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Preparation of Soluble Proteins from *Escherichia coli*

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Abstract

Purification of human IL-1 β is used in this unit as an example of the preparation of soluble proteins from *E. coli*. Bacteria containing IL-1 β are lysed, and IL-1 β in the resulting supernatant is purified by anion-exchange chromatography, salt precipitation and cation-exchange chromatography, and then concentrated. Finally, the IL-1 β protein is applied to a gel-filtration column to separate it from remaining higher- and lower-molecular-weight contaminants, the purified protein is stored frozen or is lyophilized. The purification protocol described is typical for a protein that is expressed in fairly high abundance (i.e., >5% total protein) and accumulates in a soluble state. Also, the purification procedure serves as an example of how use classical protein purifications methods which may also be used in conjunction with the affinity-based methods now more commonly used.

INTRODUCTION

Once a suitable protein expression system involving *Escherichia coli* is developed and optimized (*UNITS 5.1 & 5.2*), fermentation and induction of protein expression (*UNIT 5.3*) generates culture material from which the protein of interest is purified. Harvesting (*UNIT 5.3*) produces cell concentrate or culture medium, depending on the subcellular localization of the protein. Cell paste is the starting material for purification of proteins expressed in soluble form inside cells, such as interleukin 1 β (IL-1 β). Human IL-1 β is a 153-residue (17.4-kDa) protein cytokine of biomedical importance that plays a central role in immune and inflammatory responses. Purification of human IL-1 β is used as an example of the preparation of soluble proteins from *E. coli*. The purification of some other soluble proteins derived from *E. coli* expression, as well as issues related to soluble proteins derived from other expression systems, are discussed in the Commentary.

Bacteria containing IL-1 β are lysed, and the resulting supernatant is clarified to remove ribosomes and other particulate matter. The sample is then applied to an anion-exchange column to separate recombinant IL-1 β from cellular contaminants, such as *E. coli* proteins, nucleic acids, and lipopolysaccharides. The sample is further purified through salt precipitation and cation-exchange chromatography, and then concentrated. Finally, the IL-1 β protein is applied to a gel-filtration column to separate it from remaining higher- and lower-molecular-weight contaminants, the purified protein is stored frozen or is lyophilized.

The purification protocol described is typical for a protein that is expressed in fairly high abundance (i.e., >5% total protein) and accumulates in a soluble state. With these expression levels, only about a 20-fold overall purification is required to obtain pure protein (Fig.

6.2.1). Therefore, conventional chromatographic methods can be used, and normally only three or four purification stages are required (Fig. 6.2.2). The purification process time described can be shortened through the use of chromatography systems and fast-flow column matrices (see Table 6.2.1 and Time Considerations).

SDS-PAGE (*UNIT 10.1*) is used to monitor column fractions for the presence of IL-1 β , which is detected as a stained band (*UNIT 10.5*) at the expected 17.4-kDa location. This is common practice in recombinant protein purification, as the specific assays for many proteins, including IL-1 β , are relatively complex. The original expression and fermentation experiments involving IL-1 β (Wingfield et al., 1986) followed procedures similar to those given in *UNIT 5.3*.

CAUTION: Avoid direct contact with IL-1 β -containing solutions. Trace material from aerosols or from hand contact can cause severe inflammation of the eyes. Safety glasses and gloves should be worn.

BASIC PROTOCOL

PURIFICATION OF A PROTEIN EXPRESSED IN *ESCHERICHIA COLI* IN A SOLUBLE STATE: INTERLEUKIN 1 β

Materials—DEAE Sepharose CL-4B resin GE Healthcare Life Sciences)

Anion-exchange buffer (see recipe)

0.26% (w/v) sodium hypochlorite/70% ethanol *or* 5% (v/v) bleach (e.g., Clorox)/70% ethanol

E. coli cells (~50 g wet weight) from fermentation (*UNIT 5.3*) containing IL-1 β

Lysis buffer (see recipe)

Bovine pancreas DNase I and RNase A (Worthington Biochemical; optional, for reducing solution viscosity)

2 N sodium hydroxide

Ammonium sulfate, ground with mortar and pestle

Cation-exchange buffer (see recipe)

CM Sepharose CL-4B (GE Healthcare

Cation-exchange buffer/250 mM NaCl (see recipe)

Tris base

Gel-filtration buffer (see recipe)

Ultrogel AcA54 gel-permeation resin (Pall Corporation; Sigma-Aldrich))

Lyophilization buffer (see recipe; optional)

2- or 3-liter sintered glass funnel with fritted disc (coarse porosity) and 5-liter filter flask

Chromatography columns (preferably glass) with adjustable flow adapters: one (or optionally two) 5×50 cm and one 2.5×100 cm (GE Healthcare), Amicon, or equivalent)

RK50 packing reservoir GE Healthcare)

Peristaltic pump, UV monitor, and fraction collector (GE Healthcare; check on-line for other suppliers of equivalent items)

16×150 -mm culture tubes

40-ml French pressure cell and rapid-fill kit (Thermo Fisher Scientific – see Commentary section on breaking cells).)

French laboratory press (Thermo Fisher Scientific))

1-liter Waring commercial blender.

250, 500, and 1000-ml stainless steel beakers

Ice bucket, ~4 liter

Tissue-grinder homogenizer (Polytron Model PT 10/35, Brinkmann: various suppliers)

Ultrasonic homogenizer, 400 W, with sound enclosure (Branson or equivalent)

Preparative centrifuge: Beckman J2-21M or Avanti J series (Beckman Coulter)

Rotors for preparative centrifuge: Beckman JA-14 (capacity 6×250 ml) or JA-20 (capacity 8×50 ml)

Ultracentrifuge: Beckman Optima XL-90 or Optima L-90 k (check Beckman Coulter website for other suitable models)

Rotors for ultracentrifuge: Beckman 45Ti (capacity 6×100 ml) or 35Ti (capacity 6×94 ml)

Conductivity meter (Radiometer Analytical)

Spectra/Por 1 dialysis tubing (Spectrum Labs)

Gradient maker: Model GM-2000 (1000 ml per side with side outlet; CBS Scientific); smaller-capacity gradient makers are also available (GE Healthcare and others))

200- or 400-ml stirred ultrafiltration cell and PM10 or YM3 Ultracel Amicon ultrafiltration discs (EMD Millipore)

Millex-GV 0.22- μ m-pore-size filter units (EMD Millipore)

10- or 20-ml syringe

Additional materials and equipment for SDS-PAGE (*UNIT 10.1*) and dialysis (*APPENDIX 3B*)

NOTE: All protocol steps are carried at 4°C unless otherwise stated. Forces for centrifugation steps refer to the maximum $\times g$ (i.e., centrifugal force at the bottom of the tubes).

Prepare anion-exchange column—1. Pour 400 to 500 ml DEAE Sepharose CL-4B ion-exchange resin into a sintered-glass funnel and wash with several liters water followed by 1 liter anion-exchange buffer (pH 8.5). Measure the conductivity of the starting buffer and eluted buffer to make sure they are the same before proceeding to the next step.

The resin is supplied in 500-ml bottles as a slurry in 20% ethanol. When washing the resin, do not allow it to run dry on the filter funnel. Laboratory vacuum (e.g., water aspirator) is adequate for filtering.

2. Suspend the washed resin in anion-exchange buffer to 75% settled gel/25% buffer by volume, per manufacturer's recommendations. Degas in a filter flask and pour into a 5 $\times$ 50-cm chromatography column fitted with a filling reservoir.

