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Purification of Arsenic (+3 Oxidation State) Methyltransferase from Rat Liver Cytosol

Zuzana Drobna¹, Miroslav Styblo^{1,2}, and David J. Thomas³

¹Department of Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7461

²Center for Environmental Medicine, Asthma, and Lung Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7310

³Pharmacokinetics Branch, Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park North Carolina 27709

Abstract

Demonstrating the enzymatic basis of arsenic methylation is critical to further studies of the pathway for the conversion of inorganic arsenic into a variety of methylated metabolites. This protocol describes a procedure for the purification of an arsenic methyltransferase from rat liver cytosol. Purification of this enzyme and subsequent cloning of its gene has permitted studies of enzyme structure and function and has led to the identification of orthologous genes in genomes of organisms ranging in complexity from sea urchins to humans. These proteins are referred to as arsenic (+3 oxidation state) methyltransferases.

Keywords

arsenic; methylation; methyltransferases; protein purification; chromatofocusing; affinity chromatography

Basic Protocol

Protocol Title - Purification of rat As₃mt

Introduction—This protocol describes the purification from rat liver cytosol of arsenic (+3 oxidation state) methyltransferase (As₃mt), the enzyme that catalyzes reactions in which a substrate arsenical is methylated. Typically, the substrates for these methylation reactions are arsenite (iAs^{III}), methylarsonous acid (MAs^{III}), or dimethylarsinous acid (DMAs^{III}). In this procedure, a postmicrosomal fraction prepared from the perfused liver of adult male

Address correspondence to: David J. Thomas, MD B143-1, US EPA, 109 Alexander Drive, Research Triangle Park North Carolina 27709; telephone - 919-541-4974; fax - 919-541-1937; thomas.david@epa.gov.

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Fischer 344 rats is used as the starting material for enzyme purification. This material is initially subjected to acid fractionation followed by chromatofocusing and S-adenosylhomocysteine agarose affinity chromatography. At each step in the purification procedure, an assay based on the conversion of [^{73}As] arsenite to methylated products is used to track the enzymatic activity. This procedure achieves >9000-fold purification of protein with a mass of ~42kDa. Purification of this protein is the first step in kinetic characterization of the enzyme and cloning of its cognate gene

Materials List—Male (4 to 6 weeks old) male Fischer 344 rats (Charles River Laboratories, Raleigh, NC)

Phenobarbital (or another appropriate anesthetic agent)

70 percent Isopropanol

Trizma base - 2-amino-2-(hydroxymethyl)-1,3-propanediol

Trizma HCl - tris(hydroxymethyl)aminomethane hydrochloride

Sucrose

Glycerol

Glutathione

D,L-Dithiothreitol

Acetic acid, glacial

Sodium phosphate, monohydrate

Disodium phosphate, heptahydrate

Hydrochloric acid

Polybuffer Exchanger gel (PBE94)

Polybuffer 96

6-aminohexanoic acid agarose

S-adenosylhomocysteine

(1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride)

S-adenosylmethionine, p-toluenesulfonate salt

Cuprous chloride

Hydrogen peroxide (30%)

Ammonium hydroxide

Sodium bicarbonate

Equipment—Refrigerated ultracentrifuge – Must be able to attain $110,000 \times g$

Glass pestle with loose fitting teflon pestle

pH meter and electrode

Protocol Steps

Part 1 - Preparation of rat liver cytosolic fraction

1. Anesthetize the donor rat with phenobarbital or another appropriate anesthetic agent. Secure the fully sedated rat in the dorsal recumbent position.

Investigators should consult with animal care committees and veterinarians for advice on animal husbandry and selection of anesthetic.

2. Thoroughly clean the ventral surface from xyphoid process to pubis with 70 percent isopropanol.
3. Make midline incision through dermis and underlying tissue. Reflect the skin and secure it out of operative field.
4. Make a midline incision through abdominal wall, exposing the peritoneal membrane. Reflect the abdominal wall and secure it out of operative field. Divide the peritoneal membrane, exposing the ventral aspect of the liver. Reflect the liver and secure it out of operative field.

Investigators should consult atlases of rat anatomy to become familiar with the anatomical features critical to successful *in situ* liver perfusion

5. Expose and isolate the inferior vena cava. Place a loose ligature around the inferior vena cava below the level of liver hilus.
6. Insert a 20 gauge butterfly type needle into the inferior vena cava at about the level of the renal artery and secure it by tightening the ligature. Place a small clamp around the inferior vena cava posterior to secure the needle.
7. Section the aorta anterior to liver (at the ventral margin of the diaphragm). Sectioning of this vessel permits perfusion of liver and causes death.
8. Perfuse liver *in situ* with 120 to 180 ml of ice-cold Buffer A.

