspension of the pellet obtained from the cell lysate in Buffer 1 (20 mM Tris-HCl pH 8.0) containing 1 - 2% TX100 and incubation for sufficient time periods. To optimize this step, we re-suspended the OmpX-Om14 protein pellet obtained from the whole cell lysate directly into 20 ml Buffer 1 containing 1% or 2% TX100 by vigorous vortexing. We followed this by bath sonication using an Elmasonic P sonicator at 100% intensity and 37 Hz frequency for 5-10 min at 25 °C. After incubating this sample at 37 °C for 1 h, with mixing at 200 rpm, we pelleted the protein by centrifugation at 27,000 x *g* at 4 °C, and analysed the protein pellet on SDS-PAGE (Fig. S3A). We also derived the percentage purity of the desired protein against other impurities within each lane (Fig. S3B).

In the case of the OmpX-Om14 fusion construct, the amount of TX100 in the wash did not contribute significantly to the purity of the pellet obtained, with 2% TX100 showing an ~5 - 10% increase. Hence, for this construct 2% TX100 was chosen for the subsequent screening steps. The amounts of detergent used must, however, be adjusted cautiously, since it has previously been observed that helical membrane proteins do possess better solubility in detergents compared to β-barrels,[8] and 0.5 - 1% TX100 should be preferred for helical membrane proteins.

5.3 Unifying steps 1 and 2 for rapid lysis and lipid removal

To economize on the processing time, we incorporated the detergent wash (TX100 wash) as a part of the cell lysis. Since the presence of detergent may interfere with lysozyme activity and the subsequent sonication efficiency, we modified step 1 (section 5.1) to include TX100 after the sonication step. We flash-froze a 100 ml culture pellet in liquid nitrogen for 5 - 10 min and re-suspended it in 20 ml (1 volume) of Buffer 1, following which we incubated the suspension at 4 °C with 12.5 µg/ml lysozyme for 15 min with constant stirring. Next, we sonicated the suspension for 8 min with a 50 s ON and 10 s OFF cycle at 80% power, on a Sonics probe sonicator, using a 5 mm probe tip. We then added 20 ml (1 volume) Buffer 1 containing 4.0% TX100 to the lysate, mixed thoroughly and incubated the mixture at 37 °C for 5 - 10 min. We recovered the IBs by centrifugation at 27,000 x *g* and examined the protein preparation on SDS-PAGE; the combined protocol (lysis + detergent wash) yielded similar results as that of the two-step protocol (data not shown).

Using this unified cell lysis and wash step afforded the procedure to be completed in ~1.5 - 2 h lesser than the routine method, since the sample spin-down and re-suspension time was effectively removed. The added advantage of this combined procedure is that the difficulty involved in obtaining a nearly homogeneous suspension of a tightly pelleted IB from step 1 (cell lysis) is practically eliminated. Notably, the volume of Buffer 1 containing TX100 that is added to the sonicated sample can be increased depending on the efficiency of wash desired. The time required for processing the sample would effectively remain the same irrespective of the wash volume used, and would only be limited by the sample container and centrifuge rotor capacity. Further, we have observed that an increase in the wash volume also increases the purity of the IB pellet obtained at the end of the detergent treatment step.

5.4 Effect of DTT on cysteine-containing proteins:

The bacterial cell interior is maintained in a reducing environment, which minimizes disulphide bond formation in surface-exposed cysteines. Therefore one may anticipate that non-specific disulphides tend to form in IBs. Such covalently linked protein aggregates are compactly packed and become impenetrable to reducing agents in the buffer. This not only reduces wash efficiency, but also leads to non-specific impurities co-precipitating with the protein. Om14 has five cysteine residues and we observed significant amounts of protein impurities even after the detergent wash, which is not the case in inclusion body preparations of Cys-less proteins (demonstrated later). The buffers and steps described thus far were carried out without any reducing agent in the medium. We therefore tested the efficacy of high DTT concentrations and the method of application on the observed purity. Two DTT concentrations (50 mM and 100 mM) were introduced along with TX100, and the protein was incubated in this reducing environment for 1 h at 37 °C. Figs. S4A and S4B compare the sample purities obtained with addition of DTT during the incubation step. We observe only a marginal increase of ~10% in the purity of the protein preparation in the presence of a reducing agent (up to 50 mM).

An increase in the disulphide reduction efficiency is observed when the samples are incubated with reducing agents at 60 °C for 10 min, and is popular in processing proteomics samples.[9] To assess the effect of heat on this process, we incubated the cell lysate containing 2% TX100 at 60 °C at 200 rpm for 15 min, followed by incubation at 37 °C for 45 min. In parallel, another sample was incubated at 37 °C for 1 h. This incubation time was arbitrarily estimated based on earlier experiments, and not optimized further in this study. Figs. S4C and S4D illustrate the results obtained with 100 mM DTT treatment coupled with heating. We observe no significant increase in sample purity due to the incubation step; indeed, a marginal reduction in

the sample purity is noticeable, which may also arise from protein degradation at very high temperatures. We conclude that the addition of up to 50 mM DTT, along with an incubation time of 5 - 10 min at 37 °C, provides satisfactory removal of any trace amounts of co-precipitating covalently bound contaminants in cysteine-rich proteins. Moreover, treatment with a reducing agent is not mandatory, as sample purity levels only increase by ~10%.

A detailed report tabulating the observed results of various other permutations and reagents is presented in Table S3.

5.5 Removal of detergents and lipid contaminations:

In membrane proteins IBPs, bacterial lipids cause major problems, as large amounts of lipid contaminants adhere to the hydrophobic regions of membrane protein. Even under highly denaturing conditions and use of detergents (including TX100), it is difficult to completely eliminate the lipid contaminants. While guanidine hydrochloride is more effective for lipid-free protein preparations,[5b, 10] this salt severely limits protein purification methods to Ni-NTA (does not eliminate lipids) or gel filtration (time consuming). Earlier reports on plant protein preparations have employed chloroform : methanol mixtures to successfully remove lipids and oils.[11] It therefore seemed lucrative to extend this concept of an 'organic solvent wash' to our inclusion body preparations.

We treated samples of OmpX-Om14 fusion generated from 5.3 to 20 ml of chloroform : methanol mixtures in ratios of 2:1, 1:1 and 0:1. Visual inspection of the air-dried post-centrifugation (45,000 x *g*, 15 min) pellet (Fig. S5) confirms significant removal of lipid impurities without extensive protein loss. Qualitative estimations of the organic solvent supernatant fraction indicated maximum removal of non-proteinaceous impurities with the use of

methanol. Further, we did not obtain a well-packed protein pellet when chloroform amounts were high in the organic solvent wash. We could readily dissolve the air-dried pellets in denaturants such as guanidine or urea, whereas the untreated samples required vigorous vortexing and prolonged incubation (up to overnight incubation; eg. reference [12]). We therefore propose a quick methanol wash as our choice for near-complete lipid removal.

