

Discussion Summary. Techniques and Problems in Metal-Binding Protein Chemistry and Implications for Proteins in Nonmammalian Organisms

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Proper isolation and purification techniques for metallothionein (MT) and related proteins in nonmammalian species are essential to the study of these molecules at all levels of biological organization. There are a number of basic approaches to preparing these proteins from biological tissues that have both positive and negative aspects depending upon the subsequent scientific questions under investigation. A general flow diagram for the isolation of MT and related proteins is given in Figure 1. The following discussion will attempt to examine each of the steps in this general isolation procedure and point out both the advantages and known disadvantages in each procedure.

Homogenization Procedures

If tissues are to be stored frozen, this should be done at -70°C . Inhibition of endogenous tissue proteases during homogenization procedures is essential to preventing degradation of MT and related proteins. A number of protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF) or α_2 -macroglobulin have been commonly used to inhibit protein degradation with varying degrees of success. PMSF dissolved in ethanol and added to a final concentration of 0.5 to 1.0 mM is usually effective in preventing proteolysis by serine proteases. Since PMSF hydrolyzes quickly, it should be added directly to sample. In addition, utilization of reducing agents such as β -mercaptoethanol (β -ME) or dithiothreitol (DTT) to prevent copper oxidation of protein SH groups may be helpful. In the case of β -ME 5 to 20 mM final concentration is sufficient, whereas only 0.5 to 1.0 mM DTT is necessary (1) because of its higher reducing potential.

While addition of β -ME or DTT and use of nitrogen-

gassed buffers appears to be essential for proper study of copper-containing proteins (2,3), these agents may alter the normal intracellular metal-binding profiles for metals such as Cd, Zn, or Hg because of their ability to form complexes with metal ions. The relative importance of this knowledge depends upon which scientific questions are being asked in the system under study.

Centrifugation

Following homogenization, a centrifugation step is commonly used to remove particulate matter such as unbroken cells and various organelles prior to initiation of chromatographic procedures. One potential problem of interest in this step concerns the finding in rainbow trout liver (4) that endogenous lipids in the livers of this fish species are capable of trapping some metal-binding proteins and thus produce an artificial metal distribution during subsequent chromatographic steps. The solution to this problem simply involves centrifuging the homogenate for 2 hr at 100,000g to get maximal aqueous/lipid separation and then carefully removing and discarding the lipid layer prior to column chromatography.

Heat Treatment of Supernatant

Heat treatment (65°C) of the supernatant fraction derived from centrifugation of the homogenate is a commonly used procedure derived from the mammalian metallothionein literature. The advantage of this technique is that it preferentially removes soluble high molecular weight proteins from the supernatant leaving MT and other heat-stable proteins in solution for subsequent chromatography. This reduces the total amount of protein to be loaded on the column and viscosity of the protein solution, thereby minimizing column loading artifacts. Disadvantages of this technique are that for some proteins such as crab metallothionein (5) decreased yields of recovered protein are observed, while for others such as

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the oyster (6) no apparent differences are noted. This technique is not recommended for unknown proteins or copper-metallothioneins, since it may increase SH group oxidation.

Chloroform-Ethanol Extractions

Another technique which has proven useful for isolating mammalian MT has been to utilize chloroform-ethanol extraction of MT followed by acetone precipitation to concentrate these proteins before column chromatography. One potential problem noted for this technique involves generation of a blocked N-terminal by the chloroform if only small amounts of protein are present during this procedure.

Column Chromatography

Gel Filtration

The initial isolation of MT and other metal-binding proteins usually involves gel-filtration chromatography

with Sephadex G-75 or G-50 columns. For CuMT or similar proteins, N_2 -bubbled buffers and β -MT or DTT are used to prevent oxidation of SH groups. Aggregates are frequently a problem with SH-rich proteins and these may be usually reversed by addition of β -ME and 9 M urea. It should also be noted that G-50 is advantageous over G-75 with respect to flow rates and reduced bed packing. Many of the gel filtration resins also exhibit some metal-binding capacity due to the presence of sulfonic acid groups and thus some ion-exchange effects may also be observed during this type chromatography unless high-salt buffers are used. The effects of buffer salt concentrations on the Stokes radius of MT has also been recently reported (7) and found to influence elution position of this protein from gel-filtration columns. The advent of gel permeation columns for high-performance liquid chromatography (HPLC) has proven useful in the isolation of a number of MT-like proteins (8,9), but potential problems of metal-ion interaction with stainless steel column linings may influence results depending upon the column manufacturer and quality of steel used. Similar problems have been observed with mono-Q col-

