Metal-Chelate Affinity Chromatography

Recombinant proteins engineered to have six consecutive histidine residues on either the amino or carboxyl terminus can be purified using a resin containing nickel ions (Ni^{2+}) that have been immobilized by covalently attached nitrilotriacetic acid (NTA). This technique, known as metal-chelate affinity chromatography (MCAC), can readily be performed with either native or denatured protein. The Strategic Planning section discusses techniques for creating a fusion protein consisting of the protein of interest with a histidine tail attached (for purification by MCAC). The Basic Protocol describes expression of histidine-tail fusion proteins and their purification in native form by MCAC. Two alternate protocols describe purification of histidine-tail fusion proteins by MCAC under denaturing conditions and their renaturation by either dialysis or solid-phase renaturation. Support protocols are provided for analysis of the purified product and regeneration of the NTA resin. All of these protocols are easily adaptable to any protein expression system.

Prior to large-scale preparation, the cells should be tested for expression of the protein in soluble form (see *UNIT 16.2*). Even if protein is expressed mostly in insoluble form (i.e., bacterial inclusion bodies), there may be a small fraction that remains soluble and can therefore be purified using the Basic Protocol. If little or no soluble protein is observed, one of the denaturing protocols (see Alternate Protocols 1 and 2) should be used.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

STRATEGIC PLANNING

MCAC is designed to purify a specific protein whose complementary DNA (cDNA) sequence is available. A protein-expression system is selected and the cDNA is inserted into the appropriate expression vector (see Chapter 16). The cDNA sequence must encode a minimum of six histidines at either the amino or carboxyl terminus and must include an initiator methionine at the amino terminus and a termination codon at the carboxyl terminus. This can be accomplished by polymerase chain reaction (PCR) using primers containing unique restriction-site sequences at the 5′ end (Fig. 10.11B.1). Properly

A 5′-GGGNNNNNNATGCATCATCATCATCATCAT . . . $N_{15\text{-}30}$-3′
RE site – MetHisHisHisHisHisHis . . .

B 5′-GGGNNNNNNTTAATGATGATGATGATGATG . . . $N_{15\text{-}30}$-3′
RE site – ENDHisHisHisHisHisHis . . .

Figure 10.11B.1 Sequences of primers required to create histidine tails at protein termini. Functions (e.g., protein sequence) are marked below. Three guanines are included at the 5′ ends of each primer to facilitate restriction enzyme digestion of the PCR product prior to subcloning. NNNNNN represents a unique restriction enzyme site compatible with the selected vector. $N_{15\text{-}30}$ represents 15 to 30 additional nucleotides specific to the cDNA beginning with the second codon. Met represents an initiator methionine and END represents a termination codon. (**A**) Sequence of 5′ primer used to create a histidine tail at the amino terminus. The 3′ primer should include a second unique restriction enzyme site and the final 5 to 10 codons (including a stop codon) of the cDNA sequence. (**B**) Sequence of 3′ (antisense) primer used to create a histidine tail at the carboxyl terminus. The 5′ primer should contain a second unique restriction site and the first 5 to 10 codons of the cDNA sequence.

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designed primers will permit insertion and expression of the cDNA in the correct reading frame (see *UNITS 3.17, 15.1 & 15.7*).

An alternative to PCR is to subclone the cDNA into an existing vector, synthesize complementary oligonucleotides containing the hexahistidine sequence with compatible restriction enzyme site overhangs, and insert them into the subcloned cDNA (*UNIT 3.16*). It is important to insert the oligonucleotide into the cDNA near one of the termini in the correct reading frame.

Several companies (e.g., Qiagen, Novagen, and Invitrogen; see *APPENDIX 4*) sell expression systems that permit direct subcloning of cDNAs into vectors already containing oligohistidine tail sequences along with adjacent protease cleavage sites to allow removal of the tail after purification. Vectors have been developed for expression of histidine fusion proteins by bacteria, yeast, baculovirus, vaccinia, and various eukaryotic promoters. The protocols that follow are designed for use with a Novagen pET vector (Fig. 10.11B.2) but may easily be modified for other vectors.

NATIVE MCAC FOR PURIFICATION OF SOLUBLE HISTIDINE-TAIL FUSION PROTEINS

BASIC PROTOCOL

This protocol describes expression and purification of histidine fusion proteins in *E. coli*. A desired protein is induced in bacteria, which are then harvested and lysed. The lysate is loaded directly onto a column containing Ni^{2+}-NTA resin and the protein is eluted in nearly pure form with MCAC buffer containing imidazole.

Materials

M9ZB medium (see recipe) containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol
E. coli BL21(DE3)pLysS or other suitable strain (Novagen) containing a pET vector (Fig. 10.11B.2) expressing a histidine-tail fusion protein
0.1 M IPTG (Table 1.4.2), filter sterilized
NTA resin slurry: 50% (w/v) suspension in 20% (v/v) ethanol (Qiagen)
100 mM $NiSO_4 \cdot 6H_2O$
MCAC-0, MCAC-20, MCAC-40, MCAC-60, MCAC-80, MCAC-100, MCAC-200, and MCAC-1000 buffers (see recipe)
150× protease inhibitor cocktail (see recipe)
10% (v/v) Triton X-100
1 M $MgCl_2$
MCAC-EDTA buffer (see recipe)
DNase I solution (see recipe)

Centrifuge with Beckman JA-20 rotor or equivalent
1 × 10–cm glass or polypropylene column

Additional reagents and equipment for growth of bacteria in liquid medium (*UNIT 1.2*) and analysis and processing of purified proteins by SDS-PAGE (see Support Protocol 1)

Express the protein

1. Inoculate 10 ml M9ZB/ampicillin/chloramphenicol with *E. coli* BL21(DE3)pLysS containing a pET vector expressing a desired histidine-tail fusion protein. Grow overnight with shaking at 37°C (*UNIT 1.2*).