After settling, the height of the resin should be ~20 to 25 cm (390 to 490 ml packed resin). For details on packing columns, see *UNIT 8.4*. Because the solubility of gases decreases with increases in temperature, it is usual practice to pack the column at room temperature and then run it in a cold room or cold box.

3. Elute column with anion-exchange buffer at 100 to 150 ml/hr using a peristaltic pump. Make sure there is no compression of column contents. Monitor the absorbance of the effluent at 260 or 280 nm with a UV detector. Collect ~15-ml fractions in 16 $\times$ 50-mm culture tubes using a fraction collector. Check that the pH and conductivity of the column effluent are the same as the anion-exchange buffer applied to the column (this indicates that the column matrix is correctly equilibrated).

The bed height should not change significantly once the column is packed. Compression indicates that the pressure applied to the column is too high (see manufacturer's recommendations for maximum flow rates).

Break cells with a French press—4. Clean bench areas that may come in direct contact with cells with 0.26% sodium hypochlorite/70% ethanol.

5. Assemble the French pressure cell and chill to ~4°C either by incubation in ice or by refrigeration. Install the cell (first dried with paper towels if necessary) in the laboratory press.

It is important to cool the equipment because pressurizing will generate heat. The 20 K rapid-fill French pressure cell (1-in.-diameter piston) has a capacity of 40 ml and can be

continuously filled while installed on the press. Before using the pressure cell, replace the nylon ball at the end of the flow valve assembly or, at the very least, check it for distortion.

For small-scale work, a miniature French pressure cell (3/8-in.-diameter piston) with a 3.7-ml capacity is available.

6. Suspend thawed *E. coli* cells (~50 g wet weight) with 150 ml lysis buffer using a Waring blender. Place the suspension in a stainless steel beaker and homogenize with the Polytron tissue-grinder homogenizer until clumps are no longer detected.

IMPORTANT NOTE: Wear disposable gloves and safety glasses while working with *E. coli*. The high-pressure homogenization may generate aerosols.

The *E. coli* cells are stored frozen at -80°C as a flattened paste in heat-sealable plastic bags (UNIT 5.3). The cells are thawed at room temperature. Complete suspension of the cells with the blender is important, as any visible clumps of bacteria will block the French pressure cell. A clogged cell may have to be disassembled to clear the blockage.

7. Lyse the cells with two passes through the French press operated at 16,000 to 18,000 lb/in² (with the high-ratio setting, pressure gauge readings between 1011 and 1135). Chill the cell suspension to 4°C after each pass through the pressure cell by incubation on ice.

When filling the pressure cell, avoid drawing air into the cylinder to prevent foaming.

If a French press is not available, the cells can be broken by including 200 µg/ml lysozyme (Worthington) and 0.05% (w/v) sodium deoxycholate (EMD Millipore Calbiochem) in the lysis buffer and incubating cells ~20 min at 20° to 25°C with intermittent homogenization using the tissue grinder (Burgess and Jendrisak, 1975). Cell breakage by lysozyme treatment and sonication is described in UNIT 6.5.

8. Place the suspension (contained in a steel beaker) on an ice bath and, using an ultrasonic homogenizer, sonicate 5 min at full power with 50% duty cycle (on for 0.5 sec then off for 0.5 sec). While sonicating, stir the suspension using a magnetic stirrer.

IMPORTANT NOTE: Wear sound-protection earmuffs to protect ears from ultrasonic noise. Because sonication will generate some aerosol, use the sonicator in a microbiological hood if possible.

High viscosity reduces the rate of sedimentation of the various contaminating cellular material and thus longer (sometimes much longer) centrifugation times are required. Sonication reduces the viscosity of the suspension prior to centrifugation by shearing the released DNA and RNA. The viscosity can also be reduced by digesting the lysate 15 to 30 min at 4° to 10°C with bovine pancreas DNase I (25 to 50 µg/ml) and RNase A (50 µg/ml). If the nucleases are used, EDTA in the lysis buffer should be replaced by 5 mM MgCl_2 as DNase requires Mg^{2+} .

Clarify the lysate—9. Transfer sample from the beaker to centrifuge bottles and centrifuge the cell lysate 40 min at $22,000 \times g$ (e.g., in a Beckman J2-21M preparative

centrifuge at 12,000 rpm using JA-14 rotor or at 13,500 rpm using JA-20 rotor), 4°C. Decant the supernatants, pool, and recentrifuge 90 min at $\sim 100,000 \times g$ (30,000 rpm in Beckman Optima XL-90 ultracentrifuge using Ti45 rotor), 4°C.

Low-speed centrifugation removes unbroken cells and large cellular debris. High-speed centrifugation removes smaller particles such as ribosomes and membrane vesicles; the Beckman 70Ti rotor (capacity 8×39 ml) can be used in the ultracentrifuge for smaller-scale work. Clarification of the lysate can also be carried out by salt fractionation (see Background Information, section on determining solubility, for further details).

Pellets are usually discarded immediately; but see Critical Parameters and Troubleshooting, section on protein purification for further comments.

10. Dilute the supernatant from step 6 (~ 160 ml) 1:2 (three-fold) with anion-exchange buffer and adjust to pH 8.5 (if necessary) with 2 N NaOH. Using a conductivity meter, measure the conductivity of the diluted supernatant. If it is higher than 5.0 to 5.3 mS/cm, reduce by dilution with water.

The conductivity of the protein solution is carefully adjusted to ensure that the proteins (in this case contaminants) are bound to the matrix. Too high an ionic strength will reduce or prevent binding.

The DEAE Sepharose column is normally prepared (steps 1 to 3) before lysis of the cells is initiated. If there are any delays in applying sample to column, store the clarified lysate at 0° to 4°C (for example, in a covered beaker or flask embedded in an ice bucket). The same applies to the other chromatographic stages.

Chromatograph cleared lysate on anion-exchange resin—11. Apply the clarified lysate (480 to 500 ml) to the DEAE Sepharose column (step 3) at 150 ml/hr and elute the column with anion-exchange buffer. Continue the elution, collecting 15-ml fractions, until the effluent absorbance is close to the baseline value.

In solutions above $\sim$ pH 7.0, IL-1 β (pI 6.8, where pI is the isoelectric point) is negatively charged. Therefore, the protein should, in principle, bind to an anion exchanger (positively charged matrix) buffered at pH 8.5. In fact, under the conditions described, IL-1 β is only weakly bound to the matrix, which allows for partial resolution from proteins that do not bind to the matrix. Much of the unbound protein is of high molecular weight (or highly aggregated) and is partially separated from IL-1 β by the gel filtration effect of the matrix. IL-1 β is therefore usually located in the latter two-thirds (~ 500 ml) of the column flowthrough volume.

12. Assay every second or third column fraction by SDS-PAGE (*UNIT 10.1*).

See Figure 6.2.3A for an example of results from SDS-PAGE.

For rapid analysis, use precast gels, e.g., Novex (Life Technologies). Alternatively, if speed of purification is important, the entire flowthrough can be used, eliminating the need to analyze separate fractions. Most protein contaminants bound to the column can be removed

by step elution with 1 M NaCl in column buffer. After use, the resin should be unpacked from the column and washed on a sintered-glass funnel with 1 liter of 2 M NaCl/0.5% (w/v) Triton X-100, followed by 10 liters water. If the resin is to be stored, suspend it in 5% ethanol or 5 mM sodium azide and store at 4°C. In order to avoid potential cross-contamination, dedicate the used resin for repeat purifications of IL-1 β only.