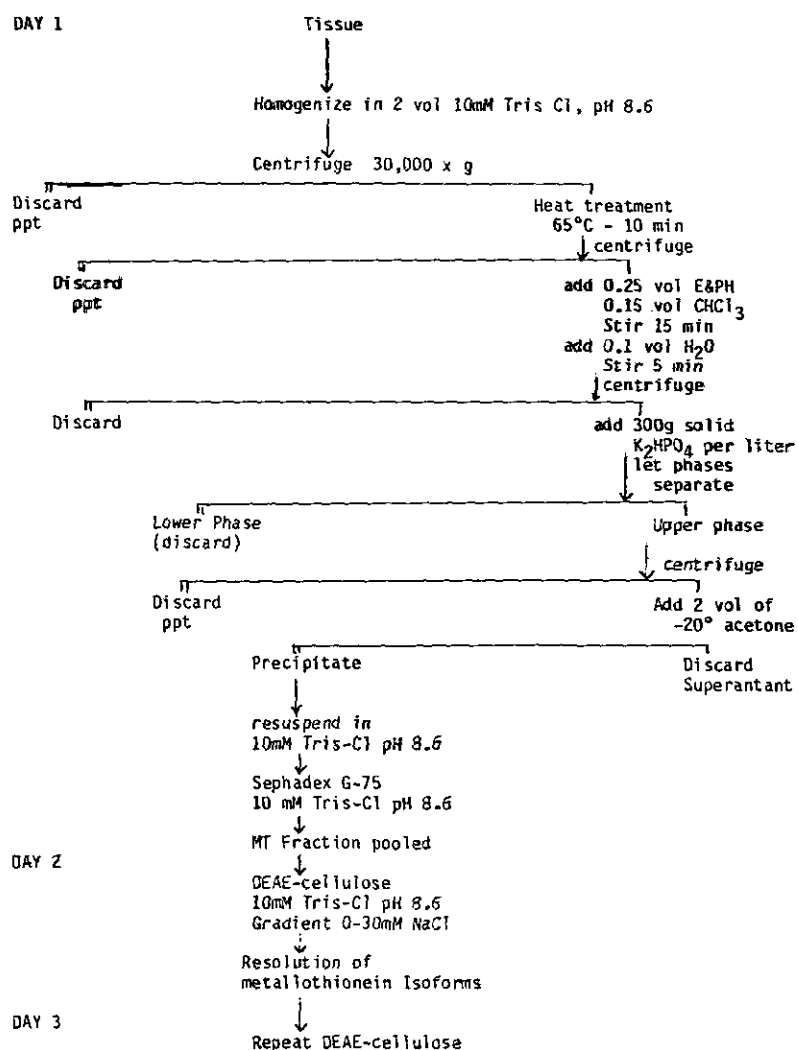


FIGURE 1. General scheme for isolation and purification of metallothionein and related proteins.

umns used for fast performance liquid chromatography (FPLC) of crab hepatopancreas MT (10).

Ion-Exchange Chromatography

The next step in the purification process usually involves DEAE-ion-exchange chromatography with elution of the protein(s) with either a Tris-Cl or NaCl gradient. Success of this procedure is dependent upon the metal composition of the protein with high Cu/Zn ratios altering the relative elution position of MT in the gradient. The use of high pH (8.6) and shallow salt gradients has been found to improve resolution of proteins. Lower pH values for buffer systems (pH 7.4) have been observed to yield "bent" conductivity gradients for Tris-Cl elution gradients. The chemical explanation of this phenomenon is unknown. Finally, ion-exchange rechromatography of peaks from initial ion-exchange column may be of great benefit in separating isoforms of the various MT or MT-like proteins.

Criteria of Purity

Electrophoresis

The object of this step is to demonstrate the lack of contaminants in the final protein preparation. There are a number of procedures currently used for this important aspect of protein purification; each with its own advantages and potential disadvantages. Electrophoresis of purified protein preparation is one of the most commonly used techniques, and it is recommended that several gel systems including both denaturing and nondenaturing gels (11) be used. The question of how much protein to load on the gel depends upon the stainability of the protein under study and whether the gels are to be stained with Coomassie blue (11) or the much more sensitive silver staining techniques (12). One problem encountered with some MT-like proteins from species such as the oyster (12,13) is loss of metals such as Cd^{2+} from the protein during electrophoresis and migration with the tracking dye front. A potential result of this phenomenon is the generation of multiple protein charge species (bands) in the gel during electrophoresis. Potential solutions to this problem may involve pre-electrophoresis of the gels to remove oxidants and use of protein carboxymethylation procedures to prevent crosslinking of SH groups following metal removal.

High-Performance Liquid Chromatography (HPLC)

HPLC involving use of reverse-phase, gel-permeation or ion-exchange columns offers another potential approach for assessing homogeneity of protein samples due to the superior resolution and sensitivity of this technique. Potential problems associated with the technique are centered around the possibility of contaminating proteins not eluting from the columns and hence being undetected.

Endgroup Analysis, Amino Acid Sequencing, Amino Acid Composition

These techniques are of great potential value providing any contaminating proteins are not blocked on the N-terminal. Acetyl or pyroglutamate are common blocking groups which could render contaminating proteins undetectable to sequencing techniques. Amino acid composition as an index of purity is highly useful for proteins such as mammalian MT, where amino acid composition is well-known, but for less-studied proteins such as those found in nonmammalian species, this approach is probably of limited value.

In the final analysis, homogeneity of purified proteins is a very difficult task, and only through the combined application of several techniques may a reasonable measure of homogeneity be assured.

Protein Quantitation

Another difficult area of protein chemistry involves reliable quantitation of the purified protein. A variety of techniques are available ranging from colorimetric methods such as the Lowry method (14), thiol titration (15), and polarographic methods (16), calculation of molar extinction coefficients (17) for apo-MT and immunochemical procedures (18). Each of these approaches to protein quantitation has its advantages and potential problems. Only through a more rigorous and complete comparison of these various procedures will the best technique for a given MT-like protein be determined. Amino acid analysis is the most rigorous method if composition of the molecule is known. Obviously this is valid only for a purified protein.

Sample Preservation

Purified protein should probably be stored at -70°C as a frozen liquid rather than as a lyophilized powder. Protein solutions are usually more stable if the solution is greater than 1 mg/mL.

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