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Detection and prevention of protein aggregation before, during, and after purification

Sarah E. Bondos* and Alicia Bicknell[1]

Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251-1892, USA

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Abstract

The use of proteins for in vitro studies or as therapeutic agents is frequently hampered by protein aggregation during expression, purification, storage, or transfer into requisite assay buffers. A large number of potential protein stabilizers are available, but determining which are appropriate can take days or weeks. We developed a solubility assay to determine the best cosolvent for a given protein that requires very little protein and only a few hours to complete. This technique separates native protein from soluble and insoluble aggregates by filtration and detects both forms of protein by SDS–PAGE or Western blotting. Multiple buffers can be simultaneously screened to determine conditions that enhance protein solubility. The behavior of a single protein in mixtures and crude lysates can be analyzed with this technique, allowing testing prior to and throughout protein purification. Aggregated proteins can also be assayed for conditions that will stabilize native protein, which can then be used to improve subsequent purifications. This solubility assay was tested using both prokaryotic and eukaryotic proteins that range in size from 17 to 150 kDa and include monomeric and multimeric proteins. From the results presented, this technique can be applied to a variety of proteins.

Keywords: Aggregation; Solubility; Inclusion bodies; Precipitation; Protein; Purification

Recombinant proteins are required in biological research to investigate enzyme activity, ligand binding, protein interactions, or other functions in vitro. Many proteins are also potential pharmaceutical agents [1–5]. One significant impediment to the study and utilization of proteins is their extreme sensitivity to solution conditions. Nonoptimal conditions during protein expression, purification, storage, or handling can alter protein structure such that the protein irreversibly aggregates, with concomitant loss of activity [4–7]. Proteins frequently aggregate at the high concentrations required for structural studies, and small soluble aggregates can be incorporated into protein crystals as defects [8–10]. Protein folding studies are often complicated by aggregation of intermediate and denatured states [9–13]. Further, point mutations or deletion mutations required for relevant protein studies may destabilize the native state and expedite aggregation [14–16]. The kinetics of aggregation may be an order of magnitude faster than folding kinetics, causing a significant fraction of the protein to be inactivated [10]. Competition between aggregation and folding can have biological ramifications: protein aggregation in vivo is implicated in a variety of disorders, including Parkinson's disease, Alzheimer's disease, and spongiform encephalopathies [15–18]. In vitro examination of the proteins involved in these diseases will require strategies to control aggregation [15,17]. Thus, protein aggregation is a problem common to biological systems, experimental research, and industrial and medical applications.

To circumvent these problems, a wide variety of buffer cosolvents that can facilitate proper protein folding and solubility have been identified. Cosolvents exert their effects by either destabilizing aggregates or enhancing native protein stability [6,12,19–27]. Examples of useful additives are listed in Table 1. Aggregate formation can be deterred by including cosolvents that

* Corresponding author. Fax: 1-713-348-6149.
E-mail address: bondos@bioc.rice.edu (S.E. Bondos).
[1] Present address: UCSD Biology Student Affairs 038, 9500 Gilman. Dr., La Jolla, CA 92093-0348, USA.

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Table 1
Agents that may promote protein solubility

	Additive	Recommended concentration range	Reference
Kosmotropes	$MgSO_4$	0–0.4 M	[19]
	$(NH_4)_2SO_4$	0–0.3 M	[19]
	Na_2SO_4	0–0.2 M	[19]
	Cs_2SO_4	0–0.2 M	[19]
Weak kosmotropes	NaCl	0–1 M	[7,19]
	KCl	0–1 M	[19]
Chaotropes	$CaCl_2$	0–0.2 M	[19]
	$MgCl_2$	0–0.2 M	[19,30]
	LiCl	0–0.8 M	[19]
	RbCl	0–0.8 M	[19]
	NaSCN	0–0.2 M	[19]
	NaI	0–0.4 M	[19]
	$NaClO_4$	0–0.4 M	[19]
	NaBr	0–0.4 M	[19,30]
	Urea	0–1.5 M	[6,12]
Amino acids	Glycine	0.5–2%	[6]
	L-arginine	0–5 M	[6]
Sugars and polyhydric alcohols	Sucrose	0–1 M	[4,11]
	Glucose	0–2 M	[21]
	Lactose	0.1–0.5 M	[21]
	Ethylene glycol	0–60% v/v	[38]
	Xylitol	0–30% w/v	[38]
	Mannitol	0–15% w/v	[38]
	Inositol	0–10% w/v	[38]
	Sorbitol	0–40% w/v	[29,30,38]
	Glycerol	5–40% v/v	[39]
Detergents	Tween 80	0–0.2% w/v	[4]
	Tween 20	0–120 μM	[5]
	Nonidet P-40	0–1%	[42]

destabilize protein–protein interactions. For example, low concentrations of charged cosolvents can screen protein electrostatic interactions that may facilitate aggregation [2,19,26]. Another strategy utilizes chaotropic species to interact with the peptide group, replacing or preventing the intermolecular interactions that lead to aggregation [6,12,25].