5.6 Improvising the modified IBP protocol for OmpX-Om14 fusion and comparison with reported methods:

As a final step to improve protein purity, we altered the centrifugation speed to minimize the centrifugal sedimentation of the non-specific impurities in the supernatant. We harvested a 1 L *E. coli* cell culture by centrifugation at 15,000 x *g* for 5 min. Following the combined lysis (1 volume, 50 ml Buffer 1) and TX100 treatment (3 volumes, 150 ml, containing ~2.6% TX100; 5 - 10 min incubation at 37 °C), we centrifuged the solution at 15,000 x *g* (reduced from 27,000 x *g* used in 5.3) for 40 min. The incubation time of the cell lysate in Buffer 1 containing TX100 and DTT was also reduced to 10 - 15 min from 1 h, since it did not affect the protein quality (data not shown). We directly re-suspended the IB pellet in 20 ml absolute methanol and bath sonicated for 5 - 10 min to obtain a homogeneous suspension. When we centrifuged this solution at 45,000 x *g* for 15 min, the protein pellet we obtained provided us with an overall purity of ~75% for the OmpX-Om14 construct (see Fig. S6A), which is an ~30% improvement over our earlier report.[1]

We conclude that the use of a single step cell-lysis coupled with detergent treatment, followed by a clean-up of the protein pellet with methanol, is sufficient for the near-complete removal of protein and lipid impurities and provide us with highly pure forms of inclusion bodies in an overall preparation time of ~1.5 h.

SUPPLEMENTARY TABLES

Table S1. Summary of proteins used in this study.

Protein	Size (MW); Structure[1]	Source; main function[2]	Reference[2]	Reported protocol[3,4]
OmpX-Om14	30 kDa; NA	A fusion construct for over production of yeast protein Om14	1	3, 5a, 12
Ail	~17 kDa; 8-stranded β-barrel	OMP of *Y. pestis;* adhesion and internalization of the bacterium into host cells	5b; 3QRA	3, 5a, 12
Rv1698c	~30 kDa; NA	OMP of *M. tuberculosis* H37Rv; drug transport	13	13
hVDAC-2 WT	~31.5 kDa; NA	Outer mitochondrial membrane protein of *H. sapiens*; nucleotide and metabolite transport	4	4, 12, 14
hVDAC-2 C0	~31.5 kDa; NA	Cys-less mutant of hVDAC-2 WT	4	4, 12, 14
OmpX	~16.5 kDa 8-stranded β-barrel	OMP of *E. coli*; neutralization of host complement system	5a; 1QJ8	3, 5a
PagC[4]	~18 kDa; NA	OMP of *S. typhimurium;* essential for survival within macrophages	15	3, 5a, 12
PagN[4]	~24 kDa; NA	OMP of *S. typhimurium;* host cell adhesion/invasion	16	17
PagP-Ec	~19 kDa; 8 stranded β-barrel	OMP of *E. coli*; Antimicrobial peptide resistance and lipid A acylation	18; 1MM4/1MM5	18
PagP-St	~19 kDa; NA	OMP of *S. typhimurium;* Antimicrobial peptide resistance and lipid A acylation	19	18

[1] NA = Structural information not available.

[2] Reference provided for information on source and function; PDB IDs (http://www.rcsb.org/pdb/) of structures, where available, are also included.

[3] References of published work from where the IBP protocols for the respective proteins were obtained.

[4] Since there was no reported IBP protocol available for PagC and PagN, we used the protocols described for OmpX and NspA (Neisserial surface protein A), respectively.

Table S2. List of primers used in this study.

Gene	Primer[1]	Primer sequence (5' → 3')
rv1698c	**Forward**	CATATGGATACTTTGCTGTCCAGCTTGCGTAGC
	Reverse	GGATCCCTACTGGGAAACCGTGACTGACATCG
pagC	**Forward**	GTTGCACATATGGATACTAACGCCTTTTCCGTGG
	Reverse	GCAGCCGGATCCTCAGAAACGGTATCCAACCCCGACG
pagN	**Forward**	CTGCATCACATATGGAAGGGATCTATATCACCGGGAAAGC
	Reverse	GCAGCCGGATCCTTAAAAGGCGTAAGTAATGCCGAGC
pagP-Ec	**Forward**	CATATGAACGCAGATGAGTGGATGACAACG
	Reverse	GGATCCTTAGAAGCGCATCCAGGCAAAGTACAC
pagP-St	**Forward**	CCTTCAGTCCATATGGCGGATAAAGGGGGGTTTAACACG
	Reverse	GCAGCCGGATCCTCAAAACTGGAAACGCATCCAGG

[1] Primers were used to directly amplify and clone the genes from the respective bacterial genomes, listed in Table S1.

Table S3. Detailed results of the effect of initial screening conditions on purity of IBP.

