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A sparse matrix approach to the solubilization of overexpressed proteins

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Many biophysical experiments depend on large amounts of pure, soluble protein. Indeed, the revolution in structural biology has depended on molecular biology's potential to make experiments possible by allowing the overexpression of normally rare proteins in a heterologous host. All too often, however, overexpressed proteins are poorly soluble in buffers that attempt to mimic physiological conditions. Often in such cases the overexpressed protein is assumed to be present in inclusion bodies and hopes of obtaining the desired sample from the overexpression vector are abandoned. We have developed a sparse matrix approach to the solubilization of such proteins that is often successful. This approach relies on well accepted theories of protein solubility and folding to build a sparse matrix that samples 'solubility space' effectively. The buffers of the sparse matrix are used to make crude extracts that are rapidly assayed for soluble protein using gel electrophoresis. We describe our approach and give examples of its application.

Keywords: inclusion bodies/protein folding/protein overexpression/protein solubility

Introduction

The preparation of large samples of purified proteins usually begins with cloning and overexpression in a host such as *Escherichia coli.* Unfortunately, overexpressed proteins often appear to be insoluble when made in a heterologous host. Although some proteins may be extracted in a denatured form and refolded, it is often wiser to extract the protein of interest under nondenaturing conditions. Over the years protein chemists have explored the solubility of proteins extensively; nevertheless, we often fail to apply this wealth of information effectively.

Hofmeister noticed the differential solubility of proteins in various salts more than 100 years ago. We call the ordering of anions and cations according to their ability to solubilize and stabilize proteins the Hofmeister series and arrange ions from the least to the most chaotropic. Thus, ammonium sulfate both stabilizes the folded state and tends to drive proteins out of solution while guanidinium chloride tends to denature proteins while holding them in solution. The differential effect of salts is the most important variable when searching for conditions that will give good solubility of a recalcitrant overexpressed protein. At low concentrations salts generally 'salt in' (Green, 1932; Arakawa and Timasheff, 1985), but any individual protein will behave according to its own chemistry (Retailleau *et al*., 1997). Since proteins are such complex electrolytes, it is appropriate to think of each as having its own characteristic solubility. In our experience, balancing stabilizing versus destabilizing effects (Lin and Timasheff, 1994), modified by the direct interaction of some salts with the protein (Arakawa and Timasheff, 1982b, 1984; Arakawa *et al.*, 1990a), can result in enhanced yields of active, soluble protein. One may screen varying concentrations of nonchaotropic, slightly chaotropic and moderately chaotropic salts as well as low concentrations of chaotropic salts to find the best conditions.

Other additives or 'co-solvents' which modify the structure of water or bind to proteins also differentially affect protein solubility (Arakawa *et al.*, 1990b). Small organic molecules such as isopropanol can be used at low concentrations to break up nonspecific aggregation (Asakura *et al.*, 1978). Glycerol can promote solubilization and stability and is generally not denaturing at any concentration (Gekko and Timasheff, 1981; Arakawa and Timasheff, 1985). Polyvalent ions like spermidine or dextran sulfate (Hedman and Gustafsson, 1984) may be able to intervene in nonspecific interactions with polysaccharides, nucleic acids or abundant highly charged host proteins such as histones. Sugars and other polyhydric alcohols may be able to act in a similar way and have an additional advantage of tending to stabilize folding (Gerlsma, 1970; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a). Detergents can be helpful in overcoming aggregation (Womack *et al.*, 1983), perhaps by binding to hydrophobic surface patches on the protein of interest.

Because these considerations lead to a large number of conditions that might be explored, we have developed a system that allows rapid sampling of this large 'solubility space'. An initial sparse matrix screen is followed with a systematic search of conditions to determine the best buffer with which to extract an overexpressed protein. Our method relies on the physical chemistry of protein folding and solubility and is inspired by the success of a sparse matrix approach to protein crystallization (Carter and Carter, 1979; Jancarik and Kim, 1991). We describe the design and application of our method and present examples of its use.