An alternate approach is to stabilize native intramolecular protein interactions, thus out-competing the intermolecular interactions that lead to aggregation. To this end, kosmotropes generally stabilize the native state of proteins [25–28]. Because kosmotropes increase the surface tension of the solvent, they are excluded from the protein–solvent surface. Therefore, the entropic cost of disturbing the distribution of small molecules to form the solvent cavity increases. Generally, the native state of globular proteins creates the smallest solvent cavity; therefore, kosmotropes raise the cost of hydrating intermediate or denatured states relative to the native state. Sugars and polyhydric alcohols, in particular, interact with the protein more weakly than water [26]. Optimization of the number of strong water–cosolvent interactions forces the cosolvent to be excluded from the protein surface, thus stabilizing the state with the smallest surface area [21,26,27,29,30]. The addition of such cosolvents not only stabilizes many proteins but also deters ice formation, thus inhibiting the deleterious effects of freezing on protein structure [4,5,26,27]. Finally, small amino acids are also preferentially excluded from the protein surface. However, charged amino acid salts may also interact with the protein at certain pH and salt concentrations [2,26].

Other types of buffer additives may also facilitate protein solubility. Dithiothreitol and β-mercaptoethanol are reducing agents that prevent aggregation of some proteins by inhibiting the formation of nonnative disulfide bonds. Importantly, reduced glutathione is not as effective; the reduced form often contains a small percentage of oxidized glutathione which, ironically, may be sufficient to oxidize the protein [24]. Compounds such as trifluoroethanol or trichloroacetic acid prevent aggregation by stabilizing α-helical structure [23]. Ethanol has been used to stabilize a folding intermediate by weakening hydrophobic interactions that facilitate aggregation [13]. Membrane proteins may require detergents or micelles to form their native structure [20,22].

The list of potential cosolvents that influence protein solubility may appear staggering to one who must determine a starting point. The following strategy may be useful to rapidly identify optimal solvent conditions for a given protein. First, cosolvents or additive concentrations that interfere with subsequent biochemical assays should be eliminated. Previous experience with the protein or a member of the protein family may suggest cosolvents that are likely to succeed. For example, many nucleic acid binding proteins often require higher salt concentrations, as demonstrated in this paper. For novel proteins, a good choice is to select one cosolvent from each category in Table 1, using a concentration in the middle of the suggested range. Chemicals and concentration ranges within a promising category can then be optimized in a subsequent assay.

When choosing a cosolvent, determination of the optimal concentration is critical. High concentrations of chaotropes will denature proteins, while high concentrations of kosmotropes will salt proteins out of solution. Consequently, removing ions from the buffer may enhance protein stability [17,31]. A list of many additives and appropriate concentration ranges can be found in Table 1. Because any additive has the potential to alter protein conformation or activity, the effects of specific conditions on protein structure and function should be investigated by varying cosolvent concentration or comparing with a second stabilizing cosolvent [26]. A more extensive list of protein stabilizing reagents and descriptions of their mechanisms of action can be found in several excellent reviews [25–27].

The large number of potentially stabilizing cosolvents and the dependence of cosolvent effects on concentration complicates determination of optimal buffer conditions for a given protein. Often, such efforts rely on trial and error during protein purification. Alternately, sample turbidity can be measured to assay for protein aggregation. However, turbidity measurements require high concentrations of protein and cannot detect low concentrations of aggregates or small, soluble aggregates [3,32]. Further, turbidity requires purified samples, prohibiting its use for proteins that aggregate during expression or purification.

Here, we describe a facile method to identify buffers that maintain soluble, native protein. This technique can distinguish precipitates and small, soluble aggregates from native protein. Multiple buffers or proteins can be screened in just a few hours. Importantly, this solubility assay can be applied to protein mixtures and crude lysates, allowing assessment of protein stability throughout a protein purification protocol. This solubility assay was demonstrated for a variety of applications, including screening buffers to inhibit aggregation in functional assays, screening mutant proteins for aggregation, screening for solubility of a single protein in heterogeneous mixtures, assaying aggregated proteins for stabilizing conditions, and screening inclusion bodies in crude cell lysates for stabilizing conditions. Both prokaryotic and eukaryotic proteins were tested, including monomeric and multimeric proteins ranging in size from 17 to 150 kDa. Therefore, this solubility assay can be utilized for a wide variety of proteins.

