Recombinant Proteins Can Be Isolated from *E. coli* Cells by Repeated Cycles of Freezing and Thawing

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Repeated cycles of freezing and thawing are sufficient to separate highly expressed recombinant proteins away from the cellular milieu of *E. coli*. Freezing and thawing liberates recombinant proteins from the bacterial cytoplasm, but does not release the bulk of endogenous *E. coli* proteins. Furthermore, protein secretion is not required. Fractionation of overexpressed proteins by freeze/thaw treatment does not depend on the identity of the recombinant protein and has been observed for thirty-five different recombinant proteins expressed in *E. coli*. These include proteins originally found in plant, animal or microbial sources, as well as several proteins designed *de novo*. Freezing and thawing typically yields ~50% of the recombinant protein in relatively pure form. Thus the freeze/thaw treatment can be utilized as a general method for the isolation of recombinant proteins from *E. coli*.

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Advances in recombinant DNA technology have made it possible to express large quantities of virtually any protein in convenient bacterial hosts[1]. Once expressed, recombinant proteins are typically purified for biochemical or physical studies. While high level expression systems have certainly facilitated protein purification, separating the desired recombinant protein from a background of endogenous bacterial contaminants remains an essential and sometimes difficult task.

Several expression strategies have been used to initially separate a recombinant protein away from cellular contaminants. The three most commonly used strategies are (i) secretion into the periplasm[2]; (ii) formation of insoluble inclusions bodies[3]; and (iii) fusion of the desired protein to a carrier peptide that can be purified by affinity chromatography[4]. The first two methods sequester the desired protein away from the cellular contaminants *in vivo*, while the third method relies on separating the protein *in vitro* after the cells have been lysed. Although all three methods are used extensively to purify recombinant proteins, each of them has limitations: Secretion is not possible for all proteins, inclusion bodies cannot always be refolded into native protein structures, and fusion proteins must be cleaved from their carrier proteins following purification.

Following expression of a recombinant protein in the cytoplasm of *E. coli*, the bacterial cells are typically lysed by enzymatic treatment or mechanical disruption[5]. While such treatment effectively liberates the desired recombinant protein, it also releases the bulk of endogenous proteins and nucleic acids thereby contaminating the recombinant protein with undesired cellular material.

Here we demonstrate that simply freezing and thawing cells releases 40% to 90% of an overexpressed recombinant protein in high purity and without cell lysis. Liberation of the recombinant protein does not depend on the use of extraneous sequences such as signal peptides or fusion proteins, nor does it require secretion or segregation *in vivo*. The treatment is extremely straightforward, appears to be generally applicable, and has been used to isolate from *E. coli* a variety of recombinant proteins ranging from cloned plant and mammalian proteins to artificial proteins designed *de novo*.

Results

Recombinant proteins are released by freezing and thawing of cells. Repeated cycles of freezing and thawing are sufficient to separate highly expressed recombinant proteins away from the majority of endogenous bacterial contaminants. *E. coli* cells expressing the desired protein are grown and harvested as described in the Experimental Protocol. Precipitated cell pellets are frozen and thawed 3 times and the sample is then resuspended in water or a suitable buffer. This simple and gentle procedure liberates a substantial fraction of the over-expressed protein, but does not lyse the cells. Consequently, the bulk of endogenous cellular proteins is easily removed by centrifugation of the remaining cells.

The procedure is general and we have used it successfully to isolate a variety of different recombinant proteins. Among these recombinant proteins are 5 natural proteins, including examples originating from mammalian, plant, and microbial sources (Table 1). In addition, we have used this procedure as a first step in purifying 30 different novel proteins designed *de novo* in our laboratory (Table 1). Figure 1 shows that following the freeze/thaw method, the desired recombinant protein is seen as the predominant (and in some cases, the only) band on Coomassie stained gels. In all cases, the freeze/thaw protocol removes the bulk of cellular contaminants and yields the desired recombinant protein in a form that is relatively pure.

