telling the program where the tubing should go next (vial number or B for buffer) and how long it should stay there. These robot control programs can be saved on disk and loaded into memory to be executed when required.

Figure 1 shows that two oocyte rigs can be simultaneously perfused by the robot. The Hamilton HV3-3 valve located just above the platform allows for either or both to be perfused with buffer. The robot cannot, however, perform different experiments on each rig simultaneously. The sample tracings obtained using hand vs. robotic transfer of perfusion tubing are shown in Figure 2. In this experiment, *Xenopus* oocyte nuclei were injected with 0.3 ng/30 nL human glycine α_1 receptor subunit cDNA, and the oocytes were treated as described in Mihic et al. (1). Figure 2 shows two sets of tracings using 50 μ M, 75 μ M and 100 μ M glycine, one obtained after transferring the perfusion tubing by hand, and the second using the robot. The peak heights observed were virtually identical.

The use of robotic techniques as well as multiple oocyte rigs allows for more rapid data acquisition, subject of course to the nature of the experiment and the quality of oocytes. It may be especially useful for the automated testing of large numbers of compounds on defined receptors. Furthermore, this inexpensive robotic technique can be used not only for *Xenopus* oocyte perfusion, but in any assay system involving the pumping of various buffers.

A schematic of the rotary table Lucite disk drill holes and the executable code of our robot control software may be freely obtained upon request. The QuickBasic 4.0 source code will also be provided, except for the Arrick Robotics core subroutines, which are provided by the manufacturer upon the purchase of an MD-2 dual-stepper motor system.

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Single-Step Purification/ Solubilization of Recombinant Proteins: Application to Surfactant Protein B

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Metal-chelate affinity chromatography of polyhistidine-tagged proteins is a powerful tool for purification of recombinant proteins from *E. coli*-based expression systems (4). Eukaryotic proteins, however, are frequently not folded correctly when synthesized in prokaryotes resulting in an insoluble, non-native form of recombinant protein, which is usually stored in inclusion bodies. Insoluble, recombinant proteins can be purified by performing Ni-NTA agarose chromatography under denaturing conditions (3). However, attempts to refold the denatured protein have been met with limited success. Refolding strategies have included dialysis and stepwise or contin-

uous removal of the denaturant while the target protein is bound to the resin (1). In this article, we report a single-step purification/refolding procedure based on modification of existing solid-phase solubilization procedures (1,2,7). Lysates of bacteria overexpressing recombinant protein are bound to Ni-NTA resin under denaturing conditions. Solubilization of the bound target protein is accomplished in a single step by omitting the denaturant from the last wash and elution buffers. Immobilization of the target protein during the solubilization step presumably restricts interaction with neighboring molecules, thereby minimizing aggregation while promoting refolding. This procedure has been proven to be faster and more efficient than conventional solubilization procedures such as stepwise dialysis. Further, we were able to solubilize proteins that would not refold in dialysis procedures.

Constructs encoding human pulmonary surfactant protein B (SP-B) (GenBank® Accession No. M24461) proprotein (amino acids 24–381) or a processing intermediate, consisting of the NH₂-terminal propeptide and mature peptide (SP-B_ΔC, amino acids 24–279) were made by polymerase chain reaction (PCR) using the human SP-B cDNA as the template. Only data for the SP-B_ΔC construct are presented since the results were similar for both SP-B constructs. The SP-B constructs were directionally cloned into the bacterial expression vector pProEx-1 (6), (Life Technologies, Gaithersburg, MD, USA) that includes a reiterative cytosine, adenosine and thymidine sequence at the 5' end of the multicloning site resulting in the incorporation of six histidines at the NH₂-terminus of the recombinant protein. The DH10 B strain of *E. coli* (Life Technologies) was transformed, grown in 1 L of bacterial culture (LB medium containing 50 μ g/mL carbenicillin) to an OD₆₀₀ of 0.6, and recombinant protein expression was induced with isopropyl-1-thio- β -D-galactoside (IPTG) at a final concentration of 0.6 mM. Bacteria were harvested after 4 h, lysed by sonication in Tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris-HCl, pH 7.9) and the soluble and insoluble fractions (separated by centrifugation at

Benchmarks

Table 1. Composition of Buffers Used for Purification/Solubilization Studies

Buffer 1	6 M Guanidine	20 mM Tris-HCl (pH 7.9)	500 mM NaCl	4 mM Octylglucoside
Buffer 2	6 M Urea	20 mM Tris-HCl (pH 7.9)	500 mM NaCl	20 mM Imidazole
Buffer 3		20 mM Tris-HCl (pH 7.9)	150 mM NaCl	
Buffer 4		20 mM Tris-HCl (pH 7.9)	150 mM NaCl	50 mM EDTA

The pellet from 1 L of bacterial expression culture was lysed in 50 mL buffer 1, sonicated and centrifuged at 20 000× *g* for 30 min. The supernatant was applied to a column (2.5 cm diameter with a flow rate of 20 mL/h) containing 4 mL of Ni-NTA agarose pre-equilibrated in buffer 1. Washing and solubilization steps were performed with 50 mL of buffers 2 and 3, respectively, and soluble recombinant protein was eluted with 25 mL of buffer 4. Yields of eluted protein were quantitated by the method of Lowry et al. (5), and purity was assessed by SDS-PAGE and silver staining.