Materials and methods

Protein expression and purification

The proteins used to test the aggregation assay are *Bacilllus anthracis* ATXA, *Eschericia coli Lac*I, *Drosophila melanogaster* UDK-c, *Drosophila melanogaster* Ultrabithorax (Ubx), and *Brachydanio rerio* LMO4. ATXA protein was a gift of Dr. Edward Nikonowicz from Rice University. The *Lac*I mutants L148F, S151P, G60+3-11, and Q60P-11 were provided by Hongli Zhan and Dr. Kathleen Matthews from Rice University. UDC-c was given by Daniel J. Catanese and Dr. Kathleen Matthews from Rice University. LMO4 crude lysates, purified protein, and GN5049 primary antibody were gifts of David Ji and Dr. Mary Ellen Lane from Rice University.

Ubx contaminated with proteolysis products and full-length Ubx were purified for use in the solubility assay. The Ubx expression construct pET-Ubx-3c, a gift from Dr. Phillip A. Beachy (The Johns Hopkins University School of Medicine), was transformed into the *E. coli* strain BL21(DE3)pLysS. Twelve flasks containing 1 liter of Luria broth plus 50 μg/ml carbenicillin cultures were each inoculated with 10 ml of overnight culture and grown at 37 °C to mid-log. The cultures were cooled to room temperature prior to Ubx induction with 1 mM isopropyl β-L-thiogalactoside. Cells were harvested 2 h after induction and frozen.

To purify Ubx, a cell pellet was lysed in 20 ml of 50 mM Tris–HCl, 4 mM DTT,[2] 800 mM NaCl, 20 μg/ml DNase, and 1 mM phenylmethyl sulfonyl fluoride. Lysis supernatant was treated with polyethyleneimine and centrifuged. The pH of the supernatant was adjusted to 6.8 and centrifuged to remove precipitates. The final supernatant was loaded onto a phosphocellulose column, washed with Buffer Z (10% glycerol, 0.5 mM DTT, 0.1 mM EDTA, 25 mM NaH_2PO_4, 100 mM NaCl, pH 6.8), and eluted with a 0 to 1 M NaCl gradient in Buffer Z. Ubx mixed with N-terminal proteolytic products was assayed for aggregation at this point in the purification. Fractions containing Ubx were dialyzed against 4 liters of 50 mM Tris–HCl, 100 mM NaCl, 1 mM DTT, 10%

[2] *Abbreviations used:* DTT, dithiothrectol; EMSA, electrophoretic mobility shift analysis.

Table 2
Proteins used in the aggregation assay and relevant parameters

Protein	Source	Filter used	Initial concentration	SDS–PAGE detection	Molecular weight and assembly
ATXA	*Bacillus anthracis*	100 kDa MW cut-off Microcon	0.16 mM	Silver stain	50 kDa, monomer
*Lac*I:	*Escherichia coli*	Ultrafree—MC filter unit		Silver stain	
L148F			0.67 mM		150 kDa, tetramer
S151P			0.60 mM		150 kDa, tetramer
G60+3-11			1.10 mM		65 kDa, dimer
Q60+3-11			0.84 mM		65 kDa, dimer
UDKc-his	*Drosophila melanogaster*	100 kDa MW cut-off Microcon	3.6 μM	Western blotting	47 kDa, monomer
UbxIb	*Drosophila melanogaster*	100 kDa MW cut-off Microcon	13 μM	Western blotting	40 kDa, monomer
LMO4	*Brachydanio rerio*	100 kDa MW cut-off Microcon	5 μM	Western blotting	17 kDa, monomer

glycerol, pH 8.0 for 1 h. Ni-NTA resin (Qiagen) was equilibrated in dialysis buffer, added to the dialysate, and mixed on a Nutator at 4 °C for 1 h. The resulting slurry was washed with 10 ml of buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 5% glucose at pH 8.0. plus 10 mM imidazole followed by 25 ml of the same buffer with 20 mM imidazole. Ubx was eluted in 10 ml of the buffer containing 100 mM imidazole. Protein was stored at 4 °C after the addition of DTT to 5 mM and assayed the following day.

Aggregation assay

The general strategy was to simultaneously test up to 14 solution conditions on small samples of purified or unpurified protein. Soluble protein was then separated from aggregates and precipitates by filtration. The molecular weight cut-off of the filter was chosen such that soluble protein was allowed to pass through the filter, while aggregate forms were retained.