S. No.		Experimental conditions	% Protein purity	
			Exp. 1	Exp. 2
1		**Ail / OmpX protocol**	27	
2		**Ail / OmpX protocol modifications**[1]		
	2A	Ail protocol involving two Buffer 1 and Buffer 2 washes	26	36
	2B	Ail protocol (Buffer 2 containing 50 mM DTT)	35	34
3		**VDAC protocol**	33	
4		**VDAC protocol modification (Components added in Buffer 1)**		
	4A	0.5 M urea	27	
	4B	1% TX100 + 0.5 M urea	28	
	4C	0.5 M urea + 100 mM DTT	56	65
	4D	1% TX100 + 0.5 M urea + 100 mM DTT	48	59
	4E	1.0 M urea + 100 mM DTT	49	61
5		**New Protocol modifications (Effect of DTT concentration and incubation time in Buffer 1)**		
	5A	4-fold Buffer 1 + 2% TX100; $CHCl_3$: CH_3OH, 2:1	51	50
	5B	4-fold Buffer 1 + 2% TX100; $CHCl_3$: CH_3OH, 1:1	47	54
	5C	4-fold Buffer 1 + 2% TX100; $CHCl_3$: CH_3OH, 1:0	52	52
	5D	4-fold Buffer 1 + 2% TX100; $CHCl_3$: CH_3OH, 0:0	50	55
	5E	4-fold Buffer 1 + 2% TX100 and 50 mM DTT; $CHCl_3$: CH_3OH, 2:1	57	
	5F	4-fold Buffer 1 + 2% TX100 and 50 mM DTT; $CHCl_3$: CH_3OH, 1:1	58	
	5G	4-fold Buffer 1 + 2% TX100 and 50 mM DTT; $CHCl_3$: CH_3OH, 1:0	53	
	5H	4-fold Buffer 1 + 2% TX100 and 50 mM DTT; $CHCl_3$: CH_3OH, 0:0	51	
	5I	4-fold Buffer 1 + 2% TX100 and 50 mM DTT (incubation at 37 °C for 1 h); $CHCl_3$: CH_3OH, 2:1	38	42
	5J	4-fold Buffer 1 + 2% TX100 and 50 mM DTT (incubation at 37 °C for 1 h); $CHCl_3$: CH_3OH, 1:1	37	43
	5K	4-fold Buffer 1 + 2% TX100 and 50 mM DTT (incubation at 37 °C for 1 h); $CHCl_3$: CH_3OH, 1:0	34	49
	5L	4-fold Buffer 1 + 2% TX100 and 50 mM DTT (incubation at 37 °C for 1 h); $CHCl_3$: CH_3OH, 0:0	33	52
	5M	4-fold Buffer 1 + 2% TX100 and 50 mM DTT (incubation at 60 °C for 15 min and 37 °C for 45 min); $CHCl_3$: CH_3OH, 2:1	35	46
	5N	4-fold Buffer 1 + 2% TX100 and 50 mM DTT (incubation at 60 °C for 15 min and 37 °C for 45 min); $CHCl_3$: CH_3OH, 1:1	39	41
	5O	4-fold Buffer 1 + 2% TX100 and 50 mM DTT (incubation at 60 °C for 15 min and 37 °C for 45 min); $CHCl_3$: CH_3OH, 1:0	30	48
	5P	4-fold Buffer 1 + 2% TX100 and 50 mM DTT (incubation at 60 °C for 15 min and 37 °C for 45 min); $CHCl_3$: CH_3OH, 0:0	33	55
6		**New Protocol modification (Effect of wash volume)**		
	6A	New protocol involving 1 round of wash of Buffer 1 containing 2% TX100 (5-fold volume of wash; incubation at 37 °C)	48	
	6B	New protocol involving 2 rounds of washes using Buffer 1 containing 2% TX100 (5-fold volume of wash; incubation at 37 °C)	51	
	6C	New protocol involving 1 round of wash using Buffer 1 containing 2% TX100 and 100 mM DTT (5-fold volume of wash; incubation at 37 °C)	46	
	6D	New protocol involving 2 rounds of washes using Buffer 1 containing 2% TX100 and 100 mM DTT (5-fold volume of wash; incubation at 37 °C)	52	

[1] Buffer 2 = 2% TX100 in Buffer 1.

Table S4. Secondary structure content estimation from CD spectra.

Protein	Processing method	Secondary structure estimation (%)[1]				
		Helix	Beta	Turn	Random	RMS Value
Ail	Purified	0.0	48.1	15.0	36.9	8.9
	Crude	0.0	48.3	13.7	38.1	6.8
	Crude (R)	0.0	47.7	14.3	38.0	7.2
OmpX	Purified	0.0	44.3	11.1	44.6	4.9
	Crude	0.0	47.3	10.0	42.7	3.1
	Crude (R)	0.0	47.6	8.9	43.5	2.6
hVDAC-2 WT	Purified	1.9	49.7	7.0	41.4	1.8
	Crude	7.6	49.3	5.5	37.6	1.8
	Crude (R)	4.8	51.2	4.4	39.6	2.2
hVDAC-2 C0	Purified	1.6	51.4	4.3	42.7	2.9
	Crude	3.8	49.9	6.0	40.3	2.3
	Crude (R)	6.3	49.9	4.8	39.0	1.8
PagP-Ec	Purified	7.0	39.8	23.6	29.6	16.9[2]
	Crude	3.9	55.6	26.0	14.5	14.4[2]
	Crude (R)	8.2	39.6	20.2	31.9	13.6[2]
PagP-St	Purified	0.0	61.8	38.2	0.0	18.5[2]
	Crude	5.9	39.3	27.6	27.2	19.5[2]
	Crude (R)	8.3	38.5	18.6	34.7	10.1[2]

[1] All estimations were carried out using Reed's method;[20]

[2] High RMS values are because the secondary structure estimation equation does not account for the interference from the 231 nm peak, corresponding to tertiary CD arising from aromatic interactions.

Table S5. Gel quantitation of the protein preparations shown in Figure S7

Protein	Protein purity (%)			~Processing time saved (h)
	Crude (R)[1]	Crude	Purified	
OmpX-Om14	45.2 ± 7.9	74.7 ± 2.4	-[2]	3.5
Ail	57.2 ± 12.2	83.4 ± 2.4	99.44 ± 1.29	3.5
Rv1698c	39.8 ± 10.0	94.8 ± 3.2	97.07 ± 4.21	5.0
hVDAC-2 WT	51.6 ± 7.3	57.5 ± 6.7	91.07 ± 0.70	5.0
hVDAC-2 C0	56.2 ± 14.6	50.7 ± 1.2	90.74 ± 4.76	5.0
OmpX	61.8 ± 2.0	75.9 ± 6.4	97.61 ± 4.04	3.5
PagC	50.3 ± 14.2	67.3 ± 4.7	94.08 ± 5.18[3]	3.5
PagN	63.5 ± 3.7	81.3 ± 8.0	95.56 ± 1.06	3.0
PagP-Ec	57.8 ± 1.7	76.3 ± 11.7	96.51 ± 2.92	3.0
PagP-St	43.8 ± 8.6	66.1 ± 7.8	98.30 ± 0.14	3.0

[1] Corresponds to the crude inclusion body prepared using reported methods.

[2] Purity level for purified form of OmpX-Om14 fusion has not been mentioned, since the construct has not been purified by chromatographic techniques.

[3] Two samples from the same purification profile have been used to calculate purity level.

SUPPLEMENTARY FIGURES

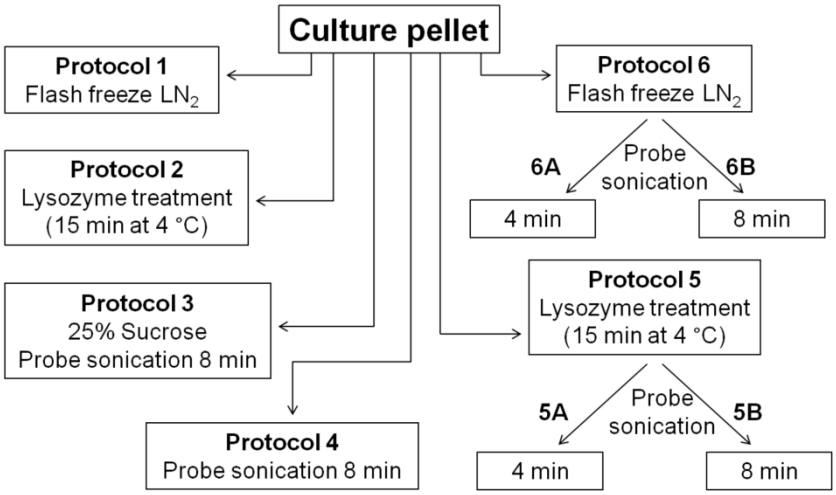


Figure S1. Summary of different cell lysis protocols. Equal volume of cells re-suspended in Buffer 1 (20 mM Tris-HCl pH 8.0) was subjected to the different lysis protocols 1 - 6B. OmpX-Om14 fusion was chosen to optimize the lysis method as expression levels of Om14 were relatively low and had five cysteines, making its purification even more cumbersome.