As shown in Figure 1, the method is effective for proteins ranging in size from 8.5 kD (*de novo* protein Rand-Alph #F) to 29 kD (carbonic anhydrase). Furthermore, both monomeric and dimeric proteins can be isolated. The method does not require the use of a particular expression system: protein has been isolated from both the T7 and Tac systems (data not shown). It is not strain-dependent, as equally high yields are obtained for several strains of *E. coli*, including both K stains and B strains[6]. In addition, high yields of protein are obtained following growth in either rich or minimal media (data not shown).

The precise yield of recombinant protein varies somewhat depending on which protein is being studied. In Figure 2 the protein released by the freeze/thaw method is compared to the profile of proteins that remain in the cell. The actual yields for each protein were determined by scanning these or similar gels and are shown on the bottom of the figure. In general, approximately 50% of the overexpressed protein was recovered by the freeze/thaw treatment, and in some cases as much as 90% could be recovered. Given both the relative purity of the desired protein, and the speed and simplicity of the procedure, a 50%

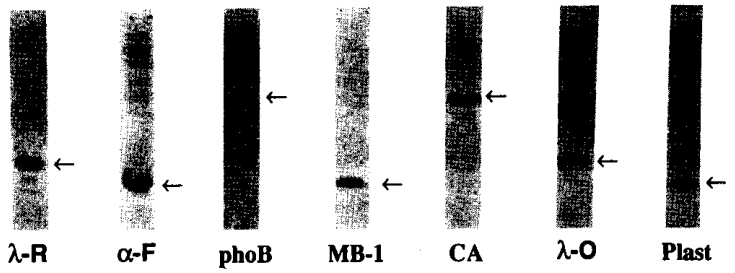

FIGURE 1. Freeze/thaw supernatants. From left to right: λ-Repressor N-terminal domain (λ-R), Rand-Alph-F (α-F), phoB, *de novo* metal binding protein (MB-1), carbonic anhydrase (CA), λ O protein (λ-O), and poplar plastocyanin (Plast). Descriptions of these proteins are given in Table 1.

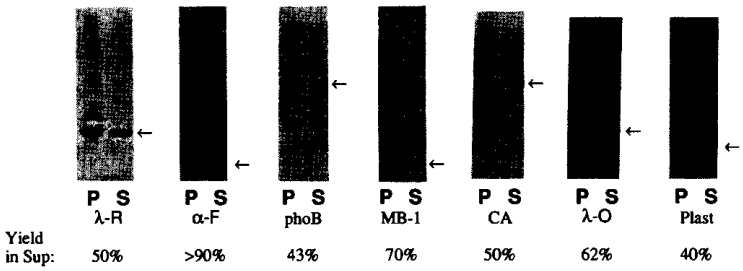

FIGURE 2. Freeze/Thaw supernatants (lanes marked 'S') compared to freeze/thaw pellets (lanes marked 'P'). The concentration of cells was held constant for each supernatant-pellet pair. Proteins are the same as described in the legend of Figure 1 and in Table 1. The percentage of recombinant protein released into the supernatant is given below the corresponding lanes.

TABLE 1. Proteins isolated by the freeze/thaw method.

Protein	Mass	Natural Host	Function
phoB	29,000	*E. coli*	DNA Binding
Carbonic anhydrase	29,000	Human	Hydration of CO_2
λ Repressor N-terminal domain	14,000	Bacteriophage λ	DNA Binding
Plastocyanin	10,700	Poplar leaves	Electron Transport
Lambda O	16,000	*E. coli*	DNA Binding
MB-1	10,000 (Dimer=2×5,000)	*de novo* Design	Metal Binding
Rand-Alph #F*	8,000	*de novo* Design	Model for Design

*Rand-Alph #F is one of 29 different four-helix bundle proteins proteins designed *de novo* using a binary pattern of polar and non-polar amino acids[14]. All 29 proteins were expressed in *E. coli* and all have been isolated by the freeze/thaw procedure described here.

yield is more than adequate for many applications—particularly since it is now fairly routine to express proteins in *E. coli* at extremely high levels. For example, in the case of MB-1, a 70% recovery is sufficient to yield >70 milligrams of pure protein per liter of cell culture[7]. The procedure is effective both for analytical and for preparative scale isolations.

The freeze/thaw procedure is extremely gentle. Since samples are kept cool and are not subjected to chemically or mechanically harsh treatment, proteins will typically be isolated in native and active forms. This was demonstrated explicitly for recombinant plastocyanin, which binds copper and displays native spectroscopic features following its isolation by the freeze/thaw treatment[6].