In more detail, protein was diluted or dialyzed into a series of buffer conditions such that the final volume was 100–120 μl. Initial protein concentrations ranged from 3 to 1 mM (see Table 2). The diluted protein was incubated at room temperature in the test buffer for 1 h. Soluble protein was then separated from aggregated protein using a Microcon concentrator (Millipore), with a molecular weight cut-off of 100 kDa. The Ultrafree–MC 0.1-μm filter unit (Millipore) was used for experiments on *Lac*I mutants to accommodate the larger proteins. The Microcon or filter unit was spun in a desktop centrifuge at 16,000*g* for 15 min. Aggregated protein retained on the membrane was resuspended in 30 μl of dH_2O, pipeting repeatedly across the membrane to ensure that as much protein as possible is removed. Samples of 30 μl soluble protein and 30 μl aggregated protein were each mixed with 10 μl of 4× sample buffer (250 mM Tris–HCl, 40% glycerol, 140 mM SDS, 0.6 M β-mercaptoethanol, pH 6.8) and heated to 85–90 °C for 10 min prior to SDS–PAGE. Because soluble protein and aggregated protein were assayed separately, small changes in either population can be readily observed. Detection of aggregates is especially sensitive because nearly half of the recovered aggregated protein retained by the filter is loaded on the gel. The final protein concentration required is dependent on the detection method used. Coomasie staining detects 0.05–0.5 μg of protein per band, silver staining detects 1–5 ng of protein per band, and Western blotting detects less than 1 pg of protein per band ([23], www5.amershambiosciences.com).

Results

The solubility assay was developed for use in situations spanning the lifetime of a protein from cell lysis, purification, and exchange into assay buffers. For simplicity, the straightforward application of assaying soluble protein for conditions that diminish aggregation is presented first. The subsequent experiments, including analyzing solubilization of aggregated protein and analyzing protein mixtures, increase in complexity. The section concludes with assaying inclusion bodies in crude cell lysates for conditions that will allow protein solubilization. This last application is the most useful, allowing optimization of cosolvents prior to purification.

Example of screening buffers to enhance solubility of purified protein during functional assays

Buffer conditions required for column binding and protein elution are often incompatible with functional and structural analysis. Initial purifications of the *B. anthracis* protein ATXA resulted in precipitation of some product. Further, gel retardation assays of DNA binding by the soluble protein fraction exhibited density in the wells, indicative of ATXA aggregation. Buffer conditions were screened for stabilization of native ATXA and prevention of aggregation in DNA binding assays (Fig. 1). Buffer additives were limited to salt and glycerol, which are normal components of buffers in DNA binding assays, and cosolvent concentrations

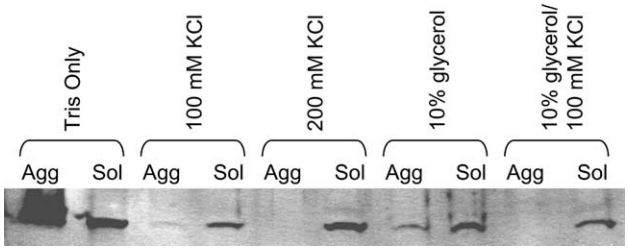

Fig. 1. Solubility assay of ATXA protein to identify an appropriate DNA binding buffer. To dilute the protein into assay buffers, 10 μl of 0.16 mM protein was added to 100 μl of the five test buffers. All buffers contain 20 mM Tris–HCl, pH 7.5. ATXA was detected by silver staining. The soluble, native protein fraction is indicated by Sol, and the aggregated protein fraction is indicated by Agg. While ATXA aggregates in buffer containing 10% glycerol or 100 mM KCl, no aggregation was observed in buffer containing both 10% glycerol and 100 or 200 mM KCl. The variation in total protein observed for each buffer condition is repeatable and thus most likely reflects adhesion to the filter used in the assay.

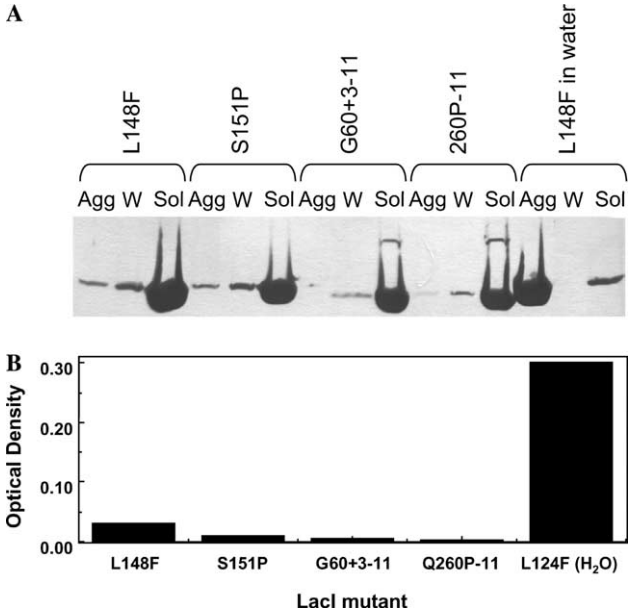

Fig. 2. (A) Solubility assay of *Lac*I tetrameric and dimeric mutants. Each mutant was diluted 1:100 into buffer containing 20 mM Tris–HCl, pH 7.5, 100 mM KCl, and 10% glycerol. Initial protein concentrations ranged from 0.6 to 1.1 mM. The positive control contains the L148F mutant diluted into Millipore water. *Lac*I proteins were visualized by silver staining. The soluble protein is indicated by Sol, the filter unit wash by W, and the aggregated protein by Agg. (B) Turbidity of each sample, as measured by the optical density at 600 nm. The turbidity results correlate with the density in the corresponding Agg lane.