Materials and methods

Constructing the sparse matrix

The task of adequately sampling 'solubility space' is analogous to the task of growing crystals of macromolecules. A useful technique for the latter task, which might be called the sampling of 'crystallization space', is to construct a very coarse matrix sampling the conditions that have some probability of success. To construct an analogous sparse matrix of buffer conditions for making crude extracts of overexpressed proteins, we made three lists. The first is a list of buffers varying the pH over

Table I. Thirty reagents for solubilization of overexpressed proteins

1.	100 mM Tris, 10% glycerol, pH 7.6
2.	100 mM Tris, 50 mM LiCl, pH 7.6
3.	100 mM HEPES, 50 mM $(NH_4)_2SO_4$, 10% glycerol, pH 7.0
4.	100 mM HEPES, 100 mM KCl, pH 7.0
5.	100 mM Tris, 50 mM NaCl, 10% isopropanol, pH 8.2
6.	100 mM K_2HPO_4/KH_2PO_4, 50 mM $(NH_4)_2SO_4$, 1% Triton X-100, pH 6.0
7.	100 mM triethanolamine, 100 mM KCl, 10 mM DTT, pH 8.5
8.	100 mM Tris, 100 mM sodium glutamate, 10 mM DTT, pH 8.2
9.	250 mM KH_2PO_4/K_2HPO_4, 0.1% CHAPS, pH 6.0
10.	100 mM triethanolamine, 50 mM LiCl, 5 mM EDTA, pH 8.5
11.	100 mM sodium acetate, 100 mM glutamine, 10 mM DTT, pH 5.5
12.	100 mM sodium acetate, 100 mM KCl, 0.1% *n*-octyl-β-D-glucoside, pH 5.5
13.	100 mM HEPES, 1 M $MgSO_4$, pH 7.0
14.	100 mM HEPES, 50 mM LiCl, 0.1% CHAPS, pH 7.0
15.	100 mM KH_2PO_4/K_2HPO_4, 2.5 mM $ZnCl_2$, pH 4.3
16.	100 mM Tris, 50 mM NaCl, 5 mM calcium acetate, pH 7.6
17.	100 mM triethanolamine, 50 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$, pH 8.5
18.	100 mM Tris, 100 mM KCl, 2 mM EDTA, 1% Triton X-100, pH 8.2
19.	100 mM sodium acetate, 1M $MgSO_4$, pH 5.5
20.	100 mM Tris, 2M NaCl, 0.1% *n*-octyl-β-D-glucoside, pH 7.6
21.	100 mM Tris, 1 M $(NH_4)_2SO_4$, 10 mM DTT, pH 8.2
22.	100 mM sodium acetate, 50 mM LiCl, 5 mM calcium acetate, pH 5.5
23.	100 mM HEPES, 100 mM sodium glutamate, 5 mM DTT, pH 7.0
24.	100 mM triethanolamine, 100 mM sodium glutamate, 0.02% *n*-octyl-β-D-glucoside, 10% glycerol, pH 8.5
25.	100 mM Tris, 50 mM NaCl, 100 mM urea, pH 8.2
26.	100 mM triethanolamine, 100 mM KCl, 0.05% dextran sulfate, pH 8.5
27.	100 mM KH_2PO_4/K_2HPO_4, 50 mM $(NH_4)_2SO_4$, 0.05% dextran sulfate, pH 6.0
28.	100 mM HEPES, 50 mM LiCl, 0.1% deoxycholate, pH 7.0
29.	100 mM Tris, 100 mM KCl, 0.1% deoxycholate, 25% glycerol, pH 7.6
30.	100 mM potassium acetate, 50 mM NaCl, 0.05% dextran sulfate, 0.1% CHAPS, pH 5.5

the range where proteins are stable. The second is a list of salts for achieving a full range of chaotropicities. The third is a list of additives that sometimes improve protein solubility due to either binding effects or their effect on the nature of the solution. In a random selection, a buffer from list 1 was combined with zero or one item from list 2 and zero to two items from list 3. Combinations were inspected to eliminate incompatibilities and to ensure bias toward their likelihood of yielding folded soluble proteins. Salt concentrations were varied over a range appropriate for giving folded, soluble protein, and additive concentrations were set either at the high end of the range we have found effective or at the concentration we have most commonly used in assays and purification. We limited the final set of buffers to 30 (Table I), which we have found to be a manageable number for one experiment of about 3 h duration. To apply the sparse matrix we devised a two step procedure. In the first step, a soluble fraction of cell lysate is made in each of the 30 buffers. In a second step, the results from the first step are analyzed to suggest a further set of test buffers with which to complete the optimization.