Fractionate sample with ammonium sulfate—13. Pool IL-1 β -containing fractions from the DEAE Sepharose column, record the volume (~500 ml), and transfer the solution to a 1-liter beaker (preferably stainless steel). Add 30.2 g (NH₄)₂SO₄ per 100 ml solution at 0°C (51.3% saturation or 2 M final concentration): add powdered (NH₄)₂SO₄ slowly over 30 min, mixing gently with a magnetic stirrer, and then allow a further 30 min of mixing.

Methods for calculating the percent saturation of ammonium sulfate solutions, which specifically refer to 0°C, have been described by Wood (1976; also see APPENDIX 3F). When using solid ammonium sulfate, calculations must include volume increases on addition of the solid to fixed volumes (as carried out above).

14. Centrifuge the slightly cloudy solution 30 min at 22,000 $\times g$ (12,000 rpm in JA-14), 4°C. Decant the supernatant into a beaker and add an additional 17 g (NH₄)₂SO₄ per 100 ml solution (77% saturation or 3 M final concentration). Equilibrate with stirring and centrifuge 30 min at 22,000 $\times g$, 4°C.

For the addition of (NH₄)₂SO₄ follow the same method as described in step 13.

15. Decant the supernatant and drain the pellets by inverting the tubes on a paper towel. Save the pellets.

Dialyze the fractionated sample—16. Suspend the pellets in ~300 ml cation-exchange buffer and dialyze, using Spectra/Por 1 dialysis tubing, against 5 liters cation-exchange buffer. Change the dialysis buffer at least once.

The dialysis step is conveniently performed overnight; the CM Sepharose column used in step 18 can be prepared during this period.

The dialysis tubing is prepared by heating 30 to 60 min at 90° to 95°C in 5 mM EDTA. The tubing is then washed well with water and stored in 10% ethanol at 4°C prior to use. A suitable length of tubing is filled to about one-half to three-quarters capacity with solution (to allow for expansion) and sealed with two knots at each end. Use gloves when handling the tubing, check for leaks before use, and make sure the magnetic stir-bar does not rub against the tubing. See APPENDIX 3B for further information concerning dialysis.

17. After dialysis, remove the slightly cloudy solution from the tubing and centrifuge 30 min at 22,000 $\times g$ (12,000 rpm in JA-14), 4°C. Save the supernatant.

Chromatograph dialyzed sample on cation-exchange resin—18. Prepare ~200 to 225 ml CM Sepharose CL-4B resin by washing on a sintered-glass funnel first with water,

then with cation-exchange buffer (pH 5.7; wash as in step 1 except using different buffer). Pack the degassed resin into a 5 × 50-cm column as in step 2.

The packed column will have a bed height of ~11 to 12 cm. The comments made in the annotation to step 3 also apply here.

19. Elute the column using cation-exchange buffer at 100 to 150 ml/hr with a peristaltic pump. Monitor the column effluent at 280 or 260 nm using a suitable UV detector. Check that the pH and conductivity of the column effluent are the same as for the buffer applied to the column.

20. Check the pH and conductivity of the dialysate supernatant from step 17 and, if necessary, dilute with water so that the conductivity is in the range 1.0 to 1.2 mS/cm (at 4° to 6°C). Apply the clear solution to the CM Sepharose column at a flow rate of 150 ml/hr. When the UV absorbance of the column effluent approaches baseline, proceed to the next step.

21. Prepare a 0 to 250 mM NaCl gradient in cation-exchange buffer by adding 500 ml buffer to the inner chamber of the gradient maker and 500 ml buffer/250 mM NaCl to the outer chamber. Apply the gradient to the column at 150 ml/hr. Collect 15-ml fractions.

The total volume of the gradient is 1 liter (~4.5 column volumes).

At pH 5.7, IL-1 β (pI 6.8) is positively charged and binds to the negatively charged cation-exchange resin. IL-1 β is eluted from the column with ~100 mM NaCl, and it will be located in the major absorbance peak (see Wingfield et al., 1986, for typical elution profile).

22. Monitor the progress of the gradient using an in-line conductivity meter positioned after the absorbance flow cell. Assay column fractions for IL-1 β by SDS-PAGE (*UNIT 10.1*) and pool fractions containing IL-1 β .

Deciding what fractions to pool is dictated by the fact that the remaining purification stage is gel filtration, a method that will not remove contaminants with sizes close to that of IL-1 β (17.4 kDa). As IL-1 β is a well-expressed protein (>10% total protein), one can afford to be conservative and pool for purity rather than yield.

The used CM-Sepharose matrix can be cleaned up and stored as described in the annotation to step 12. CM Sepharose Fast Flow or SP Sepharose Fast Flow (a strong cation exchanger; both resins are also from GM Healthcare) can be used instead of CM Sepharose CL-4B. Similar results are obtained with either matrix, with the advantage of faster flow rates.

Concentrate the proteins—23a. *To concentrate proteins by ultrafiltration:* Adjust the CM Sepharose pool (150 to 250 ml) to pH 7.5 with Tris base. Assemble a 200- or 400-ml stirred ultrafiltration cell containing a washed ultrafilter membrane, either YM3 (3-kDa cutoff) or PM10 (10-kDa cutoff). Pressurize the cell with nitrogen according to manufacturer's recommendations. Collect the effluent from the cell into a measuring cylinder and occasionally record the absorbance at 280 nm to check that the membrane is not leaking. When the volume remaining in the cell is ~15 ml, depressurize the cell and

carefully remove the solution using a Pasteur pipet with a small length of polyethylene tubing attached to the end so as not to scratch the membrane. Use ~3 ml gel-filtration buffer to wash the membrane, adding washings to the main concentrate.

Exposure to solutions below pH 4.5 and above pH 10.5 to 11.0 often causes protein denaturation. Hence, when adjusting the pH of protein solutions, there is less chance of overshooting the required pH by using concentrated buffer components instead of pure acid or base. For example, Tris base is used rather than dilute NaOH.

Select the ultrafiltration membrane pore size based on the size of the protein. The membrane can be reused; store in 5% ethanol at 4°C. It is good practice to reserve a membrane for use with a particular protein (compare comment on resin usage) and to wash the concentration cell carefully after use.

23b. To concentrate proteins by salt precipitation: Adjust the CM Sepharose pool to pH 7.5 with Tris base and slowly add 53.9 g (NH₄)₂SO₄ per 100 ml solution at 0°C (82.2% saturation or 3.2 M final concentration). Follow the basic salt precipitation method (steps 13 to 15) and suspend the pellets in 18 to 20 ml gel-filtration buffer.

Conduct gel-filtration chromatography—24. Prepare ~480 ml Ultrogel AcA54 resin by washing on a sintered-glass funnel with water, then gel-filtration buffer (as in step 1); suspend washed resin in gel-filtration buffer to 75% settled gel/25% buffer by volume and degas (step 2). Pour a slurry of degassed resin into a 2.5 × 100-cm chromatography column fitted with a filling reservoir. Pack the column at ~35 ml/hr.

UNITS 6.3 & 8.3 should be consulted for further details on the preparation and elution of gel-filtration columns. The column should be prepared in advance so that the protein samples can be applied as soon as the concentration step is completed.

