Separation of Protein Inclusion Bodies from *Escherichia coli* Lysates Using Sedimentation Field-Flow Fractionation

S. Kim Ratanathanawongs Williams, Gregory M. Raner, Walther R. Ellis, Jr., J. Calvin Giddings

Field-Flow Fractionation Research Center and Department of Chemistry, University of Utah, Salt Lake City, UT 84112, USA

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TRIBUTE TO PROFESSOR J. CALVIN GIDDINGS

I am very fortunate to have had the opportunity to work closely with Professor J. Calvin Giddings for the past 10 years. During this time, he has been both a mentor and a friend. The guidance and freedom that he has given me over the years have greatly impacted my professional growth and research directions. His insightful approaches to scientific problems, his thoroughness, and his good naturedness during times of stress are lessons that will always be remembered.

Cal Giddings approached recreational activities with the same zeal as he did work. Our skiing, river running, and mountain biking expeditions were both enjoyable and intense. The path that Cal inevitably took was the one where all others had failed or avoided because of its difficulty. Just as with work, there were no obstacles that were unsurmountable. They were challenges that could be overcome. Over the years, this has also become my personal philosophy.

I will miss him a great deal.

Kim Ratanathanawongs Williams

Abstract: Sedimentation field-flow fractionation has been used to separate myohemerythrin inclusion bodies from components of growth media, soluble proteins, and unlysed cells that are present in *Escherichia coli* cell lysates. Collected fractions were concentrated and then analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis to confirm the presence of myohemerythrin inclusion bodies and to determine their position in the elution sequence. The fractograms of samples prepared using two different cell lysing methods were compared.

Key words: inclusion bodies; refractile bodies; cell lysates; recombinant proteins; field-flow fractionation

Correspondence to: S. K. R. Williams

Kim Ratanathanawongs Williams has been with Cal Giddings for the past 10 years, first as a postdoctoral research associate and then as an adjunct assistant professor, and the assistant director of the Field-Flow Fractionation Research Center.

Present address: Gregory M. Raner, Department of Biological Chemistry, School of Medicine, University of Michigan, Ann Arbor, MI 48109.

Present address: Walther R. Ellis, Jr., National Center for the Design of Molecular Function, Utah State University, Logan, UT 84322-4630.

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INTRODUCTION

_Escherichia coli_ continues to be the most commonly used host in the expression of recombinant proteins that do not require posttranslational processing (e.g., glycosylation or prenylation). Frequently, heterologous expression levels of 10–15% of the total cell protein can be achieved. This circumstance has led to a high level of interest in the development of more efficient strategies for the purification of recombinant proteins from a crude cell lysate. Much of the work reported to date [1] has focused on new stationary supports for liquid chromatography, including immobilized metal affinity chromatography (IMAC) and immunoaffinity methods. Expressions of desired proteins fused to another protein (e.g., β-galactosidase) that provides an affinity “handle” or fused to a histidine-rich peptide for IMAC [2] have also been reported. High-performance liquid chromatography (HPLC) and free zone capillary electrophoresis have been used to separate human growth hormone from _E. coli_ cell paste extracts [3, 4].

The expression of foreign, particularly eukaryotic, genes in _E. coli_ frequently results in the formation of inclusion bodies (IBs), which consist of insoluble aggregates containing denatured heterologous and host proteins [5–8]. The initial cell disruption step may involve either milling with glass beads, osmotic shock/detergent treatment, chemical (e.g., acetone) treatment, mechanical shearing (via the use of a Manton–Gaulin homogenizer or French press), or ultrasonication. Typically, the IB recovery procedure involves several cycles of centrifugation and pellet resuspension in order to separate the IBs from soluble components and insoluble cell debris [5, 9, 10].

Preparative scale recovery of IBs is achieved using disk stack centrifugation [11–13]. Analytical and laboratory scale techniques include disk centrifugation, photodensitometry [11, 13], crossflow filtration [14], and electrical zone sensing [8]. Disk centrifugation can be very time consuming if the particulate matter is small and of low density. Middelberg et al. [15] estimated that a 0.01-μm particle can take up to 60 h to reach the detector of a Joyce–Loebel disk centrifuge. Using the homogenous start technique and a scanning detector head, the analysis time can be drastically reduced but with an accompanying loss in resolution. Even so, a reasonably well resolved analysis of 10–500-nm particles with a density of 1.3 g/mL could take of the order of hours. Fouling is a potential problem with crossflow filtration and electrical zone sensing.