Using the sparse matrix

Step 1: the 30 buffers

A 600 ml culture of *E.coli* containing the overexpression vector is grown, and expression of the foreign protein production is induced. Before and after induction 1 ml of cells are removed, collected by centrifugation and the pellet is resuspended and boiled in 100 µl 1% SDS, 2 M urea, 1.25% 2-mercaptoethanol, 2.5% glycerol, 15 mM Tris, pH 6.8. This treatment results in complete protein solubilization, allowing assessment of the total amount of overexpressed protein generated in the experimental sample. After induction is complete, the cells from the 600 ml culture are collected by centrifugation. The cells are resuspended in 30 ml 10 mM Tris, pH 8.5, 100 mM NaCl and 1 mM EDTA. The total volume of the suspension is typically 32–33 ml. The suspended cells are divided into 30 1 ml portions in 1.5 ml microcentrifuge tubes and are again pelleted by centrifugation. This step serves to wash the cells and to apportion them for treatment with the 30 buffers. When the wash step is omitted, solubility in each of the 30 buffers is more variable, and the results of step 1 are less reproducible (data not shown). The 30 pellets are then suspended in 1 ml of one of the buffers in Table I. Lysozyme is added to each sample and the suspensions are incubated on ice for 5 min. The cells are then disrupted by sonication. After incubation at 4°C for about 10 min with gentle mixing, the extracts are centrifuged at 16 000 *g* for 10 min to obtain a soluble fraction.

The result of this procedure is 30 1 ml crude cell extracts that contain variable amounts of the overexpressed protein of interest. Equal volumes of each sample are run on SDS polyacrylamide gels and the samples are compared to determine the amount of the overexpressed protein extracted. Although we performed densitometry on some gels in this study, we have found that simple visual inspection is sufficient for the comparison. As controls, the complete extract made in denaturing conditions before and after induction of gene expression are also analyzed on the polyacrylamide gels. The loadings of these samples are adjusted to represent the same volume of initial culture medium as the test samples.

Step 2: optimization

In a second step a less random, more systematic approach is taken. From a careful study of the polyacrylamide gels from step 1, the effect of the various buffer components is assessed. Using the components that seem to promote solubilization and avoiding conditions giving obvious negative effects, a second set of buffers is designed that contain reassortments of apparently helpful components. The new set of buffers along with the best one or two of the buffers from the original 30 are then used in a second experiment following the same procedure as the first. This two step procedure usually yields a buffer formula giving much improved solubility.

We have applied this approach to a number of proteins. All example experiments were done with proteins expressed in *E.coli*, although the same matrix should be useful with other expression hosts. We shall describe in detail the use of the matrix to improve the solubility of a C-terminal section of human α-tubulin (residues 309–451). This section of tubulin exhibits expected properties of a functional, independently folding domain (Mandelkow *et al.*, 1985; Nogales *et al.*, 1998). When expressed as a recombinant polypeptide in *E.coli,* the α-tubulin fragment at first appeared to be mostly insoluble (Figure 1).

From inspection of SDS polyacrylamide gels resulting from step 1 (Figure 2), we deduced that buffers 25 and 26 gave superior solubility, although others including 1, 2, 4, 7, 10, 18 and 29 gave better than average solubility. The total amount of tubulin fragment produced can be estimated from the extract made under denaturing conditions (Figure 2, lane B). Although the 30 extracts varied widely in how much tubulin domain they contained, the majority solubilized at least some of the protein. The identity of the solubilized band was confirmed by Western blot (data not shown). No buffer solubilized all of