The resin should be free-flowing yet concentrated enough to produce a packed bed with one pouring. A freshly packed gel-filtration column can be checked for packing irregularities by prerunning the column with a few colored markers. Blue dextran will be excluded from the gel matrix and will elute at the void volume (V₀, which equals 30% to 35% of the total column volume); cytochrome c (red; 12.4 kDa) will elute close to the expected position of IL-1β; and potassium dichromate (yellow) will be fully included in the gel matrix and will elute at the included volume (V_i, ~480 ml).

Superdex 75 gel-filtration matrix (GM Healthcare) can be substituted for Ultrogel AcA54. The former allows higher flow rates and thus is more compatible with the GE Healthcare AKTA systems.) Both Superdex and the cation-exchange Fast Flow resins mentioned above can be purchased in various prepacked columns that are useful for method development.

25. Filter the concentrated protein using a Millex-GV 0.22-μm filter unit attached to a 10- or 20-ml syringe and apply to the gel-filtration column. The sample can be applied either directly to the top of the column using a Pasteur pipet (care is required to prevent breaking the tip and contaminating the column) or via a three-way valve and syringe without removing the top flow adapter.

The volume of sample applied to the column (20 ml) represents ~4% of the total column volume. For columns with different dimensions, apply the same proportionate volume of sample. The column should be 60 cm long. For analytical separations, the sample volume should not exceed 2.5% of the column volume.

26. Elute the column at 35 ml/hr with gel-filtration buffer and collect 10-ml fractions. The major eluting peak contains the IL-1 β ; monitor the fractions by SDS-PAGE (*UNIT 10.1*) and pool fractions that contain pure protein. Save side fractions that contain small amounts of contaminants; this material can be rechromatographed after concentration as described in step 23a or 23b.

See Figure 6.2.3A for an example of results from SDS-PAGE.

Once the sample has been run into the column, it can be eluted with any buffer or even with a suitable column storage solvent such as 5 mM sodium azide in water.

Concentrate and store purified protein—27. Concentrate the purified protein, if required, by ultrafiltration (see step 23a). Determine the protein concentration of purified IL-1 β by measuring absorbance at 280 nm.

A molar absorbance coefficient (ϵ) of 10.61 mM⁻¹ cm⁻¹ is used. This corresponds to an absorbance of 0.63 for a 1 mg/ml solution using a 1-cm-path length cell.

28. For short-term storage (12 months), filter the protein with a Millex-GV 0.22- μ m filter unit, divide the solution into sterile plastic vials, and freeze aliquots rapidly with dry ice/ethanol. Store at -80°C. For long-term storage of IL-1 β (>12 months), lyophilize the protein. Dialyze the sample using a volatile buffer such as 50 mM ammonium bicarbonate or a nonvolatile buffer such as lyophilization buffer.

To circumvent the dialysis step, the phosphate-based lyophilization buffer can be used for gel filtration instead of the Tris-Cl gel-filtration buffer.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2E**; for suppliers, see **SUPPLIERS APPENDIX**. Buffer pH and conductivities are for solutions at 4° to 6°C. Units of conductance are given in siemens ($S = \Omega^{-1}$).

Anion-exchange buffer (50 mM Tris-Cl, pH 8.5)—Dilute 1 M Tris-Cl, pH 8.0 20-fold with water and adjust to pH 8.5 with NaOH. Make immediately before use.

Conductivity of the solution is 1.57 mS/cm.

Cation-exchange buffer, 10 $\times$ (15 mM sodium phosphate, pH 5.7/1 mM sodium azide)—19.4 g NaH₂PO₄·H₂O

1.4 g Na₂HPO₄·2H₂O

0.65 g sodium azide

H₂O to 1 liter

Store up to 1 month at 4°C

Dilute 10-fold immediately prior to use

Conductivity of the solution is 0.9 mS/cm. Sodium azide is an antibacterial agent.

Cation-exchange buffer (1×)/250 mM NaCl—14.61 g NaCl

100 ml 10× cation-exchange buffer (see recipe)

H₂O to 1 liter

Gel-filtration buffer (100 mM Tris·Cl, pH 7.5/1 mM sodium azide)—Dilute 1 M Tris·Cl, pH 8.0 10-fold with water and adjust to pH 7.5 with HCl. Add sodium azide from a 1 M stock solution (65 g/liter). Make immediately before use.

Lyophilization buffer (25 mM sodium phosphate, pH 7.5/0.5 mM sodium azide)
—2.2 g NaH₂PO₄·H₂O

11.9 g Na₂HPO₄·2H₂O

1.3 g sodium azide

H₂O to 4 liters

Make fresh and use immediately

Usually, 2 liters is required for dialysis, with one buffer change (4 liters total). Gel filtration requires smaller volumes.

Lysis buffer—100 mM Tris·Cl, pH 8.0

2 mM EDTA, pH 8.0

5 mM benzamidine·HCl (780 mg/liter)

Make immediately prior to use; alternatively, make ahead of time and store up to several days at 4°C.

Conductivity of the solution is 1.57 mS/cm.

It should be noted that a 1°C decrease in temperature increases the pH of the Tris buffer by ~0.03 pH units. Both Tris·Cl and EDTA stock solutions are commercially available.).

Benzamidine·HCl is a water-soluble serine protease inhibitor. Better protection is provided by using a protease inhibitor cocktail, e.g., Complete EDTA-Free, EASYpack(Roche

Applied Science): dissolve one tablet in 50 ml lysis buffer (see recipe for buffer). Also see Table 6.2.2.

Commentary

Background Information

The purification of IL-1 β detailed in this unit uses classical protein purification methods: ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration. Given high-level expression of soluble protein, it is very often the case that these methods will be adequate for purification. Figure 6.2.4 lists the most commonly used protein purification methods for recombinant proteins. The current trend is to express proteins with affinity tags, typically with hexahistidine [abbreviated 6 $\times$ His or (His) $_6$] and glutathione *S*-transferase (GST) tags, and then use affinity chromatography for their purification. This approach has been detailed elsewhere (*UNITS* 6.3, 6.6, & 9.4). It has obvious merit in terms of simplifying the purification process, and moreover can be applied to proteins expressed in eukaryotic hosts, such as yeast and insect cells. The downside of using tagged proteins is that the purification kits and matrices used are expensive compared to conventional chromatography media; more importantly, the tags can influence the physical and biological properties of the protein. Furthermore, if the tags are to be removed following purification using proteolytic or chemical cleavages, then the original purification advantage is somewhat negated. There are situations where the need for expression of a tagged protein supercedes purification issues. Inefficient translation initiation during expression in *E. coli* can result in poor or no accumulation of recombinant protein. One solution is to fuse a highly translated *E. coli* gene to the coding sequence, e.g., the aforementioned (His) $_6$ or GST tag (Bass and Yang, 1997). Another situation is where the expression of soluble protein is toxic to the host. This can be circumvented by expression of a fusion partner that drives inclusion-body formation, thus allowing accumulation of the target protein without cell lysis and death. Similarly, expression of an unstable soluble protein that is susceptible, for example, to proteolytic processing during extraction and purification can also be protected by the formation of inclusion bodies. Another convenient feature of tagged proteins is that antibodies against many of the commonly used fusion moieties are commercially available, thus allowing purification to be monitored using immunological methods. A fairly comprehensive listing of fusion adducts is given in *UNIT* 9.9.