2. Inoculate 100 ml M9ZB/ampicillin/chloramphenicol with 1 ml of the overnight culture and grow with shaking at 37°C to OD_{600} = 0.7 to 1.0.

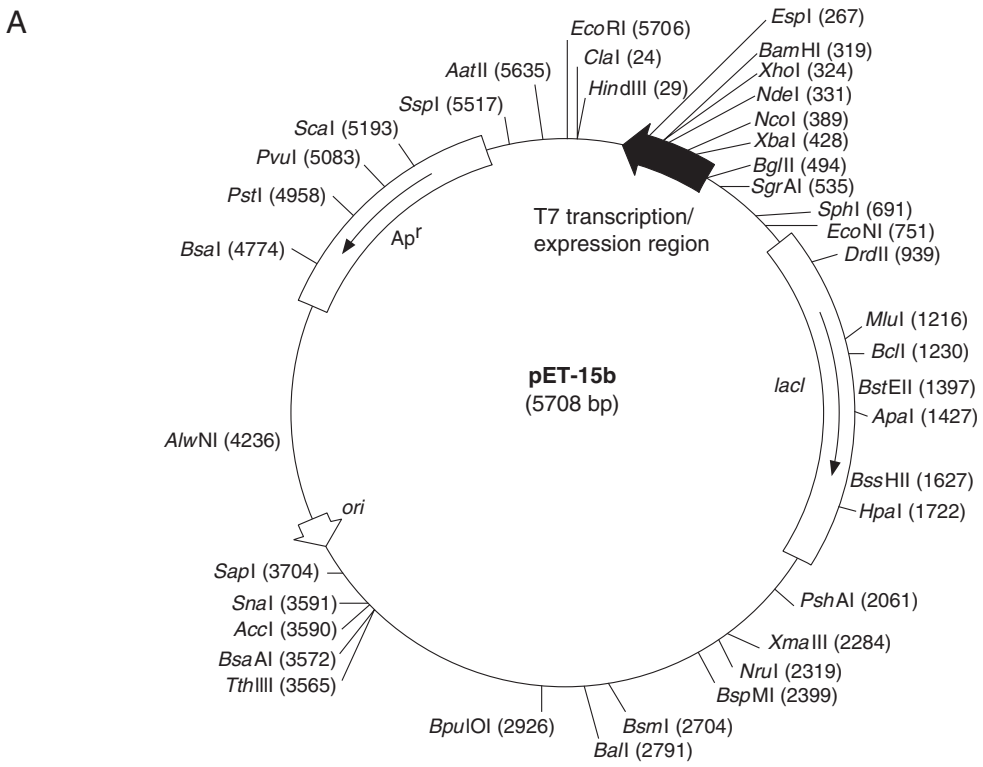

Figure 10.11B.2 pET-15b, one of a series of pET vectors designed for cloning, expression, and purification of recombinant proteins. Reprinted with the permission of Novagen. (**A**) pET-15b vector; (**B**) sequence of pET-15b cloning/expression region. The target gene is cloned into the pET plasmid such that its expression is under the control of bacteriophage T7 transcription and translation signals (see *UNIT 16.2*). pET-15b encodes an amino-terminal His·Tag leader that allows purification of the resulting recombinant protein over Ni^{2+}-NTA resin. Following purification, the His·Tag can be removed by thrombin cleavage (see *UNIT 16.7*). The pET series are derivatives of vectors originally described by Studier et al. (1990) and are available from Novagen. Figure reproduced by permission of Novagen, Inc.

3. Add 1 ml of 0.1 M IPTG (to 1 mM final) and continue shaking incubation 1 to 3 hr at 37°C.

   Incubation time will depend on the solubility of the expressed protein. A 3-hr incubation will allow maximal expression but may result in mostly insoluble protein. A 1-hr incubation will produce less protein but more of it may be soluble.

4. Centrifuge 10 min at 4400 × *g* (5000 rpm in a Beckman JA-20 rotor), 4°C. Discard supernatant. Freeze pellet at −70°C.

   It is not necessary to wash the cell pellet. The wet weight of the cell pellet will be ~0.5 g. The pellet can be stored indefinitely at −70°C before proceeding. Alternatively, extract preparation (steps 9 to 13) can be carried out immediately and the column prepared during the centrifugation at step 13.

Prepare the affinity column

5. Add 0.2 ml NTA resin slurry to a 1 × 10–cm column and allow liquid to drain.

   During this and subsequent column washes, liquid should be allowed to drain to the top of the packed resin bed that forms as resin settles (packed bed volume should be 0.1 ml) and resin should not be allowed to dry.

6. Wash column with 1 ml (5 bed volumes) deionized water.

7. Charge column by washing with 1 ml (5 bed volumes) of 100 mM $NiSO_4 \cdot 6H_2O$.

8. Wash column with 2 ml MCAC-0 buffer.

   NTA resin is nitrilotriacetic acid covalently coupled to Sepharose CL-6B. When charged with nickel ions, it has a light blue-green color, and when stripped of nickel, it is white. See supplier's instructions for resin preparation.