The bulk of endogenous *E. Coli* proteins are not released by the freeze/thaw procedure. The release of overexpressed proteins by a simple freeze/thaw protocol prompted us to question the extent to which our freeze/thaw fractions are contaminated by endogenous *E. coli* proteins. In particular, might the freeze/thaw protocol lead to complete cell lysis and thereby allow wholesale leakage of cytoplasmic proteins?

Two lines of evidence demonstrate that the freeze/thaw method does not lead to wholesale release of endogenous cytoplasmic proteins. First, by comparing the profile of proteins released by the freeze/thaw method (Lanes marked 'S' in Fig. 2) with those left behind in the precipitated cells (marked 'P' in Fig. 2), it is evident that in each case, the freeze/thaw supernatant is significantly enriched for the recombinant protein. Indeed the desired protein looks to be almost pure in some of these freeze/thaw supernatants. The vast majority of *E. coli* proteins are left behind in the precipitated cells. If total cell lysis had occurred, we would have expected to see many different *E. coli* proteins in the freeze/thaw supernatants. However, this is clearly not the case; the bulk of bacterial proteins simply do not escape into the freeze/thaw supernatants.

The second line of evidence arguing against wholesale leakage of endogenous *E. coli* proteins was provided by comparing the β-galactosidase activity liberated by the freeze/thaw method with the β-galactosidase activity left behind in the precipitated cells. The results of these assays are summarized in Table 2. In all cases, the vast majority of β-galactosidase activity remains in the precipitated cells and very little activity is detected in the freeze/thaw supernatants. These results demonstrate that wholesale leakage of endogenous *E. coli* proteins into the freeze/thaw extracts is relatively minor and cell lysis does not occur.

The freeze/thaw method releases proteins from the bacterial cytoplasm. Does the freeze/thaw method release recombinant proteins from the bacterial cytoplasm, or does it merely release proteins that had been localized into the periplasm *in vivo*?

To demonstrate that the recombinant proteins liberated by the freeze/thaw method originated in the bacterial cytoplasm and that secretion is not required, we compared the profile of proteins from the freeze/thaw extracts with those liberated by a mild spheroplasting procedure that selectively releases periplasmic proteins. Figure 3 shows three representative examples including a designed protein (MB-1), a plant protein (plastocyanin), and an *E. coli* protein (λ-repressor's N-terminal domain). In each case the recombinant protein was released by the freeze/thaw procedure ("F/T") and not by the spheroplasting procedure, which liberates only periplasmic ("PR") proteins. These results demonstrate that the recombinant protein must originate in the intracellular cytoplasm. As a control, cytochrome b562, which is synthesized with a signal sequence and is known to be secreted into the bacterial periplasm[8,9], is readily released by both methods (first two lanes in Fig. 3). These results demonstrate that the freeze/thaw method can release recombinant proteins expressed in the bacterial cytoplasm, and that secretion is not required.

Comparison with traditional methods of cell lysis. A variety of methods have been used in the past to separate a desired soluble protein away from cellular contaminants. Typically, these methods involve total lysis of bacterial cells followed by

removal of insoluble cellular debris by centrifugation. Since cell lysis releases virtually all soluble proteins, the desired protein is typically contaminated with large amounts of unwanted cellular proteins.

Traditional methods of cell lysis include (i) French press, (ii) sonication, and (iii) enzymatic lysis. The proteins released by these methods are compared to proteins released by the new freeze/thaw method in Figure 4. As can be seen by comparing lanes 2-4 with lane 5, traditional lysis procedures release a vast array of cellular proteins while the freeze/thaw method (lane 5) yields an extract that is substantially enriched in the recombinant protein of interest. Thus, as an initial step towards the purification of recombinant proteins, the freeze/thaw method seems far more appealing than traditional methods of total cell lysis.

Discussion

We have demonstrated that highly expressed recombinant proteins can be separated from the bulk of endogenous *E. coli* proteins by a simple freeze/thaw procedure. The desired recombinant protein is released in a state that is surprisingly pure for such a straightforward procedure. Since the vast majority (both in type and quantity) of endogenous bacterial proteins are not released, the freeze/thaw procedure represents an attractive alternative to traditional methods for isolating recombinant proteins from bacterial cells.