Expression of soluble proteins—The basic stages involved in preparing soluble proteins from *E. coli* are achieving protein expression, harvesting and breaking cells (or collecting culture medium, in the case of secreted proteins), purifying proteins, and characterizing the purified protein. When proteins are expressed using other expression systems, the actual purification methods used are the same. Differences are evident, however, in the methods used to disrupt the cells. In the case of mammalian cells, the protein of interest is often secreted, necessitating a different purification strategy than that used with proteins recovered from the intracellular space.

Optimizing the Expression System—The basic requirements for protein expression in *E. coli* are discussed in *UNIT* 5.1. Experience has shown that it is usually possible to express

most proteins in *E. coli*, but whether the protein will be expressed in a soluble and unmodified state is unpredictable. Tinkering with the various elements of the expression plasmid, changing the *E. coli* host strains, and altering the fermentation conditions are carried out to optimize the expression of unmodified soluble protein (*UNIT 5.1*). Use of secretion vectors and fusion protein constructs to prevent aggregation has been discussed elsewhere (Choi and Lee, 2004; *UNITS 5.1 & 6.1*). Even if efforts to express soluble protein fail and inclusion bodies are produced, there is often a good chance that the protein can be extracted and folded into native protein (see *UNITS 6.4 & 6.5*). In a noteworthy study, a combination of methods for optimizing *E. coli* expression of soluble protein is described (Ito and Wagner, 2004). The methods include codon optimization, chaperone co-expression and rational mutagenesis.

There are situations where the intrinsic properties of a protein can preclude its expression in a soluble and stable form. Examples include the following: (1) individual subunits of a heterodimer, which may be unstable and may degrade or aggregate when expressed separately (coexpression of the subunits may solve the problem; Nash et al., 1987); (2) proteins derived from activated precursors (*UNIT 6.4*; Hlodan and Hartl, 1994); and (3) proteins in which the native (authentic) sequences have been modified by site-directed mutagenesis (Chrnyk et al., 1993).

Breaking cells—Some commonly used methods for cell disruption are shown in Table 6.2.3. Methods for breaking cells and selectively extracting proteins are also reviewed in *UNIT 6.1*. In the protocol for purifying human IL-1 β , cells are disrupted with a French press. This method is the most efficient way to break *E. coli* cells and is the method of choice. It should be noted that Thermo Fisher Scientific, who originally acquired the French press from Aminco, no longer sell the equipment as of 2009. This is unfortunate because as mentioned, this is the best system for laboratory scale cell breakage. It is of some comfort to those who already have this equipment that it appears almost indestructible; in the authors laboratory the press is still in good working condition after 25 years and counting. An alternative system is the EmulsiFlex-C5 homogenizer used in the protein expression and purification core facility of the EMBL where it is used to break *E.col* and Yeast cells.

Cell breakage with lysozyme (*UNIT 6.5*) and/or sonication is also frequently used; such methods are especially suited to small-scale work (reviewed by Hopkins, 1991). In addition, several manufacturers supply cell breakage kits that are best suited for small-scale work. Although the actual composition of the solutions in these kits is not given, a check of patents filed indicates that the detergent octylthioglucosidase is the active reagent.

It is important to consider whether the cell breakage method influences the final solubility and stability of the recombinant protein in the cell lysates. A soluble recombinant protein constituting ~12% of the total bacterial protein will be present in the cytoplasmic space at a concentration of at least 30 mg/ml, assuming a total protein and RNA concentration in the cytoplasm of ~340 mg/ml in nontransformed cells (Zimmerman and Trach, 1991) and a 2.7:1 weight ratio of protein to RNA (Neidhart, 1987). Cell breakage can be expected to increase the solubility of the recombinant protein by a dilution effect (discussed by Zimmerman and Trach, 1991). Cell lysis procedures, however, especially when mechanical

shear is used (e.g., French press and sonication), can produce local heating that may have a denaturing effect and lead to aggregation. Mechanical shearing will also be expected to decrease the size of nucleic acids and release polyanionic lipopolysaccharides; both classes of compounds can bind nonspecifically to proteins, especially basic proteins, thereby reducing their solubility. Including detergents in the lysozyme treatment may also release potent membrane-bound proteases.

Despite these potential pitfalls, the recovery of soluble protein is usually not dramatically influenced by the cell breakage method, but there is always the potential that it might be. Protein recovery as a function of cell breakage methodology is rarely systematically studied or reported. Johnson and Hecht (1994) reported that freezing and thawing of cells selectively releases recombinant proteins located in the cytoplasm. The freeze-thaw procedure, in principle, should be the best method for releasing soluble proteins in an unmodified state, but its general applicability remains to be seen.

Once conditions have been found that result in the expression of soluble protein, the next hurdle is to recover the protein in an unmodified form. Avoiding proteolysis and chemical modification during the isolation process is crucial. To minimize proteolysis, it is often a good idea to add various inhibitor mixtures to the cell breakage buffer. Protease inhibitor cocktails (commercially available from Sigma, Roche, and others) can be tailored to the particular expression system used. Table 6.2.2 lists the composition of inhibitors most appropriate for various host systems.

Determining Solubility—The solubility of recombinant protein in the cell lysate or extract is usually established by differential centrifugation (*UNITS 6.1 & 5.3*). Alternatively, the extract can be clarified by filtering and applied to a small gel-filtration column. An HPLC gel-filtration separation in conjunction with a rapid screening method for assaying column fractions (e.g., SDS-PAGE can produce a rapid determination of both solubility and size distribution. As mentioned in *UNIT 6.1*, screening kits are also available for optimizing cell breakage/extraction buffer composition.

Purifying Protein—A basic checklist of the issues involved in protein purification is given in Figure 6.2.5. Many of these points are self-evident and are discussed in other units. The purification of soluble recombinant proteins from various host systems is similar following cell breakage and extraction. With mammalian expression systems, the recombinant proteins are often secreted, necessitating capture from large volumes of culture medium. Expanded-bed chromatography is a very useful method for binding protein from crude cell culture media (see *UNIT 8.8* and the handbook “Expanded Bed Adsorption: Principles and Methods” available from GE Healthsciences web site)). Following cell breakage, centrifugation is often one of the first steps used to clarify the cell extract prior to chromatographic stages (Fig. 6.2.6).

Most soluble proteins that are well behaved—i.e., do not contain excessive charge heterogeneity and are non-self-associating—can often be purified by two stages of ion-exchange chromatography and one or more polishing steps, one of which should be gel filtration. The purification of human IL-1 β is a typical example (see Fig. 6.2.2 and Table

6.2.1). Some other examples include the HIV-1 protein Nef and the anti-HIV protein MAP30, whose purifications are summarized in Figure 6.2.7 (also see *UNIT 6.1* for further discussion). An example of a small DNA-binding protein is also given in Figure 6.2.8. In this example, the expressed protein was highly susceptible to proteolytic processing, which was minimized by selection of a host cell deficient in the Lon protease (see Allet et al., 1988 for further detail).

Specific accounts of the various chromatographic methods are found in Chapters 8 and 9; Scopes (1994) particularly emphasizes first principles. In addition, very useful handbooks covering some commonly used chromatographic methods can be downloaded as pdf files from GE Healthcare Handbooks. The following journals might also be useful: *Journal of Biotechnology*, *Protein Expression and Purification*, and *Current Opinion in Biotechnology*.