   Charged resin can be stored at 4°C. If column is to be stored >1 day, wash with 10 bed volumes of 20% ethanol and add 1 bed volume of 20% ethanol prior to storage. Keep column sealed to prevent evaporation.

Prepare the extract

IMPORTANT NOTE: Beginning with this step, all procedures should be performed on ice or in a cold room unless otherwise indicated.

9. Thaw cell pellet (from step 4) on ice. Add 5 ml MCAC-0 buffer and 33 µl of 150× protease inhibitor cocktail and resuspend by pipetting, sonication, or homogenization.

   All MCAC buffers contain phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor; to reduce expense, protease inhibitor cocktail is added only to the crude extract.

10. Add 0.05 ml of 10% (v/v) Triton X-100 (0.1% final). Mix thoroughly and subject the sample to 3 cycles of freezing at −70°C and thawing on ice.

    Ionic detergents may interfere with binding of the protein to the resin and should not be used.

    Cell lysis is evidenced by a visible increase in viscosity. The pLysS plasmid in these cells encodes an endogenous lysozyme that eliminates the need for exogenous lysozyme treatment to disrupt the bacterial cell wall.

11. Add 0.05 ml of 1 M $MgCl_2$ (final concentration 10 mM) and 0.05 ml of DNase I solution (final concentration 10 µg/ml DNase I). Mix gently and incubate 10 min at room temperature.

    The DNase I treatment reduces the viscosity of the lysate.

12. Centrifuge 15 min at 27,000 × *g* (15,000 rpm in JA-20 rotor), 4°C. Decant the supernatant into a clean container on ice and discard the pellet. Set aside and freeze a 10-µl aliquot at −70°C for later analysis by SDS-PAGE (see Support Protocol 1).

The supernatant can be frozen at −70°C indefinitely before continuing with the procedure.

Purify protein

13. If extract is frozen, thaw on ice. Load onto Ni^{2+}-NTA column and allow to flow through at a rate of 10 to 15 ml/hr. Collect column flowthrough and save for SDS-PAGE (see Support Protocol 1).

Charged NTA resin has a capacity of 5 to 10 mg histidine-tagged protein per milliliter of packed resin. The amount of extract that can be loaded on the column will depend on the amount of soluble histidine-tagged protein in the extract.

14. Wash column with 5 ml MCAC-0 buffer at a flow rate of 20 to 30 ml/hr. Discard flowthrough.

15. Wash column in stepwise fashion with 5 ml each of MCAC-20, MCAC-40, MCAC-60, MCAC-80, MCAC-100, MCAC-200, and MCAC-1000 buffers at a flow rate of 10 to 15 ml/hr. Collect 0.5-ml fractions and save on ice for SDS-PAGE (see Support Protocol 1).

Alternatively, the column can be eluted with a 5-ml linear gradient (UNIT 10.10) of 0 to 400 mM imidazole in MCAC buffer.

The second and third fractions of each wash will contain most of the eluted proteins. Most proteins with hexahistidine tails will remain bound in 60 mM imidazole (MCAC-60) and elute with 100 to 200 mM imidazole (MCAC-100 or -200); therefore, the purified protein will elute in MCAC-100 or -200. Proteins with longer histidine tails (e.g., 10 residues) bind to Ni^{2+}-NTA with greater affinity and require higher imidazole concentrations for elution. However, optimum washing and elution conditions must be determined for each protein.

Once optimum washing and elution conditions are established, it is possible to prepare the crude extract in a buffer that contains the highest imidazole concentration in which the histidine tail remains bound to the Ni^{2+}-NTA (e.g., MCAC-40 or -60 buffer). This decreases nonspecific binding of proteins to resin and permits use of a single buffer for extract preparation, column loading, and column washing.

16. Elute column with 1 ml MCAC-EDTA buffer at a flow rate of 10 to 15 ml/hr, collecting 0.5-ml fractions.

The blue-green color of the column will disappear as nickel is removed by EDTA. More tightly bound proteins may be found in these fractions. The resin can now be recharged (repeat steps 6 to 8) and the column reused.

If protein is eluted adequately with imidazole (as determined by overall yield for subsequent preparations), EDTA washing can be omitted. The column can be reequilibrated with MCAC-0 buffer and the purification repeated. The same column can be used three to five times before EDTA stripping and nickel recharging are necessary. Only one protein should be purified on any given column.

17. Analyze fractions for the presence of eluted protein.

An ultraviolet (280-nm) absorbance flow monitor is helpful for following column elution but is not necessary. An alternative is to measure the OD_{280} of individual fractions to identify protein-containing fractions. However, imidazole will also absorb at 280 nm.

A quick and easy method to determine which fractions contain eluted protein is to place 2 µl undiluted Protein Assay Dye Reagent Concentrate (Bio-Rad) on a piece of

Parafilm, add 8 µl from fraction to be tested, and mix by pipetting up and down. Immediate appearance of blue color indicates that the fraction contains protein. This does not work in the presence of Triton X-100 because the detergent itself produces an intense blue color; for this reason, Triton X-100 is excluded from the washing and elution buffers.

18. Combine the fractions containing eluted protein and remove a 10-µl aliquot for SDS-PAGE (see Support Protocol 1). Freeze the remainder in smaller aliquots at −70°C or in liquid nitrogen.