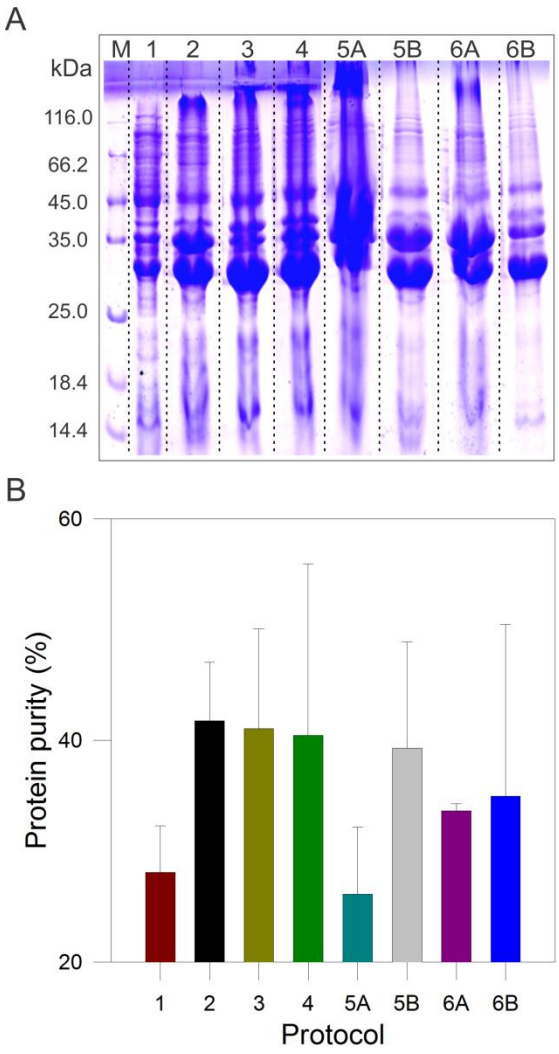


Figure S2. Quantification of pellet purity from the different cell lysis protocols. (A) Representative SDS-PAGE gels of the OmpX-Om14 protein pellet obtained by centrifugation of the cell lysis protocols 1 - 6B (Fig. S1), at 27,000 x *g* for 40 min. The pellet was dissolved in 8.0 M urea and loaded on 15% gels. Dotted lines separate independent gels that are presented together for comparison. M = Molecular weight marker. (B) Quantitation of the desired protein purity shows 5 - 15% variation in the lysate purity from the different protocols. Error bars represent mean of two datasets. Flash freezing using liquid nitrogen come out as quick and efficient cell lysis method equally complemented by lysozyme treatment and sonication. While

the use of sucrose increased cell lysis efficiency due to osmotic effect in protocol 3, the associated solution viscosity posed difficulties in the subsequent sample processing and efficient recovery of the IB pellet and resulted in substantial loss of the desired protein in the lysate supernatant, and was therefore not pursued further. Based on percentage purity obtained from densitometry analysis of the SDS-PAGE, step 2, 4 and 6B were combined together to generate an efficient cell lysis method.

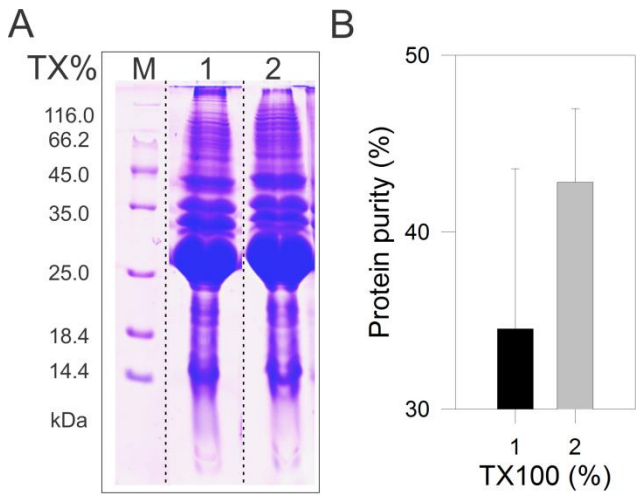


Figure S3. Effect of TX100 concentration on removing protein impurities from OmpX-Om14 fusion. (A) 15% SDS-PAGE gels show no significant difference in protein purity level upon treatment of IBs with 1% and 2% TX100 (TX) in Buffer 1. Dotted lines separate independent gels that are pieced together for comparison. (B) Densitometry analysis of protein purity shows a marginal increase of <10% when 2% TX100 is used. Hence, for this construct 2% TX100 was chosen for the subsequent screening steps. The amounts of detergent used must, however, be adjusted cautiously, since it has previously been observed that helical membrane proteins do possess better solubility in detergents compared to β-barrels,[8] and 0.5 - 1% TX100 should be preferred for helical membrane proteins. Errors indicate a mean of two datasets.

