Protein Folding Using a Vortex Fluidic Device

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Abstract

Essentially all biochemistry and most molecular biology experiments require recombinant proteins. However, large, hydrophobic proteins typically aggregate into insoluble and misfolded species, and are directed into inclusion bodies. Current techniques to fold proteins recovered from inclusion bodies rely on denaturation followed by dialysis or rapid dilution. Such approaches can be time consuming, wasteful, and inefficient. Here, we describe rapid protein folding using a vortex fluidic device. This process uses mechanical energy introduced into thin films to rapidly and efficiently fold proteins. With the VFD in continuous flow mode, liters of protein solution can be processed per day with 100-fold reductions in both folding times and buffer volumes.

Key words: Protein Folding, Vortex Fluidics, Continuous Flow, Misfolded Proteins, Inclusion Bodies, Bacterial Protein Expression

1. Introduction

Protein overexpression provides important tools for a wide range of applications ranging from therapeutics to laboratory reagents ***(1-3)***. Bacterial protein overexpression capitalizes upon the low cost, high growth rate, and versatility of *Escherichia coli* ***(4, 5)***. Although many proteins correctly fold during overexpression in *E. coli*, larger or very hydrophobic proteins often generate misfolded and insoluble aggregates, which are directed into inclusion bodies ***(6-8)***. Recovering such aggregated proteins often involves chemical denaturation of the inclusion bodies, then multi-day dialysis to remove the denaturant ***(9, 10)***. An alternative method applies high pressure to drive proteins into their folded states ***(11)***. Illustrative of the wasteful inefficiency of conventional protein folding, liters of buffer are typically used to refold small quantities (mg) of protein ***(12, 13)***. To address this issue, we have developed an approach to harness mechanical energy supplied by a vortex fluid device (VFD) to rapidly fold proteins *in vitro* under standard conditions.

The VFD, a commercially available benchtop device, can drive a range of chemical transformations in its thin films ***(14-19)***. Rapid rotation of a solution in a glass sample tube creates a dynamic thin film with ≈230 μm thickness ***(16)***. Within this film, reagents experience micromixing, shear stress, and vibrational effects ***(19-24)***. At specific rotational speeds, the sample tube enters a harmonic vibration that generates Faraday or pressure waves in the fluid, which can accelerate protein folding ***(25, 26)***.

The method described here is demonstrated with the folding of recombinant hen egg white lysozyme (HEWL) overexpressed in *E. coli.* The insoluble, misfolded protein results from overinduction, and a correctly folded form can be obtained with short VFD processing times. Though folding HEWL is illustrated here, the approach is generalizable to include

other proteins, as demonstrated for cAMP dependent protein kinase A (PKA) and a truncated form of the membrane protein caveolin-1 ***(27)***.

2. Materials

2.1 Buffers

All buffers are prepared at 1 L scale with double-distilled H_2O (ddH_2O) purified to a resistivity of 18 MΩ and filtered through a 0.22 μm filter. Buffers are autoclaved, and then stored at room temperature unless otherwise indicated. All reagents should be at least reagent grade and ACS-certified.

1. Buffer 1: 50 mM NaH_2PO_4 and 500 mM NaCl, pH 8.0. To a 1 L Erlenmeyer flask, add 6.00 g of NaH_2PO_4 and 29.22 g of NaCl. Add approximately 600 mL ddH_2O, and dissolve by magnetic stirring. Adjust the pH to 8.0 using 6.0 M NaOH, and transfer the solution to a graduated cylinder before bringing the volume to 1 L with ddH_2O (see **Note 1**).
2. Buffer 2 (Denaturing buffer): 20 mM Tris-HCl, 10 mM NaCl and 8.0 M urea, pH 8.0. To a 1 L Erlenmeyer flask, add 3.15 g of Tris-HCl, 0.58 g of NaCl, and 480.48 g of urea. Add approximately 300 mL of ddH_2O and dissolve the powders into solution using magnetic stirring. Adjust the pH of the solution to 8.0 using 6.0 M NaOH, and transfer to a graduated cylinder before bringing the final volume to 1 L with ddH_2O (see **Note 2**).
3. Buffer 3 (Elution buffer): 20 mM Tris-HCl, 400 mM NaCl and 8M urea, pH 7.8. To a 1 L Erlenmeyer flask, add 3.15 g of Tris-HCl, 23.38 g NaCl, and 480.48 g of urea. Add approximately 600 mL of ddH_2O and dissolve the powders in solution using magnetic stirring. Adjust the pH of the solution to 8.0 using 6.0 M NaOH, and