    If a different buffer for the protein is desired (e.g., for proteolytic removal of the histidine tail), the protein should be dialyzed against the buffer of choice to remove the MCAC buffer prior to storage at −70°C or in liquid nitrogen.

19. If time permits, proceed immediately to analysis of fractions by SDS-PAGE and processing of protein (see Support Protocol 1). Otherwise, freeze all samples at −70°C until ready for analysis and processing.

DENATURING MCAC FOR PURIFICATION OF INSOLUBLE HISTIDINE-TAIL FUSION PROTEINS

ALTERNATE PROTOCOL 1

High-level expression of foreign proteins in bacteria and other cells frequently results in poor solubility of the expressed protein (see *UNIT 16.4*). These insoluble proteins form inclusion bodies in bacteria, and strong chaotropic agents such as guanidine, urea, or SDS are usually required to solubilize them. These agents denature the protein and destroy the secondary structure that is essential to other affinity purification methods (e.g., maltose-binding protein or glutathione-*S*-transferase fusion proteins; *UNITS 16.6 & 16.7*). A significant advantage of metal-chelate affinity chromatography is that the oligohistidine tail will bind to the Ni^{2+}-NTA resin even when the protein is denatured. In denaturing MCAC, the protein extract is solubilized with 6 M guanidine and the entire affinity purification procedure is carried out in guanidine. The purified, denatured protein is renatured during dialysis.

Additional Materials *(also see Basic Protocol)*

GuMCAC-0, GuMCAC-20, GuMCAC-40, GuMCAC-60, GuMCAC-100, and GuMCAC-500 buffers (see recipe)
GuMCAC-EDTA buffer (see recipe)
Appropriate final buffer for protein (e.g., for proteolytic cleavage or long-term storage)
Guanidine·HCl
Additional reagents and equipment for analysis and processing of purified proteins (see Support Protocol 1) and dialysis (*APPENDIX 3C*)

Express the protein

1. Prepare the pellet of *E. coli* expressing a histidine-tail fusion protein (see Basic Protocol, steps 1 to 4).

   The pellet can be stored indefinitely at −70°C before proceeding. Alternatively, extract preparation (steps 5 and 6) can be carried out immediately and the column prepared during the centrifugation at step 6.

Prepare the affinity column

2. Prepare column (see Basic Protocol, steps 5 to 7).

3. Wash column with 2 ml GuMCAC-0 buffer.

   During this and subsequent column washes, liquid should be allowed to drain to top of packed resin bed and resin should not be allowed to dry.

Prepare cell extract

4. Thaw cell pellet (from step 1) on ice. Resuspend in 5 ml GuMCAC-0 buffer by pipetting, sonication, or homogenization.

5. Freeze 10 min at −70°C and thaw at room temperature.

   Protease inhibitors are omitted because proteases are inactivated by guanidine. Triton X-100 is not needed at this step. Freezing is not necessary but is included because it ensures complete lysis of cells.

   Subsequent steps can be performed at room temperature. However, if solid-phase renaturation is used (see Alternate Protocol 2), it is better to maintain lower temperatures throughout the process.

6. Gently mix samples for 30 min using a rocker, rotating mixer, or magnetic stirrer. Centrifuge 15 min at 27,000 × *g* (15,000 rpm in Beckman JA-20 rotor), 4°C. Decant supernatant into a clean container and discard pellet. Set aside a 10-µl aliquot for analysis by SDS-PAGE (see Support Protocol 1).

   The supernatant can be frozen at −70°C indefinitely before continuing with the procedure.

Purify protein

7. If extract from step 5 is frozen, thaw at room temperature. Load onto Ni^{2+}-NTA column and allow to flow through at a rate of 10 to 15 ml/hr. Collect flowthrough and save a 10-µl aliquot for SDS-PAGE (see Support Protocol 1).

8. Wash column with 5 ml GuMCAC-0 buffer at a rate of 20 to 30 ml/hr. Discard the flowthrough.

9. Wash column in stepwise fashion with 5 ml GuMCAC-20, -40, -60, -100, and -500 buffers at a rate of 10 to 15 ml/hr. Collect 0.5-ml fractions and save for SDS-PAGE (see Support Protocol 1).

   The second and third fractions from each wash will contain most of the unbound protein. The histidine tail binds slightly less avidly under denaturing conditions. Lower imidazole concentrations are therefore required for washing and elution than in the Basic Protocol.

10. Elute with 1 ml GuMCAC-EDTA buffer at a rate of 10 to 15 ml/hr, collecting 0.5-ml fractions.

11. Identify fractions containing the protein, pool together, transfer to dialysis tubing, and seal.

    Alternatively, fractions can be frozen at −70°C indefinitely before continuing with the procedure.

    Guanidine precipitates in the presence of SDS and must be removed by dialysis before SDS-PAGE. An alternative technique employs buffers that switch from 6 M guanidine to 8 M urea during affinity column washing (Stüber et al., 1990). This permits samples to be taken directly from urea fractions without dialysis and analyzed by SDS-PAGE or injected into animals for antibody production.

Renature purified protein by dialysis

12. Prepare appropriate final buffer for protein (e.g., for proteolytic cleavage or long-term storage) and add sufficient guanidine to bring final concentration to 4 M.