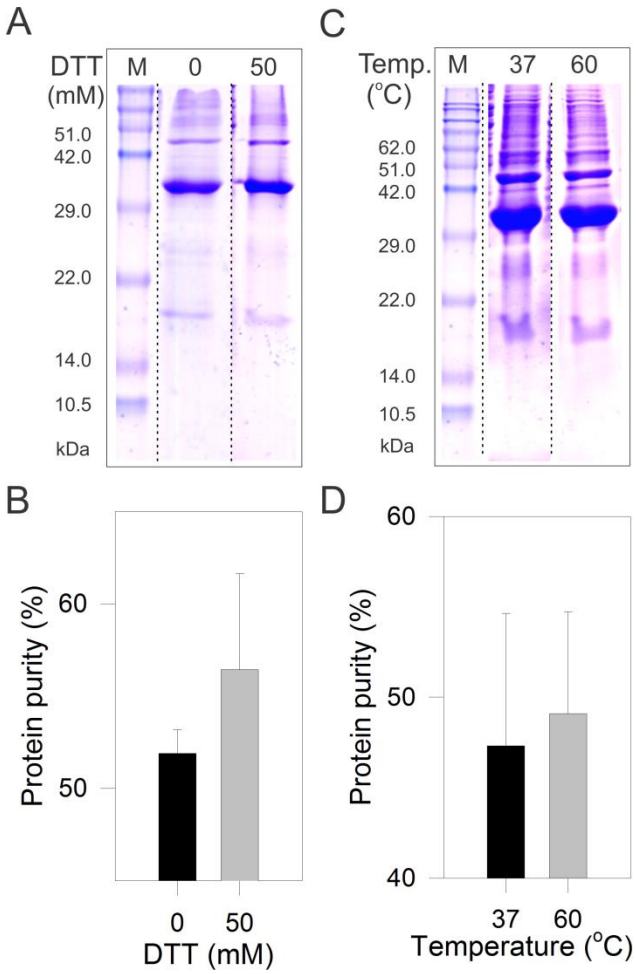


Figure S4. Effect of DTT concentration and incubation temperature on the removal of protein impurity from OmpX-Om14 fusion. (A) 15% SDS-PAGE gels showing difference in OmpX-Om14 purity levels in the absence (0 mM) and presence of 50 mM DTT. The incubation temperature was kept constant in both cases (37 °C) to clearly monitor the effect of DTT. (B) An increase in protein purity of ~10% is obtained when 50 mM DTT is included in Buffer 1, suggesting that the use of DTT helps in removing some disulphide bonded non-specific protein impurities. (C) 15% SDS-PAGE gels showing the effect of incubation temperature (Temp.) (37 = 37 °C for 1 h; 60 = 60 °C for 15 min followed by 37 °C for 45 min) in the presence of 100 mM DTT, on the purity of OmpX-Om14 IBP. We observe no significant increase in sample purity

due to the incubation step; indeed, a marginal reduction in the sample purity is noticeable, which may also arise from protein degradation at very high temperatures. We conclude that the addition of up to 50 mM DTT, along with an incubation time of 5 - 10 min at 37 °C, provides satisfactory removal of any trace amounts of co-precipitating covalently bound contaminants in cysteine-rich proteins. Moreover, treatment with a reducing agent is not mandatory, as sample purity levels only increase by ~10%. (D) Gel quantitation suggests no significant difference of incubation temperature on protein purity. Error bars (in B and D) represent the mean of two datasets.

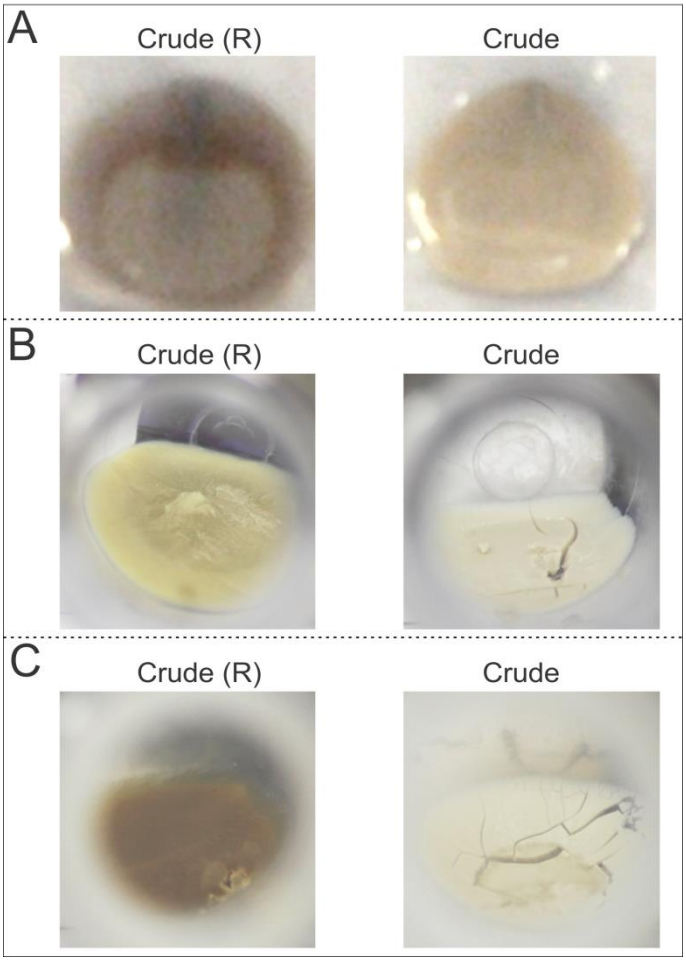


Figure S5. Efficient removal of lipid impurities by the use of an organic solvent wash. Each panel compares the IBP pellets obtained from reported (left; Crude (R)) and the modified protocol (right; Crude). Shown here are representative protein pellets subjected to methanol treatment in the modified IBP protocol. Notice that the protein pellets in the modified protocol indicate better removal of lipid contaminants, without extensive protein loss due to treatment with an organic solvent. Qualitative estimations of the organic solvent supernatant fraction indicated maximum removal of non-proteinaceous impurities with the use of methanol. Further, chloroform wash was avoided because we did not obtain a well-packed protein pellet when chloroform amounts were high in the organic solvent wash. A = OmpX-Om14 fusion; B = Rv1698c ; C = PagP-St.