Removal of residual blood from the liver is important in preparation of rat liver cytosol for use in purification of As methyltransferase activity. A well-perfused rat liver rapidly changes color from dark red brown to light tan. Uniformity of color across all lobes of the perfused liver is evidence of successful perfusion.

9. Remove perfused liver *en bloc* and clean it of adherent connective tissue and blood vessels.

10. Mince perfused liver into ~ 5 mm blocks and rinse in ice cold Buffer A. Collect and weigh minced liver.
11. Homogenize minced liver in Buffer A at a 1/5 (w/v) ratio in ice cold Buffer A, using a glass mortar-teflon pestle homogenizer with a loose fitting pestle. Cool mortar in ice water bath before addition of tissue. Homogenize liver with about 10 to 12 strokes of homogenizer, cooling mortar on ice after every 3 or 4 strokes.
12. Transfer homogenate to appropriate tubes for centrifugation. Spin homogenate at $110,000 \times g$ for 45 minutes at 4°C. This separation yields a pellet and a supernate designated as rat liver cytosol.

The supernatant fraction produced in this separation includes a floating layer of lipids at the air-supernatant interface. The lipid layer is removed with a Pasteur pipette that has been modified by introduction of a U-shaped curve in its tip. As shown in Figure 1 of **Chapter 2, “In vitro Assays of Inorganic Arsenic Methylation”** Basic Protocol - Inorganic arsenic methylation in rat liver cytosol assay system, the U-shaped tip of the pipette is carefully inserted through the lipid layer. The lipid material overlying the supernate is then slowly aspirated. This procedure can yield a cytosolic fraction with little contaminating lipid.

Part 2 - Acid Treatment of Rat Liver Cytosol: Figure 1 shows the overall strategy for purification of As3mt from rat liver. All steps in this scheme are performed at 0 to 4°C.

1. To a beaker containing the freshly prepared rat liver cytosol that is continuously stirred, add 1 M acetic acid dropwise. Monitor pH of the mixture until the target pH of 5 is reached. Centrifuge this mixture at $10,000 \times g$ for 15 minutes to produce a pellet and a supernatant fraction.
2. Transfer supernate to a beaker cooled in an ice bath and adjust the pH to 8.3 by dropwise addition of 1 M ammonium hydroxide while continuously stirring.

Part 3 - Chromatographic separations for purification of As3mt

1. Chromatofocus the pH 8.3 supernate on a column of Polybuffer Exchanger 94 (PBE 94) gel using Buffer B as eluant. Determine the As methyltransferase activity of fractions from chromatofocusing and pool active fractions for affinity chromatography.

Chromatofocusing separates proteins on the basis of isoelectric points. PBE 94 used for this separation consists of 90 μm Sepharose CL-6B particles to which secondary, tertiary, and quarternary amines are coupled by ether linkages. The eluant for PBE 94 is Polybuffer 96, a mixture of amphoteric buffers with different pI and pKa values. Polybuffer 96 generates a uniform pH gradient over the region of interest (pH 9 to pH 6). Use of chromatofocusing media is described online at <https://proteomics.amershambiosciences.com/aptrix/upp01077.nsf>).

2. Prepare S-adenosylhomocysteine (AdoHcy)-agarose following the method of Reeve and coworkers (Reeve et al., 1998). Suspend 5 ml of 6-aminohexanoic acid agarose (11.4 mmol of aminocaproate per ml of agarose) in 500 ml of deionized water. Allow the suspension to separate, decant the supernate, and place the washed 6-aminohexanoic acid agarose in a 50 ml beaker. In a test tube, dissolve 46 mg (~119 μ mol) of AdoHcy in 2 ml of deionized water. Add 1 N HCl dropwise to assure dissolution of AdoHcy. When AdoHcy is dissolved, adjust the pH of this solution to > 2 by dropwise addition of saturated solution of sodium bicarbonate. Take 0.02 ml aliquot of this mixture for use in determination of the efficiency of coupling of AdoHcy to agarose (see below). Add the AdoHcy solution to the 6-aminohexanoic acid agarose slurry. To this mixture add 100 mg (52 μ mol) of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride. Hold this mixture at room temperature for one hour with intermittent gentle stirring. Monitor pH of the mixture during this hour and, if necessary, adjust to pH 4.5 to 6 with dropwise addition of a saturated solution of sodium bicarbonate. After one hour of gentle mixing, let mixture stand at room temperature overnight. At the end of the coupling period, take 0.02 ml aliquot of the liquid phase for use in determination of the efficiency of coupling of AdoHcy to agarose. The AdoHcy-agarose is washed in sequence with 500 ml of deionized water, 500 ml of 1 M NaCl, and 500 ml of deionized water. Washed AdoHcy-agarose can be stored at 4°C in deionized water with 0.4% NaN₃.