13. Dialyze purified protein from step 11 for ≥2 hr at 4°C against 500 ml buffer/4 M guanidine (see *APPENDIX 3C*).

    The MWCO of the dialysis membrane should be chosen to be smaller than the MW of the purified protein. In most cases an MWCO of 12 to 14 kDa is sufficient.

14. Remove 250 ml buffer/guanidine and add 250 ml buffer without guanidine. Continue dialysis ≥2 hr. Repeat.

    With some proteins, renaturation by dialysis may require longer dialysis periods and more gradual decrements in the guanidine concentration of the buffer. Conditions for each protein must be determined empirically.

15. Remove dialysis bag to a container containing 500 ml of fresh buffer without guanidine at 4°C. Continue dialysis 2 hr to overnight.

16. Remove sample from dialysis bag, divide into aliquots, and freeze at −70°C or in liquid nitrogen.

    If protein precipitates during dialysis, solid-phase renaturation (see Alternate Protocol 2), in which protein bound to the column is renatured before elution, should be employed.

17. Analyze fractions and process protein (see Support Protocol 1).

SOLID-PHASE RENATURATION OF MCAC-PURIFIED PROTEINS

ALTERNATE PROTOCOL 2

In Alternate Protocol 1, removal of denaturants by dialysis will occasionally lead to precipitation of protein, possibly due in part to entanglement or aggregation of separate protein molecules as they refold. To avoid this problem, solid-phase renaturation may be attempted. In this procedure, protein extract is prepared and bound to the column under denaturing conditions. A series of washes removes the denaturing agent before the target protein is eluted and the resulting renatured protein is eluted from the column under native conditions.

Additional Materials *(also see Basic Protocol)*

1:1 (v/v) MCAC-20/GuMCAC-20 buffer (see recipes)
3:1 (v/v) MCAC-20/GuMCAC-20 buffer (see recipes)
7:1 (v/v) MCAC-20/GuMCAC-20 buffer (see recipes)

1. Prepare protein extract, bind to column, and wash with GuMCAC buffers (see Alternate Protocol 1, steps 1 to 9).

2. Wash column with 5 ml of 1:1 (v/v) MCAC-20/GuMCAC-20 buffer.

    During this and subsequent washes, liquid should be allowed to drain just to top of packed resin bed and resin should not be allowed to dry.

3. Wash column with 5 ml of 3:1 (v/v) MCAC-20/GuMCAC-20 buffer.

4. Wash column with 5 ml of 7:1 (v/v) MCAC-20/GuMCAC-20 buffer.

5. Wash column with MCAC buffers, elute proteins, and analyze (see Basic Protocol, steps 15 to 19).

    Slow elution (between 1 and 2 hr) with a 5-ml linear gradient from 100% GuMCAC-20 buffer to 100% MCAC-20 buffer may also yield efficient renaturation.

SUPPORT PROTOCOL 1

ANALYSIS AND PROCESSING OF PURIFIED PROTEINS

The success of the purification scheme (particularly during a small pilot study) should be monitored at each stage by SDS-PAGE. If the fusion protein contains a specific protease cleavage site, the histidine tail can be removed using an appropriate proteolytic procedure, if desired.

Materials

Fractions from MCAC column purification (crude extract, flowthroughs, and purified protein; see Basic Protocol or Alternate Protocols 1 or 2)
2× SDS sample buffer (*UNIT 10.2*)
MCAC-0 buffer (see recipe)

Additional reagents and equipment for one-dimensional SDS-PAGE (*UNIT 10.2*), cleavage of proteins with factor Xa *or* thrombin (*UNIT 16.4B;* optional), and dialysis (*APPENDIX 3C*)

1. Thaw aliquots of fractions to be analyzed on ice. Mix 5 µl from crude extract and crude flowthrough fractions and 10 µl from the second and third fractions from each washing step with an equal volume of 2× SDS sample buffer.
2. Load samples onto a standard SDS-PAGE gel. Run gel and visualize to identify the fractions containing purified protein (*UNIT 10.2*).

   Guanidine must be removed by dialysis prior to addition of SDS sample buffer.
3. Thaw the remaining aliquots of fractions containing purified protein, dialyze against the appropriate proteolysis buffer, and carry out cleavage procedure if desired. If necessary, after cleavage dialyze the protein against an appropriate storage buffer and freeze in aliquots.

   The size of the cleaved histidine tail will generally be <3 kDa, depending on the design of the fusion protein. This fragment will usually be removed by the dialysis. It may also be removed by ultrafiltration (which will also concentrate the protein), size-exclusion chromatography, or a second MCAC (see Basic Protocol, steps 13 to 19). The advantage of a second MCAC is that the histidine tail and any uncleaved protein will bind to the column and only the cleaved protein will be found in the flowthrough from step 12. Aliquots of each fraction should be analyzed by SDS-PAGE to verify recovery of pure protein.

   If an expression vector other than pET has been used, and the fusion protein does not contain an appropriate cleavage site for factor Xa or thrombin, or if the histidine tail will not interfere with subsequent studies, omit the cleavage procedure. Presence of the histidine tail will not generally affect the protein's biologic functions (see Background Information).

SUPPORT PROTOCOL 2

NTA RESIN REGENERATION

NTA resin can be repeatedly charged with Ni^{2+} and stripped. Over time, however, some protein residue will accumulate and decrease the efficiency of the resin, resulting in slow flow rates or lack of blue-green color after charging with Ni^{2+}. Periodic regeneration (e.g., after 5 to 10 cycles of charging and stripping) permits recycling of resin for long-term use.