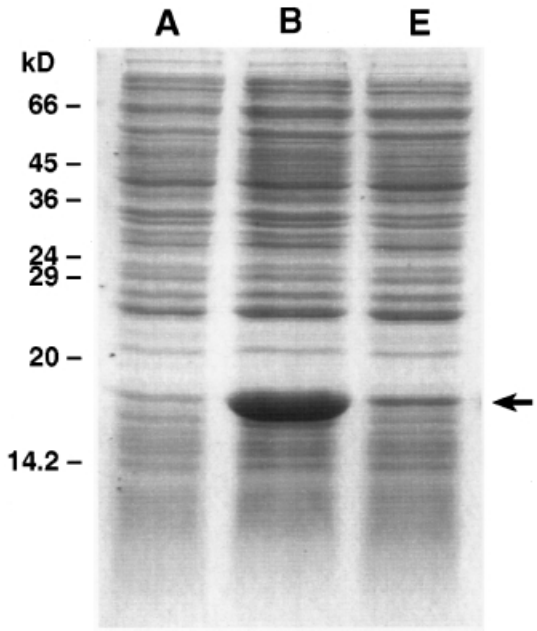

Fig. 1. Initial solubility of the C-terminal domain of human α-tubulin. Lanes A and B, fully solubilized cell extracts made by boiling samples in 1% SDS, 2 M urea, 1.25% 2-mercaptoethanol, 2.5% glycerol, 15 mM Tris, pH 6.8 (SDS polyacrylamide gel loading buffer). Lane A, before induced expression of tubulin domain; lane B, after induction of tubulin domain expression. Lane E, crude extract made with a buffer widely employed for purification and storage of tubulin (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8). An arrow marks the position of the α-tubulin fragment band. The construction of a plasmid for the expression of the C-terminal section of human α-tubulin in *E.coli* is described elsewhere (Chau *et al.*, 1998). Lysis of *E.coli* is carried out in 1 ml portions in microcentrifuge tubes. 0.05 mg/ml hen egg white lysozyme (Sigma) is added to each sample. Cells are disrupted with a Branson sonicator fitted with a microtip. During sonication the sample is supported in a microtube rack bathed in ice water. Sonication is for 3 min at 40% full power output, 30% duty cycle. Sodium dodecyl sulfate (SDS) polyacrylamide gels are stained with Coomassie blue R250. SDS gel sample loading buffer contains 1% (w/v) SDS, 16 mM Tris pH 6.8, 2.5% (v/v) glycerol, 2 M urea, 1.25% (v/v) 2-mercaptoethanol and 0.0125% (w/v) bromophenol blue.

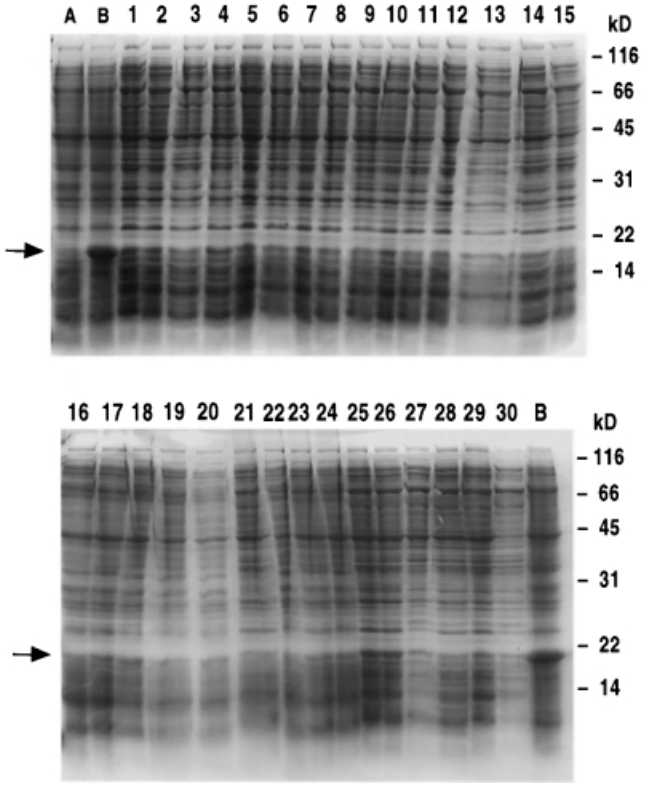

Fig. 2. Solubility in crude extracts of the C-terminal domain of human α-tubulin (step 1). Lanes A and B, fully solubilized cell extracts made by boiling samples in 1% SDS, 2 M urea, 1.25% 2-mercaptoethanol, 2.5% glycerol, 15 mM Tris, pH 6.8 (SDS polyacrylamide gel loading buffer). A, Before induced expression of tubulin domain (negative control); B, after induction of tubulin domain expression (positive control). Lanes 1–30, crude extracts made with each of the buffers 1–30 listed in Table I. An arrow marks the position of the tubulin domain band.