Characterizing Protein—Once a protein has been purified, it is usually characterized (Chapter 7) in order to establish chemical homogeneity (primary sequence) and physical homogeneity (size and conformation). Not only is this information required for a fundamental description of the protein, but it will also give insights on how best to rationalize the purification process Figure 6.2.4. Moreover, the detection of certain heterogeneities may give clues on how to avoid their occurrence. For example, chemical modifications such as deamidation may be prevented by changing one or more purification steps that expose the protein to extremes of pH. Other modifications that arise from construction of the expression plasmid itself, once recognized, can often be fixed.

Characteristics of recombinant and authentic interleukin 1 β —The cytokine IL-1 β and the closely related IL-1 α are synthesized mainly by monocytes and mononuclear cells as 31-kDa cytoplasmic precursor proteins (reviewed by Dinarello, 1989). A unique cytoplasmic cysteine protease (IL-1 β -converting enzyme, or ICE) cleaves the IL-1 β precursor in half, generating the mature form of the protein comprising the C-terminal 153 residues (Thornberry, 1992). The biological activities of IL- β (and IL-1 α) are initiated by interaction with either type I or type II cellular receptors (McMahan et al., 1991). Both receptors have three immunoglobulin-like extracellular ligand-binding domains and single membrane-spanning segments. Another form of IL-1, the interleukin 1 receptor antagonist (IL-1ra), is a natural competitive antagonist of IL-1 β . The crystal structures of the type-1 interleukin-1 receptor complexed with either IL-1 β (Vigers et al., 1997) or IL-1RA (Schreuder et al., 1997) have been solved.

Authentic IL-1 β been isolated from human monocyte cell cultures by HPLC using anion-exchange and gel-filtration (or reversed-phase) matrices. A 3000-fold purification was required to obtain 16 μ g protein from 5 liters of cell culture extract (Gery and Schmidt, 1985). The authentic protein was established to be a nonglycosylated monomer (~18 kDa), with a pI of 6.8 and containing a single N-terminal sequence (AlaProValArg) as predicted by the DNA coding sequence.

The recombinant protein produced in *E. coli* has the same properties as the natural product except that N-terminal heterogeneity is often observed. The recombinant protein usually contains a mixture of forms with Met (20%), Ala (67%), or Pro (13%) as the N terminus.

The Met form is derived by incomplete processing of the initiating Met, and the Pro form is due to cleavage of the authentic N-terminal Ala by *E. coli* protease. Despite the slight differences among the recombinant variants, partial or complete separations have been achieved (Wingfield et al., 1987; Yem et al., 1988).

Both the crystal structure of IL-1 β (Priestle et al., 1988) and its nuclear magnetic resonance (NMR) structure in solution (Clare et al., 1991) have been determined using material purified by the protocol described here. For some NMR studies it was necessary to produce material with a single N terminus, and this was fortuitously achieved by replacing the N-terminal Ala residue with a Cys residue. Purified protein from cells expressing the mutant (N-terminal CysProValArg) contain only N-terminal Pro. It should be noted that deletion of the first four residues of IL-1 β has no effect on activity; however, the presence of the unprocessed Met reduces receptor binding 10-fold.

Interleukin 1 β does not contain a disulfide—the cysteines are either buried (Cys-71) or partially buried (Cys-8)—so there is no need to include reductants in column buffers. As mentioned earlier, the authentic protein is nonglycosylated even though there is a potential N-glycosylation site (-Asn⁷-Cys⁸-Thr⁹-) near the N terminus. Interestingly, when IL-1 β is expressed in yeast, equal amounts of N-glycosylated (21 kDa) and nonglycosylated protein (17 kDa) are produced (Livi et al., 1991).

Other approaches to purifying recombinant interleukin 1 β —Because of the biomedical importance of IL-1 β , several reports have described expression and purification of the protein. Some examples are discussed.

Kronheim et al. (1986) acidified *E. coli* cell lysates to pH 4 and thus precipitated ~70% of the total protein while most of the IL-1 β remained soluble. IL-1 β was further purified by cation-exchange, anion-exchange, and dye-matrix chromatographies (UNITS 8.2 & 9.2); ~200 mg protein was recovered from 2.5 liters of culture. The noteworthy feature of this method is the acid precipitation stage. Extracts of *E. coli* contain predominately negatively charged (anionic) proteins: 60% have pI values between 5.0 and 6.0 and 80% between 4.5 and 6.7 (Sherwood, 1992). Adjustments of cell extracts to pH 5.0 and below thus result in isoelectric precipitation of substantial amounts of *E. coli* protein. This is a useful purification step as long as the recombinant protein remains soluble (or does not coprecipitate) under the conditions.

In another approach, Meyers et al. (1987) carried out ammonium sulfate fractionation directly on the *E. coli* extract. The IL-1 β protein was recovered in the fraction corresponding to 50% to 80% saturation. After desalting, the protein was further purified by ion-exchange (cation and anion) and gel-filtration chromatographies; ~400 mg protein was recovered from 250 g wet weight cells.

The high-speed centrifugation step used to remove particulate material (see Basic Protocol, step 6) can often be replaced by salt fractionation. For IL-1 β , advantage can be taken of the fact that a relatively high (NH₄)₂SO₄ concentration is required to precipitate the protein. The

disadvantage of using this approach early in the purification is that the protein must be desalted before being applied to an ion-exchange column.

Finally, it is of interest to note that IL-1 β can be released from *E. coli* cells by osmotic shock treatment (Joseph-Liauzun et al., 1990). It is well known that certain *E. coli* cytoplasmic proteins, including thioredoxin, can be released by osmotic shock.

Scale of procedure—The Basic Protocol described starts with 50 g cell paste and yields ~400 mg pure IL-1 β . For smaller (1 to 5 g) or larger (100 g) cell quantities, the method can be adapted by simply reducing or increasing, respectively, the cross-sectional area of the chromatography columns. For example, for 5 g cells, the diameters of the DEAE and CM Sepharose CL-4B columns are reduced to 2.5 cm, with column lengths of 20 and 10 cm, respectively. This results in 1:4 reductions in the resin volumes compared to those for the protocol utilizing 50 g cells. The original gel-filtration column can be used (2.5 $\times$ 100 cm); however, the volume of ~1:4 sample applied should be reduced to 8 to 10 ml. Alternately, a column of about the same length (60 to 100 cm) but smaller diameter (e.g., 1.25 cm) should be used. For discussion on scaling up column chromatography procedures, see *UNIT 8.3* (Strategic Planning and Scopes, 1994).

Critical Parameters and Troubleshooting

High-level protein expression with good cell yield (*UNITS 5.1–5.3*) is required to obtain reasonable amounts of pure protein. This is obviously true no matter how efficient (or inefficient) the purification protocol. Once the host/vector system and the fermentation conditions have been optimized and, equally important, standardized, the level and solubility of recombinant protein in the starting material should be consistent. However, it is sensible to check both the expression level and solubility before starting each protein purification. The expression level can usually be monitored by SDS-PAGE; *UNIT 5.2* describes how to prepare samples for analysis by SDS-PAGE, and *UNIT 10.1* gives electrophoresis procedures. Solubility can be easily monitored by breaking a small amount of cells (1.0 g) by sonication (*UNIT 5.3*) or by treating cells with lysozyme (*UNIT 6.5*) and microcentrifuging the resulting cell lysate to judge whether the protein is highly aggregated.