Materials

2.5 ml used NTA resin (packed volume)
Stripping solution: 0.2 M acetic acid/6 M guanidine·HCl
2% (w/v) SDS
20%, 25%, 50%, 75%, and 100% (v/v) ethanol
0.1 M EDTA, pH 8.0

1. Wash resin with 5 ml stripping solution.

   In this and all subsequent washes, liquid added to the column should be allowed to drain to top of packed resin bed and resin should not be allowed to dry.

2. Wash with 5 ml water.
3. Wash with 7.5 ml of 2% (w/v) SDS.
4. Wash with 2.5 ml of 25% (v/v) ethanol.
5. Wash with 2.5 ml of 50% (v/v) ethanol.
6. Wash with 2.5 ml of 75% (v/v) ethanol.
7. Wash with 12.5 ml of 100% (v/v) ethanol.
8. Wash with 2.5 ml of 75% (v/v) ethanol.
9. Wash with 2.5 ml of 50% (v/v) ethanol.
10. Wash with 2.5 ml of 25% (v/v) ethanol.
11. Wash with 2.5 ml water.
12. Wash with 12.5 ml of 0.1 M EDTA, pH 8.0.
13. Wash with 7.5 ml water. Proceed either to nickel charging (see Basic Protocol, step 7) or to step 14 below for long-term storage of the resin.
14. Add 2.5 ml of 20% (v/v) ethanol to resin and store at 4°C.

REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

DNase I solution

Dissolve lyophilized DNase I (U.S. Biochemical) at a final concentration of 1 mg/ml in 50% glycerol/1 mM $CaCl_2$. Store ≤1 year at −20°C.

***GuMCAC buffers** (store ≤6 months at 4°C)*

GuMCAC-0 buffer:
20 mM Tris·Cl, pH 7.9
0.5 M NaCl
10% glycerol
6 M guanidine·HCl

GuMCAC-500 buffer:
20 mM Tris·Cl, pH 7.9
0.5 M NaCl
10% glycerol
6 M guanidine·HCl
0.5 M imidazole

GuMCAC-20, GuMCAC-40, GuMCAC-60, and GuMCAC-100 buffers:

These buffers (containing different concentrations of imidazole) are made by mixing GuMCAC-0 buffer and GuMCAC-500 buffer in the appropriate ratios: e.g., for GuMCAC-20 buffer, use 96:4 (v/v) GuMCAC-0/GuMCAC-500.

GuMCAC-EDTA buffer

20 mM Tris·Cl, pH 7.9
0.5 M NaCl
10% glycerol
6 M guanidine·HCl
0.1 M EDTA, pH 8.0
Store ≤6 months at 4°C

M9ZB medium

Dissolve 10 g N-Z-Amine A (Sigma) and 5 g NaCl in 889 ml water. Autoclave, cool, and add 100 ml of 10× M9 medium (see *UNIT 1.1*), 1 ml of 1 M sterile $MgSO_4$, and 10 ml of 40% (w/v) glucose (filter sterilized). Store ≤1 year at room temperature.

MCAC buffers *(store ≤6 months at 4°C)*

MCAC-0 buffer:

20 mM Tris·Cl, pH 7.9
0.5 M NaCl
10% glycerol
1 mM PMSF (phenylmethylsulfonyl fluoride)

MCAC-1000 buffer:

20 mM Tris·Cl, pH 7.9
0.5 M NaCl
10% glycerol
1 M imidazole
1 mM PMSF

Add PMSF immediately before use from a 0.2 M stock in 100% ethanol stored at room temperature. All buffers used with Ni^{2+}-NTA resin contain high salt concentrations to reduce nonspecific electrostatic interactions between proteins and resin. Lower salt concentrations can be used but may lead to nonspecific binding of unwanted proteins to the resin.

MCAC-20, MCAC-40, MCAC-60, MCAC-80, MCAC-100, and MCAC-200 buffers:

These buffers (containing different concentrations of imidazole) are made by mixing MCAC-0 buffer and MCAC-1000 buffer in the appropriate ratios: e.g., for MCAC-60 buffer, 94:6 (v/v) MCAC-0/MCAC-1000.

MCAC-EDTA buffer

20 mM Tris·Cl, pH 7.9
0.5 M NaCl
10% glycerol
0.1 M EDTA, pH 8.0
1 mM PMSF
Store ≤6 months at 4°C

Protease inhibitor cocktail, 150×

Stock solutions:

2 mg/ml aprotinin in H_2O
1 mg/ml leupeptin in H_2O
1 mg/ml pepstatin A in methanol

Cocktail: Combine 1.5 vol of each stock solution with 5.5 vol sterile water.

Protease inhibitors can be obtained from Sigma or U.S. Biochemical. The stock solutions and cocktail are stable ≥6 months at −20°C.

COMMENTARY

Background Information

Metal-chelate affinity chromatography (also called immobilized metal affinity chromatography or IMAC) for protein purification was first described in 1975 (Porath et al., 1975). It is based on the ability of certain amino acids acting as electron donors on the surface of proteins (histidine, tryptophan, tyrosine, or phenylalanine) to bind reversibly to transition-metal ions that have been immobilized by a chelating group covalently bound to a solid support. Of these amino acids, histidine is quantitatively the most important in mediating the binding of most proteins to immobilized metal ions. Histidine binds selectively to immobilized metal ions even in the presence of excess free metal ions in solution (Hutchens and Yip, 1990b). Copper and nickel ions have the greatest affinity for histidine (Yip et al., 1989).