it, but most are more effective than the initial buffer (Figure 1). We attribute this general improvement to the washing of cells before lysis. We have often seen an improvement due to this portion of the procedure alone.

The least effective buffer appeared to be 15, which has a band for α-tubulin fragment that is not appreciably darker than that in the control sample (Figure 2, lane A), made prior to the addition of isopropylthiogalactoside. Buffer 15 is at pH 4.3, and many of the more poorly solubilized samples are at low pH. (The pI of the α-tubulin domain is approximately 4.6.) All buffers that gave better than average solubility were at pH $\geqslant$ 7.0, and this group contains all the buffers at pH greater than 8.0. High pH seems to be able to partially overcome the negative effect associated with nonchaotropic salts. The most successful buffers contained salts that were at least moderately chaotropic. For example, buffer 4 is more effective than buffer 3, and buffer 25, which contains urea, is one of the two most successful buffers. Inspection of the results also suggests that detergents have a general negative effect on the solubility. There seems to be a slight positive correlation with the presence of glycerol and dextran sulfate. No correlation with the presence of divalent cations can be made.

These observations allowed us to plan step 2: the optimization of buffer for making the crude extract. We decided to try seven new buffer combinations based on favorable buffer ingredients. All seven buffers contained Tris at pH 8.5 and either LiCl or KCl. Glycerol, urea and dextran sulfate were tested as additions. These new buffers, which we designated 31–37 to distinguish them from the matrix of 30, are listed in the legend to Figure 3. Step 2 was performed with these new buffers plus buffers 25 and 26 from the original matrix of 30 (Figure 3). All of the buffers gave improved solubility, although none solubilized all the protein produced as judged by boiling in denaturing conditions (lane B). Solubility as a percent of the total expressed tubulin fragment ranged upward from about 60% as judged by densitometry of the SDS polyacrylamide gel. One might choose among the buffers used in step 2 based on experimental considerations other than solubility alone. For purifying this protein we chose buffer 36 (data not shown; Figure 3). We found that neither the LiCl or the urea of buffer 36 was necessary to retain solubility after the protein was partially purified. The chaotropic agents in buffer 36 seem not to have harmed the protein preparation. Since this α-tubulin domain has no inherent enzymatic activity and will not polymerize to form microtubules, we have no enzymatic assay for its native fold; however, the purified protein elutes from a sizing column as a single peak and binds to the microtubule associated protein tau (Chau *et al.*, 1998), indicating that it does retain characteristics of a folded domain.

The generalizations about solubility made for the C-terminal fragment of α-tubulin are specific to that protein; we find that

each protein has a unique set of solubility characteristics. To illustrate, we made crude extracts with the 30 buffers for five other proteins. The proteins tested were the human transcription factor CTF-1, the α and β subunits of avian sarcoma virus reverse transcriptase, the p25 domain of the cyclin dependent kinase activator p35 and the neuron-specific cyclin dependent kinase 5 (cdk5). These have all shown poor solubility when expressed in *E.coli*. The solubility of all the proteins was improved in some of the buffers. We were able to solubilize most of the reverse transcriptase β subunit, virtually all of the p25 kinase, more than 50% of the CTF-1, about 20% of the cdk5 kinase, and a small amount of the reverse transcriptase α subunit (data not shown).

The solubility characteristics of all these protein are summarized in Table II. Both the α-tubulin C-terminal fragment and CTF-1 showed marked variation in solubility with pH or with the chaotropicity of the salt, but neither of the ASV RT subunits did. Instead, solubility was more dependent on the concentration of salt in the buffer and on the presence of detergents. It is interesting to note that the two reverse transcriptase subunits, which share a large portion of their sequence, have some noticeable differences in their apparent solubilities. CTF-1 extracted in the best of the 30 buffers was able to bind its DNA recognition site in a gel mobility shift assay. The ASV RT β subunit showed low but detectable activity in crude extracts, which is a typical result for reverse transcriptases.