transfer to a graduated cylinder before bringing the final volume to 1 L with ddH_2O (see **Note 3**).

4. Buffer 4 (Phosphate-buffered saline): 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, 2.7 mM KCl and 137 mM NaCl, pH 7.2. To a 1 L Erlenmeyer flask add 1.42 g of Na_2HPO_4, 0.27 g of KH_2PO_4, 0.20 g of KCl, and 8.0 g of NaCl. Add approximately 600 mL of ddH_2O and dissolve the powders into solution using magnetic stirring. Adjust the pH of the solution to 7.2 using 6.0 M NaOH, and transfer to a graduated cylinder before bringing the final volume to 1 L with ddH_2O.

2.2 VFD Components and Equipment

1. The VFD, sample tube (20 mm diameter) and delivery jet feeds are purchased from Vortex Fluidic Technologies (www.vortexfluidictechnologies.com).
2. A laboratory syringe pump with the appropriate tubing (e.g., WPI model AL-1000).
3. VFD-sample tube caps: B19 Suba-Seals® (Sigma).
4. Nachi 6005NR open bearings (Nachi-Fujikoshi) (*see* **Note 4**).
5. VFD sample tube lubricant (Dow Corning 976V High Vacuum Grease).
6. All purpose machine oil (Singer).

2.3 Protein Overexpression, Purification, and Analysis

All reagents for protein overexpression should be molecular biology grade and the appropriate measures taken to maintain sterile working conditions.

1. Super optimal broth with catabolite repression (SOC): 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$. 20 mM glucose. To a 1 L autoclavable bottle, add 20 g of tryptone, 5 g of yeast extract, 0.584 g of NaCl, 0.186 g of KCl, 0.952 g of $MgCl_2$ and 2.467 g of $MgSO_4 \cdot 7H_2O$. Bring the solution to ≈1 L with ddH_2O and swirl to dissolve the solid contents. Autoclave the solution at 121 °C for 20 min. Allow the solution to cool to room temperature, add 3.60 g of glucose, and invert the bottle to mix the contents before use.
2. Lysogeny Broth (LB): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl. To a 1 L autoclavable bottle, add 10 g tryptone, 5 g yeast extract, and 10 g NaCl. Bring the final volume to ≈1 L with ddH_2O and autoclave at 121 °C for 20 min. Allow the solution to cool to room temperature, and invert the bottle to mix the contents before use.
3. 40 mg/mL Kanamycin Sulfate: To a 50 mL plastic conical, dissolve 1.60 g of Kanamycin sulfate in approximately 35 mL of ddH_2O. Bring the total volume to ≈40 mL with ddH_2O. Filter the solution with a 0.22 μm syringe filter, and divide the solution into several 1 mL aliquots in 1.5 mL centrifuge tubes. Store the aliquots at -20 °C.
4. Sterile petri dish (Fisher).
5. LB/Kanamycin agar plate: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar. To a 1 L autoclavable bottle, add 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar. Bring the final volume to ≈1 L with ddH_2O and

autoclave at 121 °C for 20 min. Allow the solution to cool to approximately 55 °C and add 1 mL of the 40 mg/mL kanamycin sulfate from above (giving a final concentration of 40 μg/mL) and swirl to mix. Add 20 mL of this mixed solution to a petri dish (1 L of solution is sufficient for 50 petri dishes). Allow the dishes to cool until the agar has solidified (approximately 1 h), and store at 4 °C.

6. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG): In a 50 mL plastic conical, dissolve 5 g of IPTG in 15 mL ddH_2O. Transfer to a graduated cylinder, and add ddH_2O to a final volume of 21 mL with ddH_2O. Filter the solution with a 0.22 μm syringe filter, and divide this into several 1 mL aliquots in 1.5 mL centrifuge tubes. Store the aliquots at -20 °C.
7. The gene encoding for HEWL was purchased from Addgene, and was cloned into a pET28c vector (GE Healthcare).
8. $CaCl_2$ competent *E. coli* BL21(DE3) cells (EMD Millipore)
9. UNOSphere S cation exchange media (Bio-Rad).
10. Kimble-Chase Kontes FlexColumn (Fisher).
11. Pierce™ BCA Protein Assay Kit (ThermoFisher).
12. Circular Dichroism quartz cuvette (Hellma)
13. EnzChek® Lysozyme Assay Kit (ThermoFisher).

3. Method

3.1 ***Overexpression of HEWL in E. coli***

All procedures are performed at room temperature under sterile conditions unless otherwise indicated.

1. Transform a plasmid encoding HEWL into $CaCl_2$ competent *E. coli* BL21(DE3) cells as follows: Add 2 μL of HEWL DNA to 75 μL of competent BL21(DE3) cells. Incubate the mixture on ice for 30 min. Heat shock the mixture at 42 °C for

45 s, and then incubate on ice for two min. To rescue the cells, add 250 µL of SOC media to the mixture, and incubate in an incubator shaker at 37 °C and 225 rpm. for one h. Remove from the shaker, and plate cells (150 µL) on an LB/Kanamycin agar plate. Incubate the plate for 10-12 h in an incubator at 37 °C.

2. Pick a single colony of the transformed cells containing the HEWL vector into 10 mL of LB containing 10 µL of 40 mg/mL kanamycin sulfate (final concentration of 40 µg/mL). Incubate this mixture for 10-12 h in an incubator shaker at 37 °C and 225 rpm.
3. Transfer this 10 mL solution into a 2 L baffled flask containing 1 L of LB and 1 mL of 40 mg/mL kanamycin sulfate (final concentration of 40 µg/mL). Grow the culture in an incubator shaker at 37 °C and 225 rpm until the cells reach an optical density with OD_{600} of 0.8 (≈2.5 h). Then, add 1 mL of the 1 M IPTG (final concentration of 1 mM) and incubate for another 6 h at 30 °C and 225 rpm.
4. Centrifuge the culture at 5524 g for 20 min at 4 °C. Remove the supernatant and discard into 10% bleach. Re-suspend the pellet in Buffer 1 (20 mL) by mixing or pipetting the solution over the pellet, but do not vortex the pellet (*see* **Note 5**).
5. Lyse the re-suspended cells using probe sonication. For this process, incubate the cells on ice, and use eight cycles of 30 s pulses (20 Watt) with 1 min of cooling (without sonication) between cycles (*see* **Note 6**).
6. Centrifuge the viscous solution at 4 °C for 50 min at 30,996 *g* with a floor model centrifuge (eg. Beckman Avanti™ J-25) with a rotor suitable for 50 mL centrifuge tubes (eg. Beckman JA-17).
7. Discard the supernatant and retain the pellet, which contains HEWL in inclusion bodies (*see* **Note 7**). Add 30 mL of Buffer 2 to the pellet, and incubate at 4 °C with

shaking overnight to fully dissolve, denature, and solubilize any remaining protein (*see* **Note 8**).