Field-flow fractionation (FFF) is a family of techniques that is used to separate and characterize macromolecules, colloids, and particulates [16–19]. Separation takes place in a thin rectangular channel with triangular ends. Under conditions of laminar flow, a parabolic flow profile is established across the channel thickness, as shown in Figure 1. The flow velocity is fastest at the center of the channel and decreases to zero toward the walls. Differential migration is obtained by positioning the various sample components in different flow velocity streamlines. This positioning is accomplished using an external field that is applied perpendicular to the direction of flow. In sedimentation FFF (SdFFF), the external field is generated in a centrifuge. The field interacts with each sample particle in proportion to its effective mass (true mass minus mass of carrier fluid displaced) Δm. The particles are driven into the slow flowing regions near the accumulation wall by the field. This motion is opposed by diffusive transport away from the wall. At equilibrium, the higher Δm particles, which experience larger forces than lower Δm particles, will be compressed closer to the wall. Consequently, higher Δm particles are displaced downstream at slower velocities than lower Δm particles and will elute later. A theoretical analysis [17, 19] shows that the retention time _t_r of a well-retained particle is related to its equivalent spherical diameter _d_ by Equation (1):

\[
_t_r = \frac{\pi w G d^3 \Delta \rho_p t^0}{36 k T}
\]

where _w_ is the channel thickness, _G_ is the acceleration, Δρ_p is the difference between the particle density and the carrier density, _t^0_ is the channel void time, _k_ is the Boltzmann constant, and _T_ is the absolute temperature. This equation does not take into account steric effects [20, 21] and is for fixed experimental conditions. Equation (1) can be rewrit-

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**Figure 1. Schematic diagram of an FFF channel and the separation mechanism.**

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ten in terms of the effective mass $\Delta m$ or the mass $m$ of the particle:

$$\Delta m = m \frac{\Delta \rho_p}{\rho_p} = \frac{6kBT_r}{wGt^8}$$  \hspace{1cm} (2)$$

where $\rho_p$ is the particle density. SdFFF offers a simple means of isolating inclusion bodies from crude cell lysates. Furthermore, Equation (2) offers a means of measuring the effective mass or mass of inclusion bodies.

In this article, we demonstrate that SdFFF can be used to effect a one-step separation of inclusion bodies from a cell lysate. The samples consist of *E. coli* containing a Cys$^{35} \rightarrow$ Ser$^{35}$, Cys$^{99} \rightarrow$ Ser$^{99}$ myohemerythrin (Mhr), a nonheme iron oxygen carrier found in certain marine invertebrates. This mutant Mhr contains no cysteine residues, yet is exclusively found, as the apoprotein, in inclusion bodies. Using SdFFF, we also demonstrate that two methods of cell disruption, acetone powder formation and ultrasonication, yield cell lysates with different elution profiles and hence mass distributions.

**EXPERIMENTAL**

**Equipment**

Field-flow fractionation. Two SdFFF channels were used. Each was made from a Mylar spacer (with the channel volume cut out) clamped between two Hastelloy C rings [17, 19]. The dimensions of the channel used to do the cell lysate work are 0.0182 cm in thickness, 2.1 cm in breadth, and 89.4 cm tip-to-tip length. The void volume is 3.41 mL. The corresponding channel dimensions used for the latex work were 0.0127, 1, and 90 cm, with a void volume of 1.17 mL.

The assembled channel was placed in a centrifuge basket. Control of the centrifuge speed and data acquisition were accomplished using a PC compatible computer. Other components of the SdFFF instrument included a Kontron Electrolab pump (model 410, London, United Kingdom), a Valco C6W injection valve (Chrom Tech, Apple Valley, MN) with a 30-µL injection loop, and a Spectroflow 757 UV detector (Applied Biosystems, Foster City, CA) set at 280 nm.

The different stages of SdFFF analysis consist of sample injection, stopflow, elution, and detection. Sample is first injected and swept into the channel tip. This is followed by the stopflow stage in which the flow of carrier liquid is temporarily rerouted to bypass the channel and allow time for the particles to form equilibrium distributions at the accumulation wall. At the end of this stopflow stage, the liquid flow is directed through the channel and the sample components are separated and transported to the detector.

**Fraction collection and concentration.** Fractions of sample emerging from the detector were collected with an FC-80K microfractionator (Gilson Medical Electronics, Middleton, WI). In order to obtain sufficiently high concentrations for gel electrophoresis, the 5-mL fractions were lyophilized using a SpeedVac concentrator SVC100H (Savant Instruments Inc., Farmingdale, NY) and resuspended in 50–100 µL of 50 mM Tris buffer at pH 9.9.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).** A Mini-Protein II electrophoresis system with a model 200/2.0 power supply (Bio-Rad Laboratories, Hercules, CA) was used to do SDS–PAGE. The procedures used to prepare samples and solutions for SDS–PAGE are described in Current Protocols for Molecular Biology [22]. Fifty microliters of SDS/sample buffer were added to 50 µL of sample and the mixture was heated to 100°C for 1 min. A 15-µL aliquot of the mixture was loaded onto a 15% gel. A limiting current of 25 mA (and ~ 10 V) was employed during the run. Reagents for making the polyacrylamide gel were obtained from the United States Biochemical Corporation (Cleveland, OH). Silver staining was used to enhance the visibility of the protein bands on the gel.