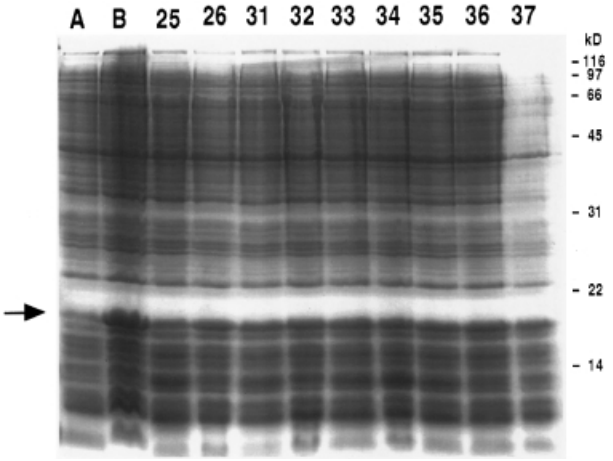

Fig. 3. Solubility test recombining components from buffers that showed increased solubility for the C-terminal domain of α-tubulin (step 2). An arrow marks the position of the tubulin domain band. Lanes A and B, fully solubilized cell extracts made by boiling samples in 1% SDS, 2 M urea, 1.25% 2-mercaptoethanol, 2.5% glycerol, 15 mM Tris, pH 6.8 (SDS polyacrylamide gel loading buffer). Lane A, before induced expression of tubulin domain; lane B, after induction of tubulin domain expression. Numbered lanes: cell extracts made with buffers containing components that showed increased solubility in the experiment in Figure 1. Lanes 25 and 26, buffers of the same number from Table I. Lane 31, 100 mM Tris, 100 mM LiCl, pH 8.5. Lane 32, 100 mM Tris, 100 mM KCl, pH 8.5. Lane 33, 100 mM Tris, 100 mM KCl, 100 mM urea, pH 8.5. Lane 34, 100 mM Tris, 100 mM KCl, 10% glycerol, pH 8.5. Lane 35, 100 mM Tris, 70 mM LiCl, 10% glycerol, pH 8.5. Lane 36, 100 mM Tris, 100 mM LiCl, 100 mM urea, pH 8.5. Lane 37, 100 mM Tris, 100 mM LiCl, 0.05% dextran sulfate, pH 8.5.

The cdk5 kinase and its activator p25 provide examples of enzymes normally soluble in eukaryotic cytoplasm but only marginally soluble when expressed in *E.coli.* Crude extracts of both kinases made in the best of the 30 buffers could phosphorylate appropriate substrates. p25 showed a dramatic range of solubility, from virtually complete solubilization to nearly complete insolubility with the matrix of 30 buffers. Interestingly, the presence of calcium consistently improved its solubility. In contrast, only a modest amount of cdk5 was solubilized in even the best of the 30 buffers. Although detergents improved its solubility, the effect was very specific. Buffers containing triton were among the best for cdk5 while buffers containing CHAPS were consistently among the worst. Neutral to nonchaotropic salts were best for both of the kinases. For all the cases summarized in Table II, it is interesting to note the individuality of each protein's behavior. The effect of the additives listed in Table II ranges through nearly all possibilities in this modest set of test cases.

Discussion

The most striking observation from the application of the sparse matrix of 30 buffers to the six example proteins is how differently each protein behaved. This variation shows why a sparse matrix approach is useful. By trying conditions from a well designed sparse matrix simultaneously a large amount of information about a given expression vector can be obtained in a single day's experiment. One might wonder whether the protein solubilized by this technique is prevented from aggregating by some buffers or whether these buffers are capable of extracting protein from aggregates formed before cells are disrupted. Although the number of proteins we have sampled is small, we think the state of the protein before cells are broken also varies.