There are many reasons why protein expression may not be as high as anticipated and, worse, why the expressed protein may switch from being soluble to insoluble. For example, faulty pH control during the fermentation may cause insolubility. Whatever the situation, one should be in a position to rationally decide whether to continue with the purification or repeat the initial fermentation.

If the protein expression level is acceptable (5% to 10%) and the protein has accumulated in a soluble state, implementing the Basic Protocol, which uses only standard purification methods, should be trouble-free. Common mistakes involve buffer preparation and other trivial errors such as pooling the wrong column fractions. As discussed later, do not discard column fractions until they have been completely analyzed.

A classic purification table (see for example, Tables 1.4.5 to 1.4.8 in *UNIT 1.4*), has not been presented, as the biological activities of IL-1 β could not be measured reliably in crude

E. coli cell extracts by assays available to the author when the method was developed (Wingfield et al., 1986). As is often the case with proteins requiring complex biological assays, protein size analysis by SDS-PAGE is used to follow purification. During the initial stages, it is of course necessary to confirm the identity of the particular band one is following, and this can be accomplished by eluting or blotting the protein from SDS-polyacrylamide gels and performing N-terminal amino acid sequence analysis. Immunoblotting with specific antibody can be used to monitor protein purification when the recombinant protein is in low abundance (e.g., for secreted proteins in the cell medium). Finally, it should be noted that several manufacturers now supply assay kits for many cytokines, including IL-1 β .

Cell breakage—Efficient cell breakage should be trouble-free as long as the French press is operated in accordance with the manufacturer's instructions. It takes a little practice to operate the flow valve. The aim is to generate as high a flow rate as possible while maintaining a pressure gauge reading of ~1000. If the flow rate is too fast, the pressure reading will drop and unbroken cells will pass into the flow stream. Toward the end of the run, the flow rate should be reduced, as it becomes difficult to control the pressure. After use the French pressure cell should be cleaned and dried, and the flow valve ball should be replaced. Store the cell at 4°C.

Protein purification—In general, troubleshooting a purification method will be much easier if fractions are not discarded until the appropriate monitoring of the purification steps is complete. When using an established method, fractions from a chromatographic run are frequently pooled on the basis of absorbance only and the remainder quickly discarded. Ammonium sulfate supernatants or pellets are often discarded on the basis of previous fractionation behavior or pilot-scale work. As stated, do not discard any fractions until they have been checked, usually by SDS-PAGE. If there has been a problem, it can usually be easily sorted out if all the fractions from the various stages are still available. If in doubt about conditions for storing fractions, freeze selected fractions at as low a temperature as possible (ideally -80°C) and discard when appropriate. When freezing material, it is worthwhile to take the extra effort to dispense small samples (<1 ml) into microcentrifuge tubes amenable for rapid analysis, if necessary, to avoid having to thaw large volumes merely to take 10 μ l for SDS-PAGE.

For reproducible results, careful recording of the pH and conductivity of starting buffers, protein solutions, and column eluents is necessary. For example, if the protein does not bind to the cation exchanger (step 19), the ionic strength of the sample or column buffer may be too high. Alternatively, the buffer pH may be too high. Errors of this kind are avoided by simply measuring the conductivity and pH of all solutions at each stage. Figure 6.2.9 lists some considerations to bear in mind when using ion-exchange resins. Other precautions and critical steps relating to chromatography procedures are mentioned in Chapters 8 and 9.

Anticipated Results

Purification results in recovery of ~8 mg purified protein per gram wet weight cells. That result is based on a 10% expression level; IL-1 β expression levels of 10% to 15% are

routinely obtained with the expression system previously described (Wingfield et al., 1986). With a 1.5-liter fermenter, at least 50 g wet weight cells/liter can be obtained using a standard complex medium (UNIT 5.3). Thus, ~400 mg protein is obtained from 1 liter of culture, and optimal conditions can lead to nearly twice that amount. The yield corresponds to an overall recovery of ~60% of the IL-1 β originally in the cells.

Figure 6.2.3 illustrates the protein content at various purification stages. The starting material for the final stage of gel filtration (Fig. 6.2.3A, lane d) is mainly contaminated with *E. coli* proteins >44 kDa in size, and these are easily separated from the IL-1 β protein (17.4 kDa) by gel filtration. Analytical rechromatography of the protein from the pooled fractions (indicated P in Fig. 6.2.3B) reveals a single symmetrical peak indicative of purity and physical homogeneity (Fig. 6.2.3B inset). A typical cation-exchange chromatogram for the purification appears elsewhere (Wingfield et al., 1986). IL-1 β , purified as described, is a single band on SDS-PAGE; however, caution should be exercised in assuming that this represents absolute purity. Chromatofocusing (UNIT 8.5) resolves this apparently pure protein into two peaks, A and B (Figure 6.2.10B), where A is protein lacking the N-terminal methionine and B is protein that has retained the N-terminal methionine. The slight difference in isoelectric point (pI) between these species arises because of the difference in the pK_a of the N-terminal amino group of Met and Ala (Met⁺ species) and this is shown in Figure 6.2.10A (see Wingfield et al., 1987, for further details).

Time Considerations

Because IL-1 β appears to be stable against proteolytic degradation and other chemical modifications during purification, the speed of purification was not critical in this case. The low-pressure chromatographic method described in the Basic Protocol requires ~4 days, which can be shortened to ~3 days or less using chromatography systems such as the AKTA (GE Healthcare) in conjunction with matrices that allow faster flow rates. The times required for purification whether using low-pressure chromatography (as described in the Basic Protocol) or medium-pressure chromatography are summarized in Table 6.2.1.

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E. coli: expression levels and protein content

Component	% dry weight
Protein	55
RNA	20
DNA	3
Lipid	10
Lipopolysaccharide	3

Reference: Neidhardt, F.C (1987)

cell dry weight = 20% wet weight

protein content of cell wet weight ~10%

1 liter *E. coli* fermentation: 50 – 80 g wet weight cells

total protein/liter = 5 – 8 g

protein expression (moderate level = 10%)

recombinant protein/liter = 500 – 800 mg

*soluble protein recovery: 40% (200 – 320 mg)

*insoluble protein recovery: 5% – 10% (25 – 80 mg)

Figure 6.2.1.

Cell component distribution and typical expression levels obtained in *E. coli*. The asterisks (*) refer to purification yields of proteins expressed in *E. coli* in the soluble or insoluble states.

Purification of human Interleukin-1 β

Figure 6.2.2.
Scheme for purifying human interleukin-1 β .

Figure 6.2.3.

Purification of IL-1 β . **(A)** SDS-PAGE analysis of samples at various stages. Analysis was conducted on a gel of dimensions 12 cm $\times$ 16 cm $\times$ 1.5 mm. Lane a, purified protein (100 μ g loaded); lane b, purified protein (10 μ g loaded); lane d, CM Sepharose pool (80%); lane e, DEAE Sepharose pool after ammonium sulfate fractionation (56%); lane f, high-speed supernatant (starting material for DEAE Sepharose column; 13.5%); lane g, cell lysate (12.0%). The percentages refer to specific IL-1 β contents of the fractions determined by densitometric scanning of the Coomassie blue-stained gel lanes. Lanes c and h contain the following protein standards (low-range standards supplied by Bio-Rad) in order of increasing migration distance: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen white lysozyme (14.4 kDa). **(B)** Analysis of results from gel filtration on Ultrogel AcA54. The excluded volume (V_0) and the fully included volume (V_i) are indicated. Inset, analytical rechromatography of the protein from the pooled fractions (indicated P in larger chromatogram).