The principle of the affinity chromatographic step is to selectively retain proteins in the eluate from chromatofocusing on the basis of their binding to AdoHcy that is immobilized on agarose. Proteins bound to AdoHcy are then eluted from the column by addition of AdoMet to the eluant. Here, an AdoHcy-agarose column initially charged with pooled active fractions from chromatofocusing is washed with starting buffer to remove proteins that do not bind (or bind weakly) to immobilized AdoHcy. The chromatographic column is then eluted with a buffer containing a competitive ligand, AdoMet. The presence of AdoMet in the eluting buffer causes the release of proteins bound to AdoHcy. For preparations starting with 150 g of rat liver, the column for AdoHcy-agarose affinity chromatography had bed dimensions of about 30 $\times$ 1.5 cm. Among these proteins is an ~ 42 kDa protein with As methyltransferase activity.

Preparation of this affinity gel requires the attachment of AdoHcy to a spacer molecule that is tethered to agarose beads. The extent of coupling of AdoHcy to agarose beads can be determined spectrophotometrically by determining the concentration of AdoHcy in the liquid phase of the coupling mixture before and after reaction with agarose. Add 0.02 ml aliquots of coupling mixture taken before or after the overnight reaction period to 0.98 ml of deionized water and determine absorption at 259 nm. Use molar extinction coefficient of AdoHcy (ϵ_{259} = 15300) to calculate moles of AdoHcy removed from the liquid phase of the reaction mixture during the coupling period. Coupling efficiency and ligand loading (mmol

AdoHcy per ml of gel) can be calculated from these data. Reeve and coworkers (Reeve et al., 1998) reported a coupling efficiency of 20% and a ligand loading of 4.8 mmol per ml of gel.

3. Transfer AdoHcy-agarose to a chromatographic column and equilibrate gel with Buffer C. Apply the pooled active fractions from chromatofocusing to the column of AdoHcy-agarose.
4. Elute gel with 5 bed volumes of Buffer C followed with Buffer D containing 1 mM AdoMet. Collect fractions and assay for As methyltransferase activity.

Figure 2 shows the pattern of purification of proteins from rat liver that possess a high level of As methyltransferase activity. A single Coomassie Brilliant Blue reactive band in lane 4 indicates that this procedure highly enriches a protein of ~ 42 kDa that catalyzes the methylation of iAs. The extent of enrichment of enzyme activity at each step in the purification process is shown in Table 1. Overall, about a 9000-fold purification was achieved. Notably, the sharp rise in the total activity between the acidification step and the chromatofocusing step suggests that an endogenous inhibitor of the As methyltransferase activity might have been removed by chromatofocusing

The procedure is based on the use of about 150 g of rat liver as starting material. Inclusion of GSH and D, L dithiothreitol (DTT) in Buffers A - D is critical to preservation of As methyltransferase activity. This procedure can be interrupted after preparation of treated supernate; this material can be stored overnight at 4°C without loss of activity in later steps. All other steps in the purification procedure are performed without extended storage of chromatographic fractions.

Determination of protein concentrations in fractions prepared in the course of purification of As3mt provides the denominator for use in calculations of the specific activities. Inclusion of GSH in Buffers A – D poses special concerns for protein quantitation. Assays for protein quantitation that involve binding of a dye to proteins (e.g., Coomassie brilliant blue binding in the Bradford assay) or the interactions between Cu ions and peptide bonds (e.g., Lowry's assay with Folin-Ciocalteu phenol reagent or assays with bicinchoninic acid) will detect GSH. Hence, using any of these assays will require use of blanks that include the appropriate amount of the GSH-containing buffers. Alternatively, proteins can be quantified in samples after dialysis to exchange the GSH-containing buffers for GSH-free buffers. However, dialysis can result in nonspecific and variable protein loss. Protein concentrations in fractions can also be estimated on the basis of absorption at 280 nm. Using this method also requires inclusion of blanks that include the appropriate amount of the GSH-containing buffers and calibration using standards containing known amounts of protein (e.g., bovine serum albumin). Direct spectrometric determination of protein concentration is an easy and quick procedure that can be used during the purification process.