**Transmission electron microscopy.** A droplet of the collected fraction was placed on a Formvar coated 200-mesh Pelco copper grid (Ted Pella, Inc., Redding, CA) and allowed to stand for 1 min. Excess fluid was siphoned off using a piece of filter paper and the grid was allowed to dry. A Philips model 201 (Arvada, CO) transmission electron microscope was used to examine the prepared grids. Typical magnifications were 10,000–20,000 ×.

**Preparation of cell lysates.** *E. coli* K38 containing the Mhr-C plasmid, which codes for Cys$^{35} \rightarrow$ Ser$^{35}$, Cys$^{99} \rightarrow$ Ser$^{99}$ Mhr was propagated in *Luria–Bertani* medium. The plasmid structure and cell culture conditions are described in detail elsewhere [23]. The IB-containing cells were lysed either by sonication or through the preparation of an acetone powder [24, 25]. Using the first method, a vial containing the *E. coli* suspension in *Luria–Bertani* medium was immersed in an ice bath and sonicated for ten 1-min intervals (with 1-min stops in between) at a power setting of 150 watts. The second method of cell lysis involved an initial centrifugation step whereby the *E. coli* cells were pelleted at 8000 rpm for 10 min using a Beckman model J2-21M centrifuge with a JA-10 rotor (Fullerton, CA). The supernatant was decanted and the remaining cell
paste was added to cold acetone (−10 to −20°C) in ca. 1-g aliquots. Powdered dry ice was continuously added to the cell paste suspension which was stirred for 1–2 h. The suspension was filtered onto Whatman #1 paper. The collected solids were resuspended in cold acetone, filtered, washed with fresh cold acetone and ether, and air dried. The sample injected into the SdFFF channel was prepared by suspending 100 mg of the acetone powder in 1 mL of 50 mM Tris buffer (pH 9.9). Freshly made samples were used because of the potential solubilization of the IBs at high pH [26].

Standards and carrier liquid. Polystyrene latex standard beads were obtained from Duke Scientific (Palo Alto, CA). The protein standard mixture, used as SDS–PAGE markers, was purchased from Sigma Chemical (SDS-70L marker kit, St. Louis, MO). This mixture of lyophilized proteins, also known as Dalton Mark VII-6, contained bovine albumin (molecular weight 66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kD), carbonic anhydrase from bovine erythrocytes (29 kD), trypsinogen from bovine pancreas (24 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin from bovine milk (14.2 kD).

The carrier liquid used to effect the separation of the latex beads was doubly distilled deionized water containing 0.1% FL-70 surfactant (Fisher Scientific, Fair Lawn, NJ) and 0.02% sodium azide (Sigma Chemical Co., St. Louis, MO). The carrier liquid used for the cell lysate separations was pH 9.9, 50 mM Tris (United States Biochemical Corp., Cleveland, OH).

RESULTS AND DISCUSSION
Polystyrene latex standards were used to confirm the separation capability of SdFFF in the size range of interest. In Figure 2(a), latex beads with nominal diameters of 0.222, 0.320, 0.398, and 0.596 μm were fractionated using fixed conditions during the run. The flow rate was set at 2.71 mL/min and the rotation rate was held at 1800 rpm throughout the run. The four components are completely resolved from one another. Constant run conditions with high field strengths are used when high resolution is a primary objective. From Equation (1), it is apparent that a 10-fold range in d would result in a 103-fold range in t. The analysis time can be shortened by using a lower field strength (rpm) and/or higher flow rate.

When characterizing samples with broad size distributions, it is necessary to program the field strength (analogous to solvent strength programming in chromatography) to achieve separation in a reasonable analysis time [27, 28]. Figure 2(b) demonstrates the use of a programmed field in the separation of the same latex mixture shown in Figure 2(a). The dotted line traces the change in rpm with time. A high centrifuge speed, used initially to retain the small particles, is reduced according to the power function introduced by Williams and Giddings [29]. This reduces the analysis time from 30 to 18 min. More dramatic reductions in run time are observed for samples with a broader size distribution or when the rpm is reduced at a faster rate.