Proteins and peptides containing phosphoamino acids (phophoserine, phosphotyrosine, or phosphothreonine) have also been purified by MCAC via selective interaction of the terminal phosphate group with Fe^{3+} (Muszynska et al., 1992). Phosphoproteins also bind to less common metals such as lutetium, scandium, and thorium (Andersson and Porath, 1986).

The chelating group that has been used most extensively for MCAC is iminodiacetic acid (IDA). The tridentate IDA group binds to three sites within the coordination sphere of divalent metal ions such as copper, nickel, zinc, and cobalt. When copper ions (coordination number of 4) are bound to IDA, only one site remains available for interaction with proteins (Hochuli et al., 1987). For nickel ions (coordination number of 6) bound to IDA, three sites are available for binding to proteins. Thus Cu^{2+}-IDA complexes are stable on the column but have lower capacity for protein binding. Conversely, Ni^{2+}-IDA complexes bind proteins more avidly, but the Ni^{2+}-protein complexes are more likely to dissociate from the solid support.

A pentadentate chelating group—*N*,*N*,*N*′-tris(carboxymethyl)ethylenediamine (TED)—has also been used for MCAC. This chelator binds five coordination sites of the metal ions, providing highly stable metal ion–TED groups. However, for metal ions with a coordination number of 6, this leaves only one site available for protein interaction.

The development of a new metal-chelating adsorbent, nitrilotriacetic acid (NTA), has provided a convenient and inexpensive tool for purification of proteins containing histidine residues (Hochuli et al., 1987). The quadridentate NTA moiety covalently coupled via a spacer arm to Sepharose CL-6B forms an extremely stable complex with metal ions possessing a coordination number of 6 (e.g., Ni^{2+}). After binding to the NTA, the Ni^{2+} ion has two sites within its octahedral coordination sphere available for binding to electron donor groups (i.e., histidine) on the surface of proteins. Thus the advantage of NTA over IDA is that the Ni^{2+} ion is bound by four rather than three of its coordination sites. This minimizes leaching of the metal from the solid support and allows for more stringent purification conditions. The NTA also binds Cu^{2+} ions with high affinity, but this occupies all of the coordination sites, rendering the resulting complex ineffective for MCAC.

The affinity of histidine residues for immobilized Ni^{2+} ions allows selective purification of proteins containing a high proportion of histidine residues on the surface. Immobilized copper or nickel ions bind native proteins with a K_d of $1–17 \times 10^{-5}$ M (Hutchens and Yip, 1990a). This affinity is enhanced significantly by designing proteins to contain short stretches (6 to 10 residues) of histidines in regions likely to be surface exposed (amino or carboxyl termini) using recombinant DNA technology. Addition of a histidine tail results in a protein that binds to the Ni^{2+}-NTA complex with a K_d of 10^{-13} M at pH 8.0 even in the presence of detergent, ethanol, 2 M KCl (Hoffmann and Roeder, 1991), 6 M guanidine (Hochuli et al., 1988), or 8 M urea (Stüber et al., 1990).

The histidine tail binds to the Ni^{2+}-NTA resin via the imidazole side chains of the histidine residues. At pH ≥7.0, the imidazole side chain is deprotonated, with a net negative charge; at pH 5.97 (the pK of the imidazole side chain of histidine), 50% of the histidines are protonated; and at pH ≤4.5, almost all of the histidines are protonated and do not interact with Ni^{2+}-NTA. Thus, there are two methods of dissociating histidine tails from the Ni^{2+}-NTA resin. The first method, presented in this unit, uses increasing concentrations of imidazole at constant pH to displace the histidine tail from the Ni^{2+}-NTA. This technique provides great versatility because it can be used for both native and denaturing MCAC and buffer preparation is simplified. The second method uses buffers

of decreasing pH to elute the histidine tail and is quite efficient for producing pure protein (Hochuli et al., 1988). Disadvantages are that the pH must be maintained accurately at all temperatures and that some proteins may not be able to withstand the extreme pH change.

Creation of a small histidine tail has advantages over other fusion protein systems. Addition of six histidines to the protein adds only 0.84 kDa to the mass of the protein, whereas other fusion protein systems utilize much larger affinity groups that often must be removed to allow normal protein function (e.g., glutathione-*S*-transferase, protein A, maltose-binding protein, and lacZ have masses of 26, 30, 40, and 116 kDa, respectively). The small histidine tail is not immunogenic and therefore need not be removed before the purified protein is injected into animals for antibody production. Histidine tail fusion proteins often retain their normal biologic functions—e.g., dihydrofolate reductase and adenylylcyclase retain their enzymatic activities (Hochuli, 1990; Taussig et al., 1993) and the TATA box binding protein retains its transcriptional regulatory function (Parvin et al., 1992). The aforementioned studies also demonstrate the technique's suitability for purification of membrane, cytoplasmic, and nuclear proteins. However, although it often is not necessary to remove the histidine tail, it is possible to include this step and still generate sufficient quantities of protein pure enough for detailed X-ray crystallographic studies (Nikolov et al., 1992).

The histidine tail can also be used to detect the recombinant protein. Ni^{2+}-NTA covalently coupled to alkaline phosphate (available from Qiagen; see *SUPPLIERS APPENDIX*) can bind directly to histidine tailed proteins, and the bound alkaline phosphatase can be quantitated by standard techniques (*UNIT 10.8*). This allows for direct quantitation of histidine-tailed proteins blotted to membranes without the need for primary and secondary antibodies (Botting and Randall, 1995).