Many different methods are commonly used to release recombinant proteins from bacterial cells[5]. The yield and purity of recombinant protein that each method releases depends on the type of damage inflicted on the bacterial envelope. Methods that destroy the bacterial envelope lead to complete cell lysis and thereby contaminate the desired recombinant protein with endogenous cellular material (see lanes 2–4 of Fig. 4). Conversely, methods that merely disrupt the outer membrane while leaving the inner membrane intact release periplasmic proteins selectively, but cannot be used to isolate cytoplasmically expressed recombinant proteins. The freeze/thaw treatment described here is unusual in that it neither releases the entire contents of the cell as is the case for total lysis, nor is it limited to the selective release of periplasmic proteins as is the case for outer membrane disruption.

The mechanism responsible for the release of proteins in the freeze/thaw treatment has not yet been elucidated. However, in attempting to understand the operative mechanism, we must consider both the state of the cell envelope following the freeze/thaw treatment and the state of the recombinant protein following high level expression.

The cell envelope is damaged by repeated freezing and thawing[10]. Studies using both intact cells and cell-free systems have demonstrated that repeated cycles of freezing and thawing disrupt the integrity of membranes and can lead to the formation of transient pores[11,12]. Thus, the freeze/thaw treatment of *E. coli* cells can liberate recombinant proteins without causing total destruction of the cell. Since the freeze/thaw treatment releases neither the bulk of cellular proteins (Figs. 2 and 4), nor β-galactosidase activity (Table 2), nor the chromasomal DNA (data not shown), it is clear that complete cell lysis does not occur.

Why are highly expressed recombinant proteins preferentially released while the bulk cell contents are not? What properties of highly expressed recombinant proteins allow them to escape into the surrounding medium?

Protein solubility is an clearly important factor governing the release of a protein by the freeze/thaw treatment. For example the Felix protein, which forms inclusion bodies[13], is not released. Felix is similar to the Rand-alph proteins in that they have similar lengths (70–80 amino acids) and were designed *de novo* to fold into four-helix bundle proteins[13,14]. Yet, while 29 soluble variants of the Rand-alph proteins were released by the freeze/thaw treatment, Felix was not. This result is consistent with the formation of small pores in the cytoplasmic membrane, which allow escape of recombinant proteins only if they are soluble at high expression levels. Does the size of a recombinant protein affect its yield in the freeze/thaw procedure? Within the molecular mass range between 8 kD and 29 kD we observed no correlation between size and yield. However, for proteins larger than 30 kD, the yields are relatively low (<20%—data not shown), consistent with the pore size being relatively small.

It is unclear why recombinant proteins are released selectively while endogenous bacterial proteins are not released. However, it seems likely that most endogenous proteins interact

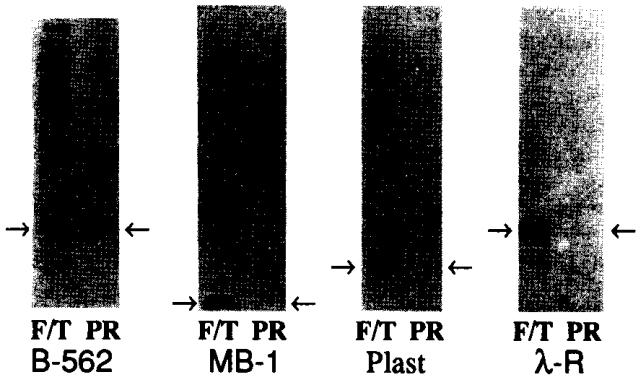

FIGURE 3. Comparison of the freeze/thaw (F/T) method to a gentle spheroplasting method that releases periplasmic (PR) proteins. Cytochrome b-562, which is known to be secreted into the periplasm, serves as a control and is released by both methods. MB-1, Plastocyanin, and λ-Repressor's N-terminal domain are not secreted and are released only by the freeze/thaw method. The concentration of cells was held constant for each pair.