8. Centrifuge the dissolved pellet at 33,264 *g* for 1 h. Retain the supernatant and discard any remaining pelleted cell debris. The supernatant should now contain denatured HEWL.
9. HEWL may then be purified using UNOSphere S-Cation exchange media in a column by hand. For this procedure, add 3 mL of the resin to a Kontes FlexColumn, and rinse several times with water (1.0 mL/min) and then 3-4 column volumes of Buffer 2 (1.0 mL/min). Incubate the denatured HEWL with the resin for 2 h at 4 °C, and then wash the resin-adhered protein with 50 mL of Buffer 2 **(**1.0 mL/min). Then elute the purified protein from the column using 50 mL of Buffer 3 (1.0 mL/min). SDS-PAGE can be used to determine the purity of the eluted HEWL (*see* **Note 9**).
10. Protein concentration in the eluted solution is then measured by measuring the absorbance at 280 nm (molar extinction coefficient for HEWL of 37970 M^{-1} cm^{-1}) or by using a BCA assay kit. The solution is then either concentrated or diluted (Buffer 3) to ≈4.4 mg/mL using a 10 kDa concentrator. This protein solution can next be folded.

3.2 Refolding HEWL using the VFD

The VFD should be set at an angle of 45° relative to the horizontal position and the bearings should be well oiled (*see* **Note 10**).

3.21 Small Scale Folding of HEWL by confined mode VFD processing (<3 mL)

1. To lower the concentration of urea from the HEWL solution in Buffer 3, disperse 10 μL of the solution in 990 μL of Buffer 4, and mix with a 1 mL pipette.

2. Add the diluted solution (≈44 μg/mL) to a 20 mm external diameter glass sample tube, and seal the sample tube with a Suba-Seal® septum.
3. Insert the sample tube into the VFD. To do this, first apply a thin layer of lubricant around the sample tube. Then, insert the sample tube gently through the upper housing unit, until it is firmly mounted in the housing unit of the lower bearing.
4. Place the safety shield on the device, and rotate the sample tube at a 5 krpm rotational speed for 5 min (*see* **Note 11**).
5. Remove the contents of the tube, and transfer into a 2 mL centrifuge tube containing 100 μL of 100% glycerol. Quickly mix, and flash freeze the sample in liquid nitrogen or a mixture of dry ice and ethanol. Store the sample at -80 °C until analysis (*see* **Note 12**).

3.22 Large Scale Folding of HEWL by Continuous Flow VFD processing

1. The solution of HEWL, currently in Buffer 3 is rapidly diluted into Buffer 4. Disperse 1 mL of the HEWL solution in Buffer 3 in 99 mL of Buffer 4 contained in a 250 mL beaker or flask. Mix this solution rapidly by swirling.
2. As shown in Fig 2, the VFD can be configured for continuous flow operation. Process the solution by flowing through the VFD at a rate of 0.1 mL/min. Collect the solution of folded protein in a beaker or flask on ice.

3.23 Analysis of Refolded HEWL

Circular Dichroism

1. Circular Dichroism (CD) is used to determine the secondary structure of the refolded HEWL samples. The sample (200 or 300 μL) is added directly to a CD cuvette. The spectrum is obtained using a spectropolarimeter such as the Jasco J-

810 spectropolarimeter scanning 20 nm/min over four accumulations. Based on previous published results and crystal structures, the expected secondary structure of HEWL is largely α-helical *(28)* (*see* **Note 13**).

HEWL Activity Assay

2. To verify the protein folding efficacy of the sample, perform a HEWL activity assay. The experiment uses the commercially available Enzcheck Lysozyme Assay Kit. Follow the manufacturer instructions to determine the activity of the refolded samples. A non-VFD-processed sample provides a direct comparison.