The example applications illustrate that, although the sparse matrix approach is often helpful, it does not by itself always lead to good recovery of the overexpressed protein. In the case of the reverse transcriptase α subunit or of ckd5, only a small fraction of the protein was solubilized. Perhaps in these cases, before cells are disrupted a large part of the protein is present in a denatured state in true inclusion bodies. Further,

Table II. Favorable conditions for the solubilization of the six test proteins

General conditions	α-Tubulin domain	CTF-1	ASV RT α	ASV RT β	p25	cdk5
Best pH	high, 8.0–8.5	moderate, 7.0–8.0	indifferent	indifferent	⩾ 6.0	7.5–8.5
Best salt	chaotropic	nonchaotropic	indifferent	indifferent	neutral to nonchaotropic	neutral to nonchaotropic
Best salt concentration	low	low	low	high	indifferent	low
Effect of selected additives[a]						
Detergent	–	–	+	0	–	+/–
Glycerol	+	0	+	+	+	0
Dextran sulfate	0	–	–	0	0	+

[a]+, Increased solubility; –, decreased solubility; 0, little or no effect;
+/–, Variable effect—see text.

the results obtained may be dependent on culture conditions in subtle ways. For example, the solubilization we achieved for the α-tubulin fragment appears to be dependent on the strain of *E.coli* used for expression. Only three out of four strains we surveyed gave solubility with the best buffers found (data not shown). In difficult cases, it may be necessary to combine this sparse matrix with other techniques that can improve solubility. For example, growth of the bacterial host at temperatures lower than 37°C occasionally improves solubility (Wulfing and Pluckthun, 1994), as does varying the length, conditions and timing of induction (Sawyer *et al.*, 1994). Success has also been reported with the co-expression of chaperonins (Caspers *et al.*, 1994; Kim *et al.*, 1998).

We have sometimes been able to take advantage of a protein's tendency to insolubility by using a first extraction in a buffer that does not solubilize the protein of interest, followed by a second extraction of the initially insoluble material in a buffer selected for maximum solubility of the target protein. When following this route, the optimal solubilization buffer is not always the same as for extracts of whole cells. It is sometimes necessary to adjust the concentrations of buffer components for this second extraction, but we have found the results of the sparse matrix procedure are always useful in designing the final buffer. A simple explanation for these observations is that the state of the protein in undisrupted cells also varies from case to case.

The application of this sparse matrix approach to solubilization may lead to the use of unusual buffers. It is often assumed that buffers for biochemistry should be at a pH near 7.0 with 100–150 mM NaCl. The inside of a cell, however, does not resemble the dilute conditions usually used for biochemical analysis but resembles more closely protein in a crystalline state. Proteins exist inside a living cell in a solution with non-ideal properties (Minton, 1981) which may actually be mimicked reasonably well by buffers containing various 'non-physiological' salts and additives (Minton and Wilf, 1981). We often find it necessary to manipulate solution conditions to achieve our experimental goals, and we should view the use of a buffer deduced by this method in context with other manipulations that lead to accepted results. For example, crystallographers subject proteins to unusual solution conditions in order to obtain crystals, but it is generally accepted that the protein visualized as a result of X-ray diffraction is a reasonable representation of the same protein in its native environment.

The method of analyzing solubility by SDS polyacrylamide gels is crude but effective. We often must examine the results carefully to observe all the solubility information they contain. One could be more quantitative by assessing the amount of the overexpressed protein in pellet and supernatant by some other method, for example with antibodies. The gel method, however, has been sufficient in our hands. Gel analysis has the advantage of being possible in all cases, even when antibodies or other means of assay are unavailable or impractical. It also meets our demands for rapid analysis.

In summary, we present a simple and generally applicable procedure, based upon fundamental principles of protein chemistry, designed to assist in identifying solubilization conditions for overexpressed proteins. The utility of this method is that it can be applied by any relatively skilled molecular biologist or biochemist without prior knowledge of the characteristics of the overexpressed protein, or, indeed, without extensive experience in protein chemistry. The sparse matrix induces even the skilled practitioner to solve a solubility problem quickly rather than by an extended set of single variable experiments. We hope that this procedure will be of value in future solubilization efforts and in understanding the range of physical properties exhibited by proteins.

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