were optimized within an acceptable range for DNA binding assays. Severe aggregation was detected in buffer containing only 20 mM Tris, pH 8.0. Aggregation was also visible when 100 mM KCl or 10% glycerol was added. However, inclusion of 200 mM KCl prevented aggregation. Thus, the protein is sensitive to low salt conditions. Interestingly, while neither 100 mM KCl nor 10% glycerol alone can prevent aggregation, a combination of both additives maintains soluble protein. The efficacy of combinations of cosolvents has been observed in other proteins also [6]. This assay provided two useful solution conditions for performing functional assays, which is important because DNA binding is profoundly affected by alterations in salt and glycerol concentrations [33–35]. Therefore, the solubility assay can successfully identify buffers that stabilize soluble protein.

Example of screening mutant proteins for aggregation

Mutations can dramatically alter the structure, stability, or aggregation properties of a protein. Even though mutant proteins may purify like wild-type protein, mutations may alter protein solubility. Proteins with mutations in the full-length tetrameric lactose repressor, *Lac*I, and the dimeric deletion mutant, −11 *Lac*I [36,37], were assayed for aggregated contaminants after storage at −80 °C (Fig. 2). Ultrafree–MC 0.1-μm filter units were used to ensure sufficiently large pore size to accommodate the 65-kDa dimers and the 150 kDa monomers. The 0.1-μm pore size is approximately 15 times the size of the −11 *Lac*I dimer. Each mutant protein was diluted 1:100 into 20 mM Tris, pH 7.5, 100 mM KCl, and 10% glycerol. As a positive control, the L148F *Lac*I mutant was also diluted into water, thus forcing aggregation and precipitation. The high protein concentrations used in this assay raised concerns that residual native protein left on the wet filter might be erroneously interpreted as aggregate. To prevent this problem, the membranes were washed by filtering with an additional 100 μl of buffer or water after separation of native and aggregated protein. Any native protein left on the filter after the wash should be below the detection limits of silver staining.

As expected, the majority of the protein in the water control was retained by the filter, indicating that the filter was capable of separating native and aggregated protein. While the different *Lac*I and −11 *Lac*I mutants were primarily native, aggregation was detected in the tetrameric L148F and S151P samples. Both −11 mutants had little or no aggregates. Most of the positive control, *Lac*I in water, was retained by the filter, demonstrating that a 0.1-μm filter can retain large amounts of aggregated *Lac*I. Concerns that small concentrations of *Lac*I aggregates might not be retained on filters with the very large pore size prompted comparison of the turbidity at 600 nm prior to sample filtration (Fig. 2B). The turbidity measurements correlate very well with the density of the band in the retentates, verifying that dimeric mutants can be assayed using the 0.1-μm filter. Thus, the solubility assay works well using filters with larger pore sizes and can be applied to multimeric proteins.

Screening for aggregation of a partially purified protein

Because solubility is monitored by SDS–PAGE, aggregation of a single component can be detected in a mixture of proteins. An interesting example is provided by the *D. melanogaster* protein Ultrabithorax Ib (Ubx). Ubx is not produced as inclusion bodies and does not precipitate during purification. However, a portion of the protein originally remained in the wells during electrophoretic mobility shift analysis (EMSA) to measure DNA binding, an indication of protein aggregation. A partially purified sample with contaminating N-terminally degraded Ubx was used to assay buffers that potentially stabilize full-length, Ubx (Fig. 3A). Full-length Ubx aggregates were detected in buffers containing low concentrations of salt. However, none of the degradation products precipitated under low-salt conditions; therefore, either the salt-sensitive region of Ubx must reside in the N-terminal region or the structure must be altered upon removal of this region. Buffers for the remainder of the purification were adjusted to include 100 mM NaCl and 5–10% glycerol or glucose. Full-length purified Ubx was also assayed (Fig. 3B). The results for purified, full-length Ubx match the protein mixture. These results indicate that interactions with proteolysis products do not influence the aggregation behavior of full-length Ubx and confirm that the N-terminus of Ubx contains a domain sensitive to salt concentration. The use of Ubx purified under the new buffer conditions for EMSA experiments abrogated the signal caused by protein trapped in the wells, thus confirming the solubility assay results (data not shown). Thus, aggregation of a single protein in a protein mixture can be reliably assessed using the solubility assay.