The protocols presented in this unit utilize protein produced in bacteria; an example protocol for the purification of HIV-1 integrase expressed in *E. coli* is presented in *UNIT 6.5*. Histidine-tail fusion proteins have also been expressed and purified from HeLa cells using transient transfection techniques (Janknecht and Nordheim, 1992; Papavassiliou et al., 1992) or a vaccinia expression system (Janknecht et al., 1991) and from baculovirus-infected insect cells (Gearing et al., 1993; Taussig et al., 1993). Therefore, MCAC can be used with any protein expression system currently available.

This technique can also be used to prepare immunoaffinity columns (*UNIT 10.11A*). Histidine tails have been covalently linked to oligosaccharide moieties of monoclonal antibodies and then subsequently bound to Ni^{2+}-NTA columns (Loetscher et al., 1992). The advantage of this method over more conventional techniques of antibody immobilization is that the immobilized antibody will retain a much greater ability to bind antigen and is recoverable from the column.

Critical Parameters

NTA can be synthesized according to the protocol provided by Hochuli (1990); more commonly, it is purchased from Qiagen, which provides detailed protocols for its use.

Binding and elution of proteins from the Ni^{2+}-NTA resin depend upon the histidine content of the protein. Each protein will have a slightly different histidine content and optimal elution profile on the Ni^{2+}-NTA resin. Therefore, it is important to establish the buffer imidazole concentrations that will give the best purification of a particular protein. As outlined in the Basic Protocol, gradient or stepwise elution of small-scale preparations should be done before large-scale preparations are attempted. It is also possible to perform batch purification by adding charged resin to a tube containing the extract, mixing for 1 hr, loading into a column, washing, and eluting.

Troubleshooting

The most common problem is low yield of purified protein in the expected fraction. This can be caused by insufficient protein loaded on the column, protein not binding to the column, or protein not eluting from the column.

Insufficient protein

It is important to verify that the desired protein is adequately expressed in the chosen system. Check the crude extract for the presence of the expressed protein by SDS-PAGE or other methods before proceeding to the affinity purification steps.

Protein in the crude lysate may undergo significant proteolytic degradation in some cell types. Always use PMSF, minimize freezing and thawing, maintain low temperatures during purification, and use additional protease inhibitors whenever practical.

Lack of binding

If the protein does not bind to the column, either the protein lacks a histidine tail, the column is inadequately charged with nickel, or the histidine tail is buried in the protein and is inaccessible to the resin. Before expression and purification are attempted, verify that the DNA sequence of the histidine tail and adjacent protein-coding sequence are in frame with each other and that initiation and termination codons are present. If the histidine tail is at the amino terminus, it may be helpful to delete the endogenous codon for initiator methionine; if this is present, translation may initiate downstream of it, producing a full-length protein without a tail. Placing the histidine tail at the carboxyl terminus ensures that only the full-length protein will bind to the Ni^{2+}-NTA resin (if protease activity is controlled). However, a histidine tail at the carboxyl terminus is more likely to be inaccessible to the resin.

Chelating agents (e.g., EDTA and EGTA) must be excluded from all solutions because they will strip the Ni^{2+} ions from the affinity column. Strong reducing agents such as dithiothreitol should also be avoided because they reduce the Ni^{2+} ions to metallic nickel, forming a brown precipitate. Some proteins will require the presence of reducing agents to maintain structure. It is possible to use up to 10 mM 2-mercaptoethanol with the Ni^{2+}-NTA resin, although the lowest possible concentration should be used.

The Ni^{2+}-NTA should retain a distinct blue-green color throughout the purification process until EDTA is applied. If the resin is white or if the blue-green color is faint after charging the column with Ni^{2+}, check all buffers and make sure no chelating and reducing agents are present. Recharge the column with Ni^{2+} and if the color is still faint or absent, regenerate the NTA resin (see Support Protocol 2) and repeat Ni^{2+} charging (see Basic Protocol, steps 6 to 8). If the resin is still white, discard it and start over with a new batch.

If the protein has or should have a histidine tail but is not binding to the resin, perform the purification under denaturing conditions (see Alternate Protocol 1 or 2) to unmask the inaccessible histidine tail. Placing the histidine tail at the opposite terminus of the protein may also be helpful.

Failure to elute

If the protein appears to be binding to the column but not eluting, make a final elution with EDTA-containing buffer. Any protein bound to the Ni^{2+}-NTA will be removed with the EDTA. Occasionally the protein bound to the resin will precipitate and not elute; if this problem is suspected, purify the protein under denaturing conditions and analyze all eluted fractions by SDS-PAGE for the presence of the expressed protein.

Anticipated Results

The maximum binding capacity of Ni^{2+}-NTA resin is 5 to 10 mg of protein per milliliter of packed resin. Under ideal conditions, as much as 80% of the bound protein can be recovered. The limiting factor in most instances will probably be the amount of protein loaded on the resin, which depends on the protein expression system.

Time Considerations

Expression of protein and preparation of crude extract require 1 day for the system described here. Column preparation requires 30 to 60 min. Loading, washing, and eluting the column require 4 to 6 hr.

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Key Reference

Hochuli, 1990. See above.

Describes basic principles of MCAC with detailed protocols.

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