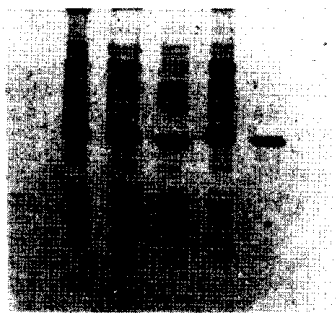

FIGURE 4. Comparison of the freeze/thaw method to various traditional methods for cell lysis. Lane 1—whole cells lysed in gel loading buffer; Lane 2—French pressure lysis; Lane 3—sonication; Lane 4—enzymatic lysis; Lane 5—freeze/thaw fractionation. The protein used in this experiment is carbonic anhydrase.

TABLE 2. β-galactosidase assays.

	β-Galactosidase Units			
Overexpressing Protein	F/T Supernatant	F/T Pellet	Total	Percent in Supernatant
Carbonic anhydrase	18	1675	1693	1.1%
Plastocyanin	51	8815	8866	0.6%
λ Repressor N-terminal domain	0.6	51	51.6	1.2%
Lambda O	2	63	65	3.6%
None (Vector pET-3a)	70	4000	4070	1.8%

β-galactosidase activity was measured in strain BL21/DE3. Activity is expressed in the units of Miller[19].

with larger structures such as ribosomes, membranes, nucleic acids, or multi-enzyme complexes, which are excluded from the relatively small pores.

In summary, we have shown that freezing and thawing cells can release recombinant proteins in good yield and high purity. Release does not require secretion. The secondary and tertiary structure of the protein does not play an important role, since both plastocyanin (all β-strand) and the N-terminal domain of λ-repressor (all α-helical) were released. The only major requirements seem to be that the recombinant protein be (i) moderately sized, (ii) readily expressed at high levels, and (iii) soluble. Since a wide variety of recombinant proteins satisfy these requirements, the simplicity of freeze/thaw procedure coupled with its high yield makes it a widely applicable first step for the isolation and purification of recombinant proteins.

Experimental Protocol

Bacterial strains, expression vectors, and recombinant proteins. *E. coli* B strain BL21/DE3 (F^-, *hsdS*, *gal*, r^-, m^-)[15], and K strain X90/DE3 (*ara*$^-$, Δ*lac-pro*, *nalA*, *argEam*, *rif*^{R}, *thil*$^-$, [F′, *lacI*^{q}, *pro*$^+$])[16] were used in this work. The lysogen DE3 is a λ derivative that expresses T7 RNA polymerase under lac control[15,17]. The recombinant proteins and the vectors used to express them are summarized in Table 1. The proteins used in this study include natural proteins originally derived from plant, animal, or microbial sources, as well as 30 different proteins that were designed *de novo*. Among these novel proteins are 29 different sequences designed to form four helix bundles[14] and MB-1, a novel protein designed to bind divalent metal ions[7].

The freeze/thaw method. *Cell growth:* Proteins were expressed in *E. coli* either in strain BL21/DE3 or X90/DE3. Cells were grown at 37°C in the presence of 100 μg/ml ampicillin. When cells had reached an OD_{600} between 0.8 and 1.0, expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Cells were grown for an additional three hours and harvested by centrifugation. *Analytical scale:* One to three milliliters of cell culture were centrifuged in a 1.7 ml microfuge tube. (3 ml were harvested by 2 successive spins of 1.5 ml.) The supernatant was removed with a Pasteur pipette. Pelleted cells were frozen by submerging the microfuge tube in a dry-ice/ethanol bath for 2 minutes. The sample was then thawed by transferring it to an ice/water bath for 8 minutes. (Thawing was done at 0°C rather than at elevated temperatures to minimize possible degradation of sensitive samples.) This cycle was repeated 2 additional times. 50 to 75 μl of resuspension buffer (see below) were then added to the sample. The pellet was resuspended by tapping the tip of the microfuge tube for 30 seconds. The microfuge tube was not vortexed, agitated, nor mixed in any other manner. A muddy brown mixture indicated successful resuspension. The mixture was then placed in the ice/water bath for an additional 30 minutes. The sample was centrifuged at 4°C for 10 minutes at 10,000×g. The supernatant, containing the overexpressed protein, was carefully pipetted off from the pellet. *Preparative scale:* Ten l of cell culture were spun down in six 500 ml centrifuge bottles via multiple centrifugations. Pelleted cells were frozen by submerging the bottles in a large dry-ice/ethanol bath for 10 minutes. Samples were then thawed by transfer to an ice/water bath for 15 to 20 minutes. This cycle was repeated 3 times. 40 ml of elution buffer were then added to each centrifuge bottle and the pellets were resuspended using a glass pipette. Samples were than placed in the ice/water bath for another 60 minutes. Periodically the samples were agitated to facilitate complete mixing. Samples were then centrifuged at 6,000×g for 15 minutes at 4°C. The supernatant containing the protein of interest was then carefully decanted away from the pellet. *Resuspension buffers:* Although we typically resuspended the freeze/thaw pellets in water, the procedure works equally well using standard buffers such as Tris/HCl (20 mM, pH=8) or sodium phosphate (20 mM, pH=7). Furthermore, salt (200 mM NaCl) can be included in the buffers or left out. Since the procedure itself does not depend on the composition of the buffer (data not shown), the choice of resuspension buffer is dictated solely by the solubility and stability properties of the recombinant protein being isolated.