20 000× *g* for 30 min) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. The target proteins were found to be in the insoluble fraction (Figure 1A), and subsequent chromatographic procedures were performed under denaturing conditions. Following induction of expression, bacterial pellets were isolated, dis-

solved in buffer 1 (Table 1) and bound to Ni-NTA agarose (Qiagen, Chatsworth, CA, USA), which selectively binds polyhistidine-containing proteins under both denaturing and non-denaturing conditions. The NTA column was washed with buffer 2 and eluted with the same buffer containing 50 mM EDTA. Subsequent removal of urea from the eluted fraction by dialysis

against TBS yielded less than 1% soluble protein (not shown). As a consequence, the purification protocol was amended to include a solubilization step immediately following the denaturing wash step (Table 1) as previously suggested for ligand-binding assays (7). The TBS-solubilization step (buffer 3) resulted in removal of urea without any detectable elution of the recombinant protein (Figure 1B, lane 6). Subsequent elution of the column with buffer 4 resulted in recovery of recombinant protein that was completely soluble in TBS and remained in solution for at least two months (Figure 1B, lane 7). The column was finally washed with buffer 1 containing 50 mM EDTA in order to recover any insoluble aggregates that might have formed during the solubilization step. No recombinant protein was detected in this wash, demonstrating that essentially all recombinant protein that had bound to the NTA resin was successfully solubilized and eluted (Figure 1B, lane 8). This finding was confirmed by purifying equal amounts of bacterial lysate in parallel, either under completely denaturing conditions (i.e., by addition of 6 M urea to buffers 3 and 4 in Table 1) or exactly by the protocol described in Table 1: Yields of recombinant protein were virtually identical for both procedures (7 mg/L of bacterial culture) supporting the efficacy of the solubilization step (data not shown). Although we have not investigated the efficiency of recovery at higher protein loads (i.e., >1.75 mg recombinant protein/mL resin), a significant increase in protein concentration would increase the probability of interactions among neighboring molecules on the column, likely resulting in less efficient solubilization.

Figure 1. Determination of solubility and purification/renaturation of SP-B Δ C. Panel A. Expression of recombinant SP-B Δ C. Lane 1, pellet from 100 μ L bacterial expression culture lysed directly in electrophoresis sample buffer. Lane 2, the pellet from 100 μ L bacterial culture was sonicated in TBS and the insoluble protein (pellet) isolated by centrifugation. Lane 3, TBS-soluble protein (supernatant from sample in lane 2). Lane 4, molecular weight markers as in panel B. SP-B Δ C was detected only in the insoluble phase. Panel B. Solubilization of recombinant SP-B Δ C: The bacterial pellet was dissolved in buffer 1 and subjected to Ni-NTA chromatography, as described in the text and Table 1. Lanes 1 and 9, molecular weight markers; lane 2, pre-induction culture; lane 3, expression culture 4 h after induction with IPTG; lane 4, flow-through fraction from NTA column; lane 5, proteins recovered after washing with buffer 2; lane 6, proteins recovered after solubilization with buffer 3; lane 7, proteins recovered after elution of the NTA column with buffer 4 (SP-B Δ C comprises more than 90% of the eluted protein); and lane 8, proteins recovered by elution of column with buffer 1 containing 50 mM EDTA. A and B: Electrophoresis was performed on 10%–20% SDS-polyacrylamide gels and proteins were detected by silver staining. The identity of SP-B Δ C was confirmed by Western blotting and NH₂-terminal sequence analyses.

Benchmarks

Similar studies were performed with other proteins, including rat thyroid transcription factor 1 (TTF-1) and chloramphenicol acetyltransferase (CAT). CAT, like SP-B and SP-B Δ C, was expressed from pProEx1 and carried the hexahistidine tag at the NH₂-terminus, whereas TTF-1 was expressed from the pET 21b expression vector (Novagen, Madison, WI, USA) resulting in COOH-terminal localization of the hexahistidine tag. TTF-1 was primarily synthesized by bacteria in an insoluble form, whereas CAT was expressed in a soluble form and could be purified under non-denaturing conditions. Dialysis of purified, denatured, recombinant protein resulted in solubilization of approximately 40% of TTF and nearly complete recovery of CAT. In contrast, the single-step purification/solubilization procedure resulted in complete recovery and solubilization of both TTF and CAT. We have used recombinant

proteins prepared by this procedure for generation of polyclonal and monoclonal antibodies, as antigens in ELISA and as substrates for enzymatic cleavage. The biological activity of the refolded recombinant proteins was not assessed, and it is therefore not certain that the solubilization step resulted in native conformation.

The protocol described here is a simple and efficient method for solubilization of His-tagged recombinant proteins. We did not find it necessary to remove the denaturant slowly or gradually as previously suggested (1), thus simplifying the procedure. Solubilization of immobilized recombinant protein was independent of the position of the polyhistidine tag. For certain recombinant proteins (e.g., CAT), conventional solubilization procedures were equally efficient to this "refolding-on-the-column" protocol, whereas other proteins could not be renatured

(e.g., SP-B) or were inefficiently renatured (e.g., TTF). Therefore, the usefulness of this procedure appears to be protein-dependent. Overall, the single-step purification/solubilization procedure represents a rapid, efficient and simple method for recovery of soluble, recombinant proteins in non-denaturing buffers.

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