4. Notes

1. NaH_2PO_4 can be difficult to dissolve and requires thorough mixing. We recommend using a stir plate, and optionally gentle heating.
2. Add all components of the buffer, apart from the urea, to 300 mL of ddH_2O as this large amount of urea will greatly increase the solution volume. The reaction is endothermic and will require gentle heating and stirring to completely dissolve the salts. The pH of this buffer is temperature dependent; thus, allow the solution to equilibrate at room temperature before adjusting the pH as detailed above.
3. As with Buffer 2, Buffer 3 requires heating with stirring. The pH should again be adjusted after allowing the solution to reach room temperature.
4. The bearings in the VFD need to be replaced every ≈3000 hours. See **Figure 1** for bearing location during replacement.
5. At this point, the re-suspended solution can be stored at -80 °C after addition of 10% glycerol.
6. Other methods of lysing cells such as using a French press are also acceptable.

7. A layer of cell debris can often be observed on the outside of the pellet. This layer can be removed and discarded along with removal of the supernatant.
8. We find it easier to dissolve the pellet in urea by breaking up the pellet into smaller pieces using a sterile spatula. We then transfer the solution into a plastic conical tube to dissolve overnight.
9. If purity is poor after this step, wash steps can be added. Slowly increase the concentration of NaCl in the binding buffer from 0 mM to 50 mM in 10 mM increments. Additionally, size exclusion chromatography can be performed to further purify the protein.
10. As shown in several publications ***(25, 16),*** any deviation from the 45° tilt angle may drastically reduce the efficiency of the VFD. Additionally, the vibrations responsible for driving protein folding are produced by vibrations inherent to the VFD. Such vibrations can be sensitive to small changes in operational parameters, such as tilt angle, rotational speed, sample tube size, and wear on housing unit and bearings. Therefore, device maintenance is critical. For example, the addition of oil (e.g. machine oil, Singer) to the bearings before each use can increase reproducibility.
11. Several factors contribute to the optimal rotational speed for protein refolding, including quality and wear of both bearings and the housing unit. If refolding is not observed, vary the rotational speed of the sample tube in 50 rpm increments around 5 krpm to optimize folding conditions. Additionally, the housing unit and bearings can be replaced easily. The link below contains a 3D printing file for the housing unit (https://grabcad.com/library/vfd-collar-1, **Fig. 1**); high density ABS plastic is sufficient for production of this part.

12. Rapid dilution is commonly used to fold proteins. For this comparison between VFD and non-VFD processed conditions, samples are flash frozen after processing to prevent further conformational changes. For the non-VFD control, identical treatments include dilution of the sample and incubation at room temperature for the same time period as VFD processing (5 min); therefore, a direct comparison can be made with the VFD-processed solution.
13. Use the Dichroweb program to analyze the CD spectrum of the proteins, and assign its secondary structure ***(29)***.
14. The jet feeds must be held firmly in the housing unit lid. If they are not held firmly, then vibration of the jet feeds against the rotating sample tube will cause the glass sample tube to shatter.

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Figure Legends

Figure 1. Schematic diagram of a VFD. A) The VFD and its rotational speed controller unit. The electric motor connects to the controller box and the lower bearing. Both the upper and lower bearings hold the sample tube in place during rotation. B) The sample tube is inserted into the upper bearing. C) The upper bearing of the device showing connection to the housing unit by six screws. D) The housing unit of the VFD. The holder to the continuous flow jet feeds, termed the housing lid, attaches to the housing unit via the screw inserts.

Figure 2. A step-by-step guide for continuous flow protein folding by VFD. A) Insert the 20 mm external diameter sample tube. For this process, we suggest a lubricant be added to the sample tube before applying any force. B) Insert a metal jet feed through the housing lid until ≈75% of the jet feed has been inserted (*see* **Note 14**). C) Add the housing lid onto the housing unit and fasten with plastic screws. Orient the jet feed with the exit facing the lower side of the sample tube. D) Draw the solution of unfolded HEWL into a 50 mL syringe, and vent any air bubbles. E) Insert the syringe first into the jet feed, and then place the syringe into the syringe pump. Select the required flow rate and start the VFD and syringe pump. For the confined mode of operation (1-3 mL), add the unfolded HEWL solution to the sample tube *via* a pipette, and cap with a Suba seal.