A cell lysate sample, prepared by the acetone powder method, was fractionated using programmed field SdFFF. The results are shown in Figure 3. The dotted line represents the change in rpm as a function of time. The large void peak appearing at time t0 is due to the elution of unretained soluble pro-
Figure 3. Fractogram of an inclusion body–containing cell lysate prepared using the cold acetone method described in the experimental section. Four fractions (I, II, III, and IV) were collected at the intervals shown. Separation conditions are 1700 rpm programmed to 100 rpm. (Power programming constants were $t_1 = 5.15$ min and $t_2 = 41.2$ min.) The flow rate was 1.01 mL/min and the stopflow time was 12 min.

The carrier liquid used initially in the cell lysate separation was pH 7.2 Tris buffer. No elution was observed, indicating that the lysate sample was adsorbed on the channel accumulation wall. This can be explained by examining the charges on the apo form of the Mhr protein and the FFF channel wall. The isoelectric points of apo Mhr and the Hastelloy C wall are 6.2 and 9.4, respectively [23, 31]. At pH 7.2, the protein and the accumulation wall are oppositely charged, leading to adsorption. By increasing the buffer pH to 9.9, sample loss is averted. In cases where increasing the pH is not an option, a polymethylmethacrylate accumulation wall has been shown to yield good recoveries for biological samples [32].

Fractions were collected at the intervals marked on the fractogram in Figure 3. The fractions were lyophilized, resuspended, and analyzed for recombinant protein by SDS–PAGE. The gel electrophoresis results are shown in Figure 4. Protein markers occupy the last lane on the right-hand side of the figure. Myohemerythrin, with a molecular weight of 13.9 kD, is clearly present in fraction II but could not be detected in any other fraction. This corresponds to the major peak eluting at 10.0 min in the
cell lysate fractogram (Figure 3). Since solubilized proteins would elute with the void peak, these results indicate that the IBs remained intact despite the acetone powder treatment.

Fraction II was collected in the time interval of 9–16 min corresponding to the elution of sample components with $\Delta m$ ranging from $6.30 \times 10^{-17}$ to $1.71 \times 10^{-16}$ g. Transmission electron microscopy of the IB-containing bacteria showed dense IB granules ranging in size from 0.10 to 0.30 $\mu$m. Using the $\Delta m$ obtained by SdFFF and $d$ measured by transmission electron microscopy (TEM), the $\Delta \rho_p$ of the inclusion bodies can be estimated. [Equations (1) and (2) are equated and rearranged to yield the expression $\Delta \rho_p = 6 \Delta m/\pi d^3$.] Assuming the IBs elute in the order of increasing diameter, the $\Delta \rho_p$ ranges between 0.012 and 0.12 g/mL. In distilled deionized water, the density range of the IBs would be 1.01 and 1.12 g/mL. These densities are comparable to those reported by Taylor et al. [8] of 1.034 g/mL for prochymosin and 1.124 g/mL for $\gamma$-interferon IBs based on a combination of electrical zone sensing and centrifugal sedimentation measurements.

In Figure 3, the peak at 45.3 min is attributed to intact $E. coli$. This identification is supported by overlapping the fractograms of unlysed and lysed bacteria, as shown in Figure 5, all of which display a peak at this location. In addition, TEM of fraction IV reveals a large number of whole bacteria.

A comparison of the fractograms in Figure 5 resulting from $E. coli$ that were lysed by sonication and by treatment with cold acetone shows that the peak at 10.0 min is conspicuously missing from the sonicated preparation. The likely explanation is that the IBs have remained attached to the cell membrane debris and thus elute later (spread over a large retention time range) than expected because of the additional mass [9, 33]. The implication is that a more vigorous sonication procedure might be required to completely fragment the cell membrane and liberate the inclusion bodies. (It should be noted that the sonication procedure was performed without removing the $E. coli$ from the fermentation broth. Since the broth consists of relatively low mass components, these components are expected to elute with the void peak.)

In this study, it has been demonstrated that SdFFF can be used to recover myohemerythrin inclusion bodies from cell lysates. The separation of IBs from soluble proteins and intact cells takes 20 min. The field can then be turned off to rapidly flush the remaining components out of the channel in preparation for the next injection of cell lysates. Alternately, the separation can be allowed to go to completion to determine the presence of unlysed cells and thus obtain information about the effectiveness of the cell lysing procedures. Similar experiments can be carried out with cell lysates produced by the Manton–Gaulin homogenizers commonly used in the biotechnology industry. Further experiments need to be done to determine the purity and the percent recovery of the SdFFF separated IBs and to track the elution of cell membrane fragments. Size, mass, and density distributions of these particulate materials can be obtained by coupling SdFFF and flow FFF [34, 35]. The exhibited utility of sedimentation FFF in the recovery of inclusion bodies from recombinant cell lysates clearly warrants further exploration.

REFERENCES