Example of assaying aggregated protein for stabilizing conditions

Many proteins precipitate upon purification to homogeneity. Therefore, it is desireable to be able to use aggregated protein to assay buffers that will stabilize the native, soluble form. The *D. melanogaster* protein UDKc, also named UbxBP1, DIP1-c, and KLETT-c, originally precipitated after purification on a Ni-NTA column (Qiagen) via a histidine tag. The microfiltration solubility assay was used on purified, precipitated protein to determine buffer conditions that would maintain native UDKc during subsequent purifications (Fig. 4A). Precipitation may be irreversible; therefore, the presence of any soluble protein indicates a condition that may prevent protein aggregation. UDKc-his, a histidine-

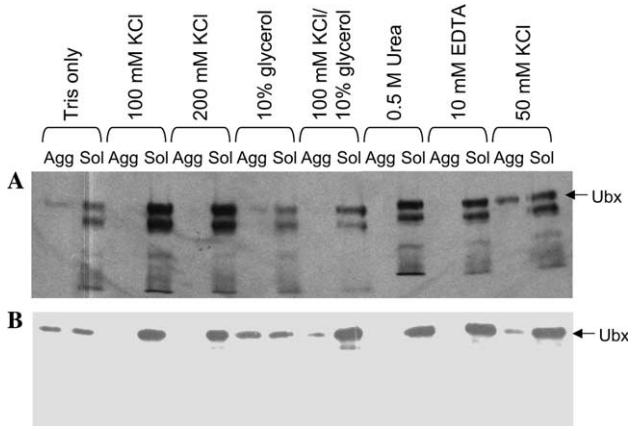

Fig. 3. Solubility assay using a protein mixture. Ubx protein bands on a 10% SDS–PAGE gel were detected by Western blotting, using FP3.38 as the primary antibody [41]. Ubx, 10 μl, was diluted with 100 μl of test buffers. All buffers contain 20 mM Tris, pH 8.0. (A) Aggregation assay of a mixture containing full-length Ubx and its N-terminal degradation products. The top band is full-length Ubx, and the bottom bands are N-terminal proteolysis products. Ubx aggregates in buffer with no additives, buffer with 50 mM KCl, and buffer with 10% glycerol. However, Ubx did not aggregate in buffers with higher salt concentrations or with EDTA. (B) Aggregation assay of purified full-length Ubx. The behavior of purified Ubx matches the behavior of the mixture of full-length in a mixture with proteolyzed Ubx. Lanes are labeled as in Fig. 1.

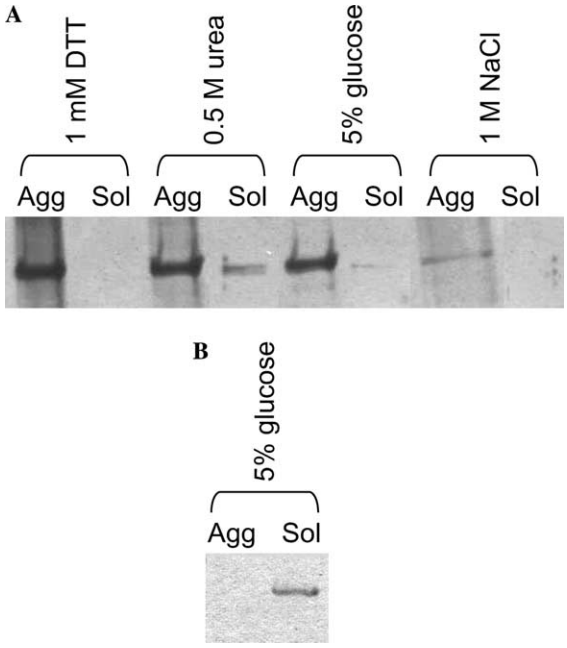

Fig. 4. Solubility assay of aggregated UDKc-his, a his-tagged RNA binding protein. Aggregated UDK-c was purified using Ni-NTA resin (Qiagen) and following the protocols therein. Protein bands on a 10% SDS–PAGE gel were detected by western blot using mouse anti tetra-his (Qiagen) as the primary antibody. (A) UDKc-his at 3.6 μM was dialyzed into four test buffers. Each buffer contains 50 mM sodium phosphate, 150 mM NaCl, 10 mM imidazole, pH 8.0, in addition to the cosolvents indicated. Lanes are labeled as in Fig. 1. Addition of both urea and 5% glucose increased the amount of soluble UDKc-his. (B) Aggregation assay of 100 μl of 5.1 μM UDKc-his similarly purified with all buffers containing 5% glucose. All of the protein is in the flow-through, demonstrating that inclusion of glucose in the purification buffers prevents aggregation of UDKc-his.