Periplasmic fractionation. For stringent periplasmic sub-fractionation via spheroplasting, we developed a procedure based on the method of Birdsell et al.[18]. Cells were grown and harvested as described above. Cell pellets were resuspended in 50 mM Tris HCl (pH=7.4) in a volume 1/10 of the original cell culture. Lysozyme was then added to a final concentration of 2 μg/ml. Following a 5 minute incubation at room temperature, EDTA was added to a final concentration of 1 mM. After incubation for an additional 10 minutes, spheroplasts were removed by centrifugation at 10,600×g for 10 minutes at 4°C. Periplasmic proteins remain in the supernatant.

Cell lysis. *French pressure cell:* Cells were grown and harvested as described above. Cell pellets were resuspended in a lysis buffer containing 50 mM Tris HCl (pH=7.4), 200 mM NaCl, 1 mM EDTA in a volume 1/5 of the original cell culture. The cell slurry was passed through a French pressure cell twice at a pressure of 16,000 PSI. Insoluble cell debris was removed by centrifugation at 10,600×g for 10 minutes at 4°C. *Sonication:* Cell pellets were resuspended in the same lysis buffer described above in a volume 1/10 of the original cell culture. The suspension was sonicated (Heat Systems Ultrasonics, Model W-385) at 0°C over 10 minute intervals pulsed at alternating cycles of 60 seconds on and 60 seconds off. Sonication was continued until cells were >90% lysed. *Enzymatic lysis:* Whole cell lysis was done using the lysozyme procedure described by Studier et al.[17].

Gel electrophoresis. Samples were electrophoresed on a Pharmacia PhastGel System using 20% homogeneous gels and SDS buffer strips. Gels were stained with PhastGel Blue R (Pharmacia). Gels were scanned using an Apple One Scanner connected to a Macintosh LCIII. The scanning program was OFOTO 1.0.1. Individual bands were quantitated using the program NIH IMAGE 1.5.1.

β-Galactosidase assays. β-galactosidase assays were performed by the method of Miller[19]. Following the freeze/thaw procedure, β-galactosidase assays were carried out both on the soluble extract (supernatant) and the residual cells (pellet). To assay the soluble extract, 7.5 μl of supernatant were added to 992.5 μl of Z buffer[19]. To assay the residual cells, the pellet was resuspended in 100 μl of 0.5 mM $MgCl_2$, and then 900 μl of Z-buffer were added. In addition, for the pellet samples, 50 μl of 0.1% SDS, and 50 μl of chloroform were added to facilitate the entry of ONPG (ortho nitrophenol β-D-galactoside) into the residual cells. Samples were equilibrated at 28°C for 5 minutes. Reactions were initiated by addition of ONPG, and stopped by the addition of Na_2CO_3. Samples containing resuspended freeze/thaw pellets were spun at 13,000×g for 5 minutes to remove any cell debris prior to analysis. OD_{420} and OD_{550} were measured using a Shimadzu UV 160U spectrophotometer. β-galactosidase activity were calculated as described by Miller[19].

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