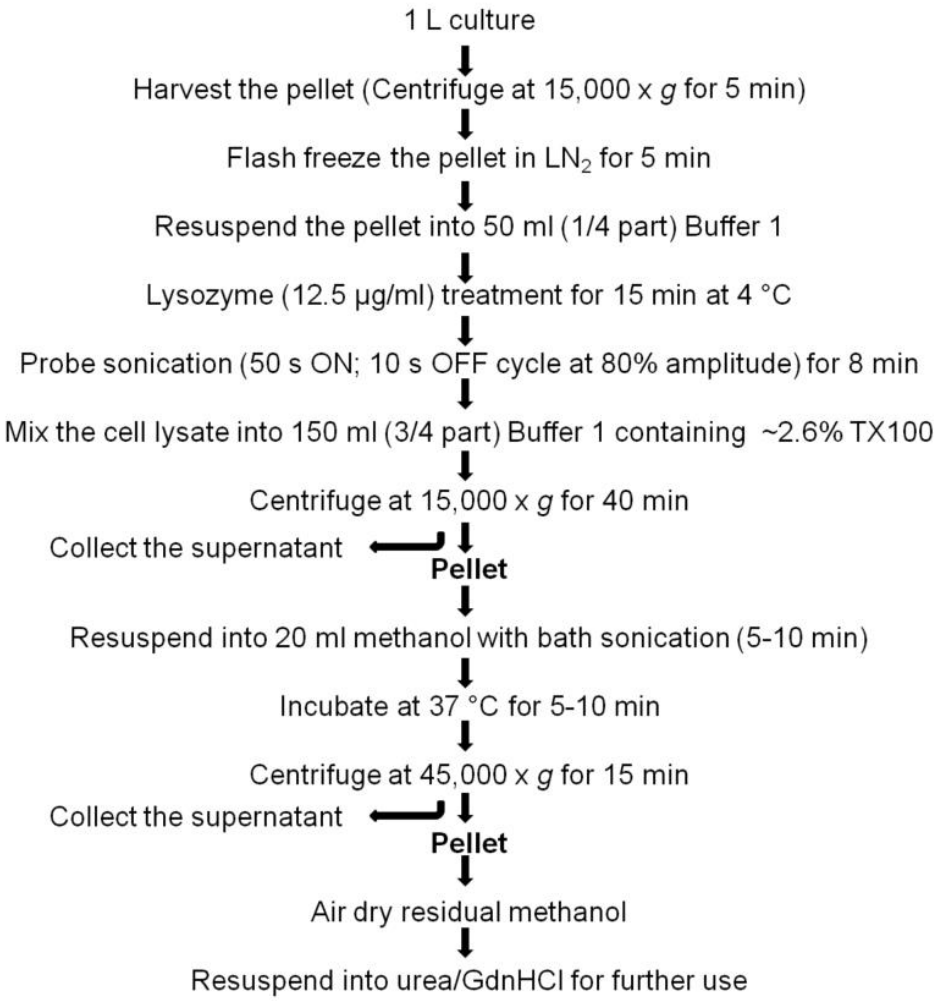


Figure S6. Compiled flowchart summarizing the modified IBP protocol. All the steps to be followed for efficient removal of protein and lipid impurities are presented here for a 1 L bacterial culture pellet. Scale-up to higher culture volumes can be readily carried out by increasing the wash volumes, as it does not significantly change the IBP time. We recommend that the supernatant after each centrifugation step be decanted carefully, and checked on SDS-PAGE prior to discarding. The wash volume, detergent and DTT concentrations can be adjusted, as described, since this affects the final protein quality. The final protein pellet can be directly re-suspended in urea, GdnHCl or any other solubilizing agent, including sodium dodecyl sulphate, for immediate use in biophysical analysis and functional assays.

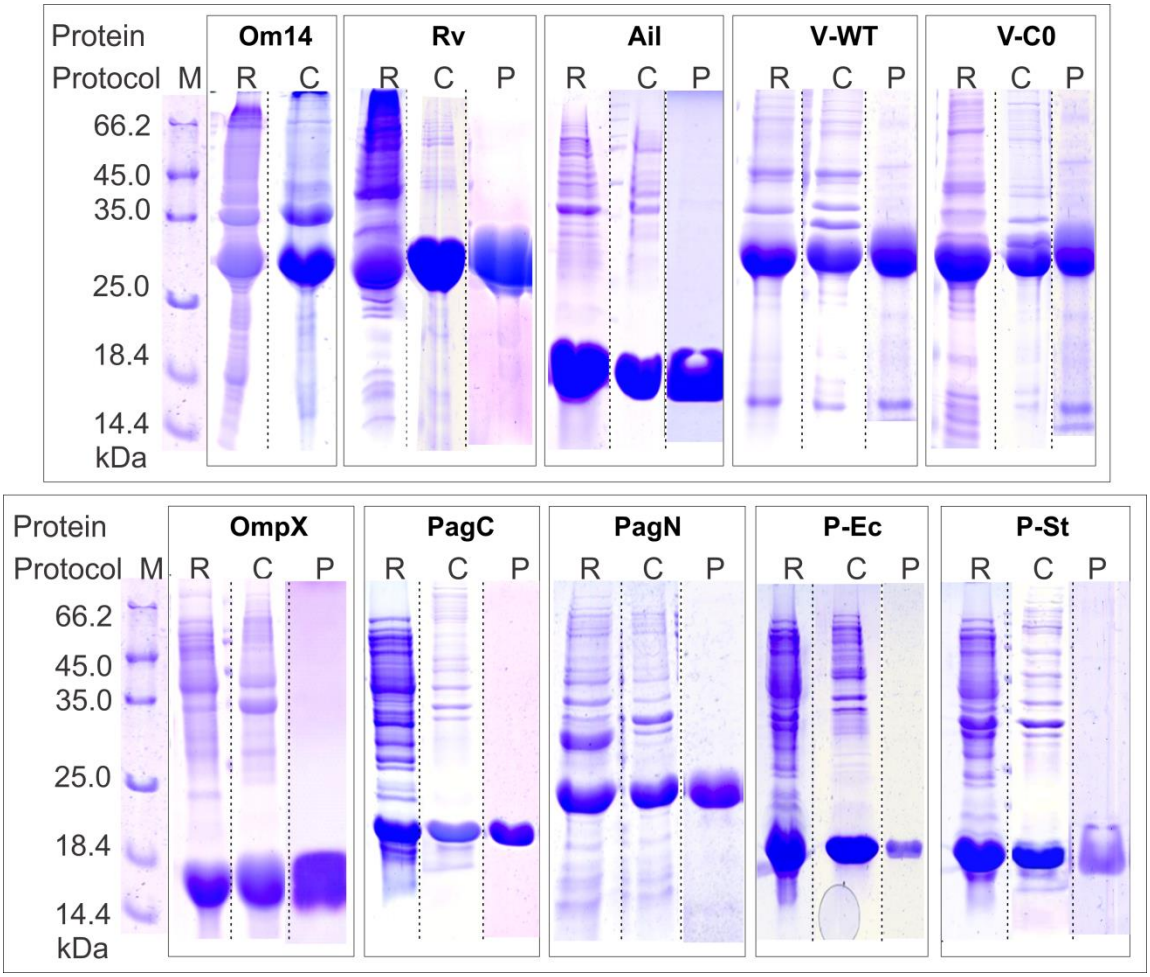


Figure S7. Comparison of protein purity with the modified IBP protocol. Representative SDS-PAGE profiles of protein pellets obtained from the crude preparation from reported protocols (R), crude preparation from modified protocol (C) and purified protein obtained after ion exchange chromatography (P), showing varying degrees of protein purity for the different proteins, ranging from marginally to vastly improved purity levels. Visual inspection of different preparations of each protein clearly indicates that purity levels improve upon changing the method of protein preparation. Densitometry analysis (Fig. 1A) yielded us an ~5 – 50% improvement in the IBP purity across various proteins using the modified IBP protocol, over the reported methods. The values obtained from densitometry analysis of different protein preparations have been separately summarized in Table S5. OmpX-Om14 fusion construct

(Om14) was not purified, and its gel is therefore not included. M = Molecular weight marker; Om14 = OmpX-Om14 fusion construct; Rv = Rv1698c; V-WT = hVDAC-2 WT; V-C0 = hVDAC-2 C0; P-Ec = PagP-Ec; P-St = PagP-St. Dotted lines separate different gels that are presented together for purposes of comparison. The same marker is shown in both panels (above and below).