tagged construct that produces a 47-kDa protein, was dialyzed into different buffers to maintain the protein concentration at detectable levels. Buffer containing urea, and to a lesser extent 5% glucose, decreased aggregation of the protein. Because urea might affect subsequent biophysical characterization, 5% glucose was selected as a purification additive for subsequent purifcations. A second solubility assay using UDKc-his protein purified in buffers containing 5% glucose revealed that the protein did not aggregate under the new buffer conditions (Fig. 4B). In a single afternoon, application of the solubility assay to precipitated protein successfully predicted conditions that would stabilize the native state of the protein, allowing protein purification and long-term storage.

Screening for solubilization of inclusion bodies

Many proteins, including the *B. rerio* (zebrafish) zinc finger protein LMO4, are expressed as inclusion bodies in *E. coli*. Purification then has the added onus of restoring the protein to its native, soluble state. Crude lysate containing 8 M urea was assayed for conditions that enhance LMO4 solubility during purification (Fig. 5A). Due to the large number and high concentration of other proteins in the mixture, Western blotting was used to visualize LMO4 after SDS–PAGE. Because the protein is produced as inclusion bodies, determination of buffer conditions that will maintain soluble, native LMO4 was expected to be difficult. Therefore 12 cosolvents that address a broad range of potential triggers for aggregation, such as exposure of hydrophobic groups, charge–charge interactions, and cysteine oxidation, were selected for examination using the solubility assay. All test buffers contained 50 mM Tris–HCl, pH 8.0, 1 mM $ZnSO_4$, and at least 100 mM KCl. The density of both aggregated and soluble LMO4 for several buffer conditions is very light. This light density is repeatable and therefore not an artifact of gel loading or aggregate resuspension. The most likely explanation is that LMO4 adopts a conformation capable of irreversibly adhering to the membrane or the plastic casing in the filtration device under these buffer conditions. The only buffer to yield a substantial percentage of LMO4 in the soluble fraction contained 10 mM DTT, indicating that protein oxidation likely triggered LMO4 aggregation.

To determine whether the proteins, lipids, and DNA in the crude lysate had influenced LMO4 solubility, precipitated LMO4 purified without DTT was assayed in the same buffers (Fig. 5B). While some LMO4 was observed in the flow-through of buffers containing urea, arginine, and trichloroacetic acid, all of the protein diluted into buffer containing 10 mM DTT was in the filtrate. Therefore, use of the solubility assay to analyze the solution behavior of proteins in mixtures and crude lysate can predict the behavior of purified proteins. Assaying crude lysates is an effective strategy for designing purification protocols for novel proteins or proteins with a history of aggregation or precipitation. Upon growth of a purification-scale batch of *E. coli*, a