Protein purification: key methods

1. ion exchange (UNIT 8.2)
2. gel filtration (UNIT 8.3)
3. IMAC: (His)₆-tagged (UNIT 9.4)
4. affinity: tagged fusions (Chapter 9)
5. bioaffinity/immunoaffinity (UNIT 9.5)
6. reversed phase/HIC (UNIT 8.4)

Figure 6.2.4.

Common methods used to purify soluble recombinant proteins. Abbreviations: IMAC, immobilized metal affinity chromatography; HIC, hydrophobic interaction chromatography.

Protein purification checklist

1. determine use of protein and how much is needed
2. determine what is known about the protein by sequence and database analyses
3. assay and detection of protein
4. source: recombinant or non-recombinant?
5. extraction and capture
6. purification (chromatography)
7. storage

Characterization: rationalize process?

Figure 6.2.5.

Checklist of issues to consider when planning a purification strategy.

First stage of the purification process:
clarification to remove cell debris

Centrifugation is the method of choice

- batch centrifugation (laboratory scale)
- continuous-flow centrifugation for large scale

Centrifugation aids: flocculating agents (e.g., polyethylene imine)

Differential centrifugation

- low speed: pellet unbroken cells and debris
 - high speed: pellet subcellular complexes
- 100, 000-g for 1 hr to clarify soluble protein

Alternate methods for the high-speed spin

- $(\text{NH}_4)_2\text{SO}_4$ precipitation (usually 0%-25% saturation; APPENDIX 3F)
- polyethylene glycol precipitation (UNIT4.5)

Methods suitable for large-scale purification:

- ultrafiltration: tangential flow (Millipore)
- batch filtration: Celite filter aid (Whatman)

Figure 6.2.6.

Initial protein purification methods typically involve centrifugation (the specific conditions vary according to culture scale and location of expressed protein), $(\text{NH}_4)_2\text{SO}_4$ precipitation, or polyethylene glycol (PEG) precipitation.

Figure 6.2.7.

Purification of the HIV-1 protein Nef and the anti-HIV protein MAP30.

Figure 6.2.8.

Purification of the DNA-binding protein Ner of bacteriophage Mu. This basic (pI ~ 10) 9-kDa protein regulates phage lysogenic and lytic activity.

Ion exchange: a few notables

buffers

anion exchanger: use anionic buffer (e.g., HTris⁺)

cation exchanger: use cationic buffer (e.g., HPO₄²⁻)

Donnan effect (H⁺ or OH⁻ attraction)

anion exchanger (+)

pH ~ 1 unit higher in vicinity of matrix

cation exchanger (-)

pH ~ 1 unit lower in vicinity of matrix

buffer additives

- urea (1-8 M)
- nonionic detergents (e.g., Triton X-100)
- zwitterionic detergents (e.g., CHAPS)

note: EDTA binds to anion-exchanger

capacity of exchanger^a

decreases with increase in molecular weight

binding

column or batch mode (large scale)

elution^b

- column: increasing salt gradient
- batch mode: step gradients

^a If the protein does not bind to anion or cation exchangers (i.e., it is in flow-through), it may be aggregated or "big."

^b If the protein binds but is not eluted with up to 1 M salt, it may be denatured
Elute with Gnd-HCl or urea

Figure 6.2.9.

Issues to consider for ion exchange chromatography.

Figure 6.2.10.

Chromatofocusing of IL-1 β . (A) Theoretical titration curve of IL-1 β with N-terminal methionine (M^+) and N-terminal alanine (M^-). (B) Chromatofocusing of a mixture of IL-1 β M^+ and IL-1 β M^- . Chromatography was performed using a Mono HR5/20 column (GE Healthcare) as described by Wingfield et al. (1987).

Table 6.2.1**Outline of Interleukin β Purification^a**

Day	Steps (low pressure)	Steps (AKTA sytem)
1	Preparation of DEAE Sepharose column (steps 1–3) Cell breakage (steps 4–8) Clarification of lysate (steps 9 and 10) DEAE Sepharose chromatography (step 11) SDS-PAGE of DEAE Sepharose fractions (step 12) (NH ₄) ₂ SO ₄ fractionation (steps 13–15) Dialysis (steps 16 and 17)	As for low pressure (steps 1–17)
2	CM Sepharose chromatography (steps 18–22) SDS-PAGE of CM Sepharose fractions (step 22)	Fast Flow (substitute for CM Sepharose; steps 18–21) SDS-PAGE of Fast Flow fractions (steps 22) Concentration (step 23a) Superdex 75 gel filtration (substitute for Ultrogel; steps 24–26)
3	Concentration (step 23a or 23b) Ultrogel gel filtration (steps 24–26)	SDS-PAGE of Superdex 75 fractions (step 26) Concentration (step 27)
4	SDS-PAGE of gel-filtration fractions (step 26) Concentration (step 27)	

^a Step numbers in parentheses refer to the Basic Protocol. Chromatography materials as well and AKTA chromatography systems are from GE Heathcare.

Table 6.2.2

Protease Inhibitor Cocktails for Cell Extracts^a

Inhibitor ^b	Specificity	Bacterial cells	Mammalian cells ^c	(His) ₆ -tagged proteins	Yeast cells
AEBSF	Serine proteases	+	+	+	+
Aprotinin	Serine proteases		+		
Bestatin	Amino peptidases	+	+	+	
EDTA	Metalloproteases	+			
E-64	Cysteine proteases	+	+	+	+
Leupeptin	Serine and cysteine proteases		+		
Pepstatin A	Aspartic acid proteases	+	+	+	+
Phosphoramidon	Thermolysin, collagenase, and metalloendoproteases			+	
1,10-Phenanthroline	Metalloproteases				+
Reductant ^d		+	+		+

^aProtease inhibitor kits available from Sigma, Roche, and others.

^bAbbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; E-64, 1-*trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane.

^cIncludes insect cells (host for baculovirus expression system; *UNIT5 5.4 & 5.5*).

^dA reductant, e.g., DTT, should not be added to extracts used for nickel-chelate chromatography, although 1 to 2 mM β-mercaptoethanol (or TCEP hydrochloride) may be tolerated for that technique.

Table 6.2.3**Methods of Cell Breakage**

Method	Principle	Stress	Scale	Cell type
Osmotic	Osmotic rupture of membrane	Gentle	Small	Bacteria
Detergents^a CHAPS ^b DOC	Solubilization of membrane	Gentle	Medium	Bacteria Mammalian cells Insect cells
Enzymes^a Lysozyme Zymolyase	Digestion of cell wall	Gentle	Medium	Bacteria Yeast
Homogenization Dounce Polytron	Cells chopped and sheared	Moderate	Medium	Mammalian cells Insect cells
Grinding	Grinding with abrasive	Moderate	Small	Yeast
Ultrasonication	Sonic cavitation	Harsh	Small	Bacteria Mammalian cells Insect cells
Ball (bead) mills	Shear force: grinding and collision with beads	Harsh	Small-large	Yeast
Pressure cell French press Manton-Gaulin	Shear force: cells under high pressure passed via small orifice with rapid decompression	Harsh	Small-large	Bacteria Yeast

^aEnzymes and detergents can also be included with mechanical breakage methods.

^bAbbreviation: CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.