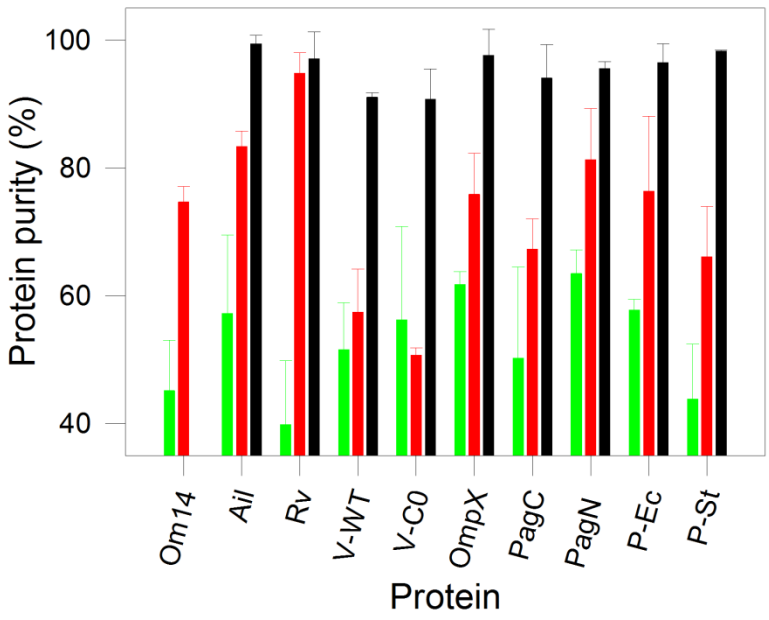


Figure S8. Comparison of purity levels using densitometry. SDS-PAGE quantification of IB preparations generated using reported methods (green bars) and the optimized (modified) IB protocol (red bars) discussed herein shows a ~5-50% improvement in purity of the preparation. Black bars denote densitometry results of protein preparations that have been chromatographically purified. Protein purity obtained using the modified IB preparation (crude) is, by and large, between 70-85%, when compared with the reported methods.

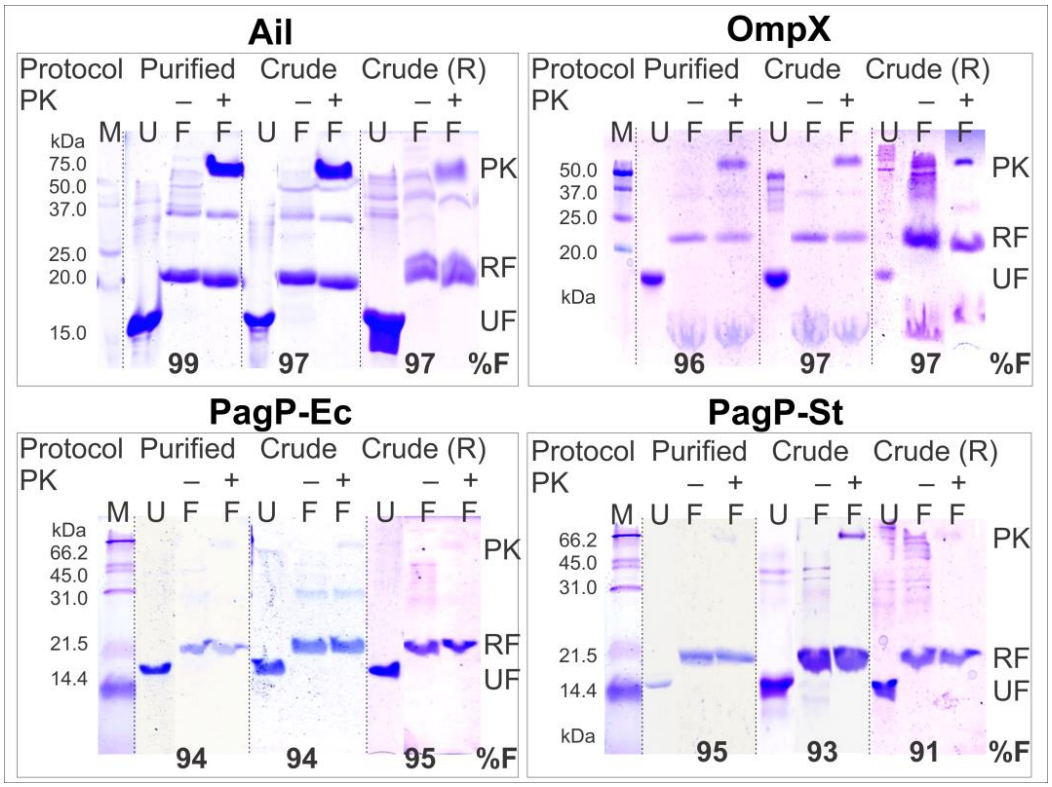
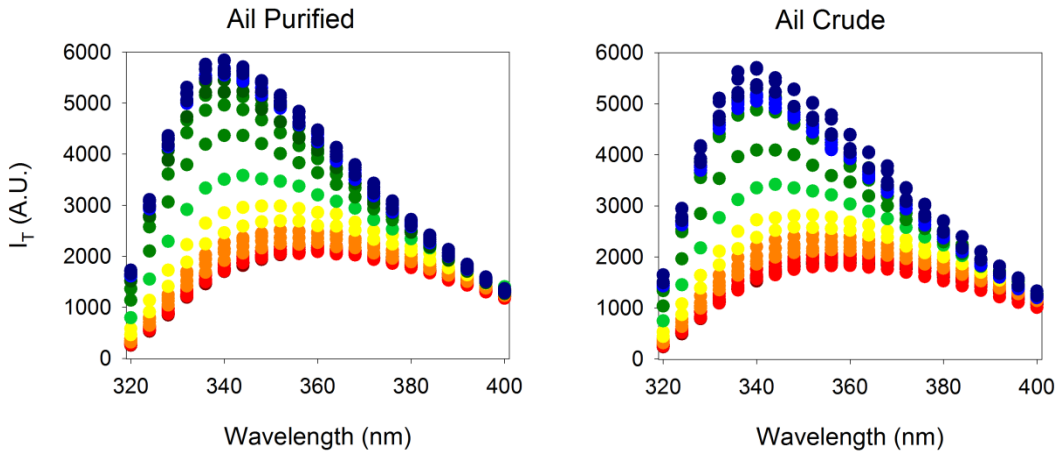


Figure S9. Refolding efficiency of the crude IB protein compares well with the purified protein behaviour. SDS-PAGE analysis of refolding efficiencies of Ail, OmpX, PagP-Ec and PagP-St, comparing purified protein behaviour to that of the crude protein generated using the modified and reported methods. Ail and OmpX were refolded in 125 mM and 100 mM LDAO, respectively, and diluted 5-fold for gel experiments. Similarly, PagP-Ec and PagP-St were refolded in 100 mM DPC and diluted 2-fold. Shown are gel mobility shifts of unboiled samples, and resistance of the folded protein to proteinase K (PK) digestion. All samples show comparable refolding efficiency and resistance to proteolysis, suggesting barrel refolding and stability in these proteins does not require purification. M = Molecular weight marker; U = Unfolded protein control in 8.0 M urea; F = Refolded protein; RF = Refolded monomer showing anomalous mobility; %F = percent refolded protein, calculated only for the monomer species. Dotted lines separate different gels that are shown together.