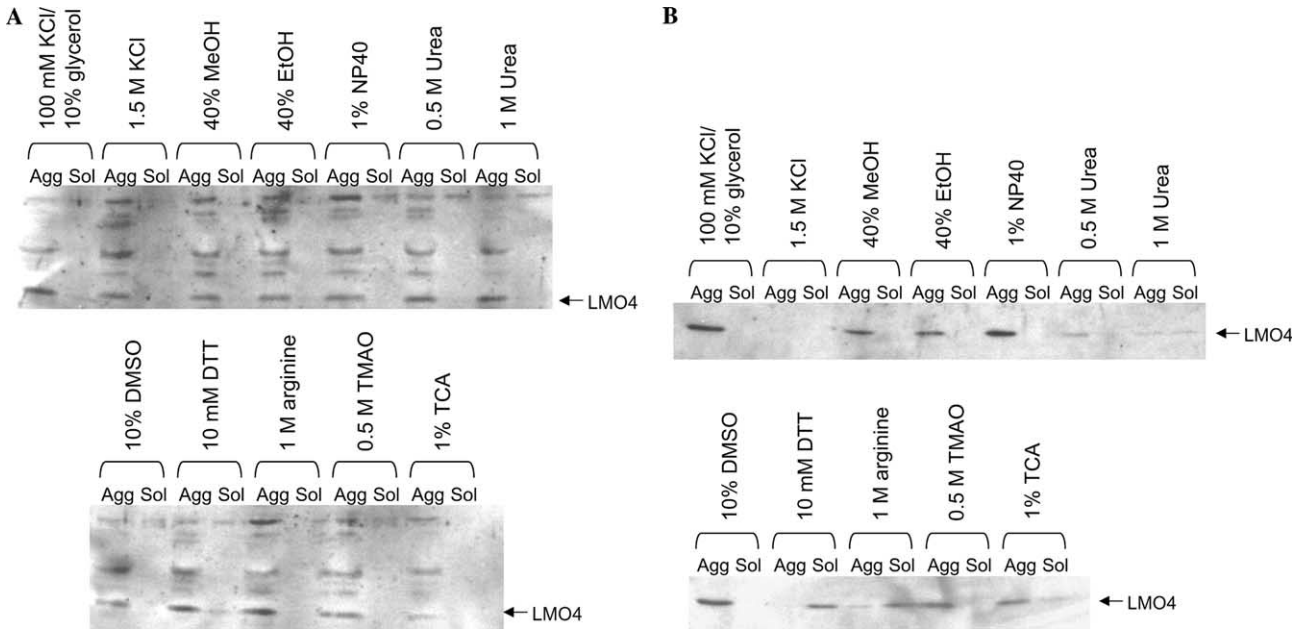

Fig. 5. Solubility assay using crude lysate. Ten microliters of both the crude lysate and the purified protein were diluted into 100 µl of test buffer. All buffers contain 50 mM Tris–HCl, pH 8.0, 1 mM $ZnSO_4$, and at least 100 mM KCl. LMO4 protein was detected by Western blot using the rabbit anti-LMO4 antibody GN5049. (A) Analysis of LMO4 in crude lysate containing 8 M urea. Only the buffer containing 10 mM DTT contains a substantial percentage of LMO4 in the flow-through. (B) Analysis of LMO4 purified in buffer containing 8 M urea. All of the LMO4 protein is in the flow-through for the 10 mM DTT sample. Lanes are labeled as in Fig. 1.

small aliquot of protein-expressing cells should be frozen separately. This aliquot can be lysed and the protein solubility tested, allowing adjustment of the purification buffers to suit the needs of the protein *prior* to the initial protein purification. This strategy saves a substantial amount of time and supplies compared to an iterative trial and error approach to protein purification.

Discussion

Effective screening of many possible additives at various concentrations requires a rapid assay for protein solubility. However, efforts to identify optimal buffer conditions often rely on repurification or functional assays, a time- and protein-consuming trial and error approach. Alternately, the turbidity, or light scattered by precipitates at a nonabsorbing wavelength, can be used to rapidly detect insoluble protein aggregates. Turbidity measurements require large volumes of at least 10 μM final protein concentration [3]. The yields of many protein preparations are too low to allow screening by buffer dilution. Because turbidity is dependent on the molecular weight and the radius of gyration, the size or shape of the aggregates influence the outcome [32]. In addition, impure protein cannot be assayed. Finally, small soluble aggregates or low percentages of aggregates can impact protein function or create point defects in crystal growth, but are not detectable with turbidity measurements [10].

Here, we describe a sensitive method to simultaneously screen a large number of conditions for soluble or insoluble aggregates in a few hours. Because the assay separates the species by size, small soluble aggregates can be separated from native protein and detected. Aggregation of a single protein in a mixture can be detected, allowing analysis of partially purified protein or unpurified lysates. The analysis of both partially purified Ubx- and LMO4-containing crude lysate matched the results from the corresponding purified protein. Thus, this solubility assay can be used to screen for aggregated protein before, during, or after protein purification. A useful approach to a novel or difficult protein purification would be to use a small aliquot of *E. coli* expressing the protein of interest for analysis using the solubility assay. The required cosolvents could then be included in the protein purification buffers, dramatically increasing the probability of success. Previously aggregated protein can also be assayed to search for conditions that will prevent aggregation during subsequent purifications.

Some proteins may adhere to the filter units used in the assay under certain buffer conditions. Even this situation would be detectable. The protein would repeatedly not be present in the flow-through or the retentate under one or more buffer conditions, reflecting irreversible loss in the filter (for example, see Fig. 5B (LMO4 in 1.5 M KCl)). Because the Durapore membrane in the Ultrafree MC filter unit binds only 4 μg/cm^2 protein, this filter may be better for "sticky" proteins than the regenerated cellulose filters used in the Microcon [40]. Proteins may also adhere to the plastic tubes used in the assay. In this case, tubes treated with chlorinated organopolysiloxane in heptane to reduce protein adhesion may be used.

The aggregation assay was successfully tested on five proteins from both prokaryotic and eukaryotic organisms. Monomeric and multimeric proteins, ranging in size from 17 to 135 kDa, were examined in these experiments. From these results, we expect this assay to be generally applicable for rapid analysis of a wide range of proteins. The total time needed to optimize purification and buffers for functional assays for all five proteins was less than 2 weeks, a tremendous savings in time and material. This assay can be applied to proteins at all stages of purification, eliminating the necessity for trial and error approaches to optimize purification buffers.

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Further reading

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