(A)



(B)

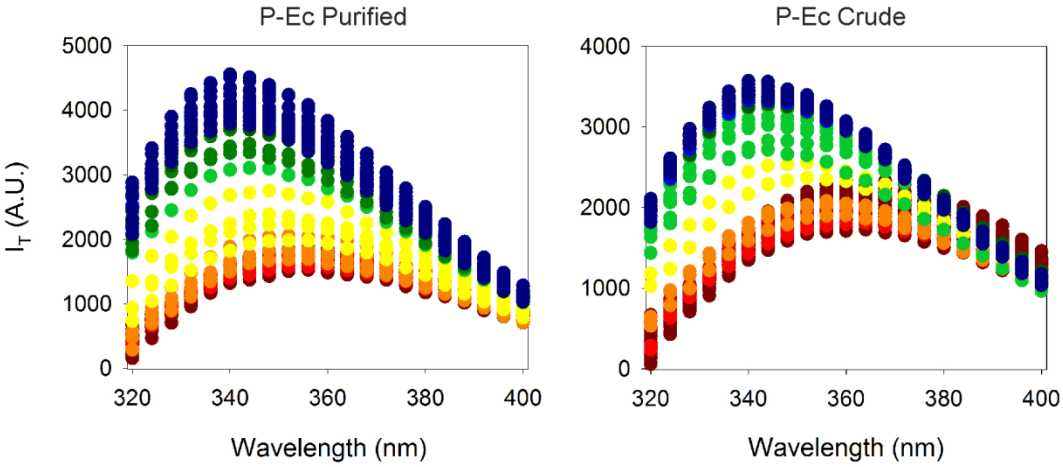


Figure S10. Comparison of fluorescence emission spectra of purified and crude preparations for Ail and PagP-Ec. (A) Fluorescence emission profiles for purified Ail and crude IB preparations show no significant difference, suggesting little or no interference from protein impurities present in crude protein. (B) In the case of PagP-Ec, we present a representative preparation wherein we observe a slight difference in Trp fluorescence between crude and purified proteins. However, this difference does not affect the equilibrium refolding profile of the crude protein (see Fig. 3 of the main text). Hence, with our modified IB method, interference from protein impurities in fluorescence measurements is minimal and does not influence our experimental results even when the protein of interest possesses few Trp residues.

References

1. A. Gupta, D. Chaturvedi and R. Mahalakshmi, *International Review of Biophysical Chemistry*, 2012, **3**, 147-156.
2. A. Gupta, P. Zadafiya and R. Mahalakshmi, *Sci. Rep.*, 2014, **4**, 6508.
3. S. R. Maurya, D. Chaturvedi and R. Mahalakshmi, *Sci. Rep.*, 2013, **3**, 1989.
4. S. R. Maurya and R. Mahalakshmi, *J. Biol. Chem.*, 2013, **288**, 25584-25592.
5. (a) J. Vogt and G. E. Schulz, *Structure*, 1999, **7**, 1301-1309; (b) L. A. Plesniak, R. Mahalakshmi, C. Rypien, Y. Yang, J. Racic and F. M. Marassi, *Biochim. Biophys. Acta*, 2011, **1808**, 482-489.
6. C. Park and S. Marqusee, *Nat. Methods*, 2005, **2**, 207-212.
7. (a) L. Burri, K. Vascotto, I. E. Gentle, N. C. Chan, T. Beilharz, D. I. Stapleton, L. Ramage and T. Lithgow, *FEBS J.*, 2006, **273**, 1507-1515; (b) S. Lauffer, K. Mabert, C. Czupalla, T. Pursche, B. Hoflack, G. Rodel and U. Krause-Buchholz, *J. Biol. Chem.*, 2012, **287**, 17447-17458.
8. (a) A. P. Middelberg, *Trends Biotechnol.*, 2002, **20**, 437-443; (b) C. Klammt, D. Schwarz, K. Fendler, W. Haase, V. Dotsch and F. Bernhard, *FEBS J.*, 2005, **272**, 6024-6038; (c) B. Alberts, *Molecular biology of the cell*, 5th edn., Garland Science, New York, 2008.
9. P. J. Walian, S. Allen, M. Shatsky, L. Zeng, E. D. Szakal, H. Liu, S. C. Hall, S. J. Fisher, B. R. Lam, M. E. Singer, J. T. Geller, S. E. Brenner, J. M. Chandonia, T. C. Hazen, H. E. Witkowska, M. D. Biggin and B. K. Jap, *J. Proteome Res.*, 2012, **11**, 5720-5735.
10. J. A. Cuesta-Seijo, C. Neale, M. A. Khan, J. Moktar, C. D. Tran, R. E. Bishop, R. Pomes and G. G. Prive, *Structure*, 2010, **18**, 1210-1219.
11. (a) M. Ferro, D. Seigneurin-Berny, N. Rolland, A. Chapel, D. Salvi, J. Garin and J. Joyard, *Electrophoresis*, 2000, **21**, 3517-3526; (b) M. Axelsson and F. Gentili, *PLoS One*, 2014, **9**, e89643.
12. R. Mahalakshmi, C. M. Franzin, J. Choi and F. M. Marassi, *Biochim. Biophys. Acta*, 2007, **1768**, 3216-3224.
13. A. Siroy, C. Mailaender, D. Harder, S. Koerber, F. Wolschendorf, O. Danilchanka, Y. Wang, C. Heinz and M. Niederweis, *J. Biol. Chem.*, 2008, **283**, 17827-17837.
14. (a) S. R. Jovel, T. Kumagai, N. Danshiitsoodol, Y. Matoba, M. Nishimura and M. Sugiyama, *Protein Expr. Purif.*, 2006, **50**, 82-88; (b) R. Ujwal, D. Cascio, J. P. Colletier, S. Faham, J. Zhang, L. Toro, P. Ping and J. Abramson, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 17742-17747.
15. W. S. Pulkkinen and S. I. Miller, *J. Bacteriol.*, 1991, **173**, 86-93.
16. M. A. Lambert and S. G. Smith, *BMC Microbiol.*, 2008, **8**, 142.
17. L. Vandeputte-Rutten, M. P. Bos, J. Tommassen and P. Gros, *J. Biol. Chem.*, 2003, **278**, 24825-24830.
18. P. M. Hwang, W. Y. Choy, E. I. Lo, L. Chen, J. D. Forman-Kay, C. R. Raetz, G. G. Prive, R. E. Bishop and L. E. Kay, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 13560-13565.
19. L. Guo, K. B. Lim, C. M. Poduje, M. Daniel, J. S. Gunn, M. Hackett and S. I. Miller, *Cell*, 1998, **95**, 189-198.
20. J. Reed and T. A. Reed, *Anal. Biochem.*, 1997, **254**, 36-40.