Support Protocol

Protocol Title - *In vitro* assays of arsenic methyltransferase activity in fractions from the purification of arsenic (+3 oxidation state) methyltransferase from rat liver cytosol

Introduction—Tracking purification of the protein which catalyses the methylation of As requires an assay system that is flexible enough to adapt to the changing properties of the various fractions (e.g., acid treated cytosol or eluate fractions from chromatographic steps) generated during the purification process. The setup of these assays follows the general procedures outlined in **Chapter 2, “In vitro Assays of Inorganic Arsenic Methylation”** Basic Protocol - Inorganic arsenic methylation in rat liver cytosol assay system. Assays of As methyltransferase activity of fractions require, at a minimum, a substrate for methylation (i.e., radiolabeled iAs^{III}) and AdoMet as a source of methyl group donors. We routinely use iAs^{III} radiolabeled with $i^{73}As^{III}$ as the substrate. Preparation of $i^{73}As^{III}$ from commercially available $i^{73}As^V$ is described in **Chapter 2, “In vitro Assays of Inorganic Arsenic Methylation”** Support Protocol – Reduction of $[^{73}As]$ arsenate to $[^{73}As]$ arsenite for use as a radiolabeled substrate in *in vitro* assays of arsenic methylation. Typically, assays are run at 1 mM AdoMet concentration. For assays of most fractions from the purification procedure, AdoMet must be included as part of the reaction mixture. However, because fractions from affinity chromatography on AdoHcy-agarose in which the eluant (Buffer D) contains 1 mM AdoMet, it is not necessary to add AdoMet to the reaction mixtures.

The radiolabeled products formed in assay mixtures which contain $i^{73}As^{III}$ as substrate have been routinely separated by thin layer chromatography as described in **Chapter 5, “Analysis of Arsenical Metabolites in Biological Samples”** Basic Protocol 1 - Analysis of arsenical metabolites by thin layer chromatography

The design of these assays is influenced by the level of As methyltransferase activity in individual fractions that are analyzed. In this purification scheme, samples from four separate steps in the purification process were analyzed. These samples differed in composition not only in terms of total protein and degree of enrichment of As methyltransferase activity (see Table 1) but also in terms of the composition of the solvent phase (Table 2). In all cases, the buffer used in sample preparation or in chromatographic separation contained a monothiol (GSH) and a dithiol (DTT) compound. However, both the buffer present and its pH differed among the samples. To provide uniformity, aliquots of samples from each step in the purification procedure were assayed for As methyltransferase activity after dilution into an appropriate buffer system. This approach reduced between sample differences in buffer composition and pH and provided data on activity that was more comparable.

Materials List—D,L-Dithiothreitol

Glutathione

Hydrogen peroxide (30% solution)

Sodium phosphate, monohydrate

Disodium phosphate, heptahydrate

S-adenosylmethionine, p-toluenesulfonate salt

Protocol Steps

1. Mix a 0.1 ml aliquot of samples from purification procedure with 0.4 ml of an appropriate Assay Buffer (see Table 2) in 1.5 ml microcap tubes. Preincubate mixture for 15 minutes at 37°C..
2. Initiate reaction by addition of up to 2 μ C of $i^{73}\text{As}^{\text{III}}$ to each microtube. Incubate mixture for up to 2 hours at 37°C.
3. Terminate reactions by addition of a volume of 0.2 M CuCl in 0.2 N HCL equal to the volume of the reaction mixture (final concentration 0.1 M Cu).
4. Secure lid of microcap tube with a lid lock or a clip. Incubate tube in a boiling water bath for five minutes.

This procedure liberates arsenicals that are bound to proteins and denatures most proteins in the reaction mixture.

5. Let tubes cool to room temperature. Centrifuge tubes at ~ 10,000 rpm in a bench top centrifuge for 10 minutes at room temperature to remove denatured proteins.
6. Transfer supernates to new microcap tubes. Mix at a 2:1 volume ratio (supernate: H_2O_2) with 30% H_2O_2 .

Hydrogen peroxide is highly reactive and corrosive. Use appropriate precautions when handling.

7. Incubate these samples at room temperature for at least several hours (or overnight).

Oxidation of arsenicals in the presence of 10% H_2O_2 converts all arsenicals to the pentavalent oxidation state. This oxidation improves separation of arsenicals by thin layer chromatography as described in **Chapter 5, "Analysis of Arsenical Metabolites in Biological Samples"** Basic Protocol 1 - Analysis of arsenical metabolites by thin layer chromatography.

Reagents and Solutions—*Physiological Saline* - 154 mM NaCl

Dissolve 9 g of NaCl in 1000 ml of deionized water. Store at 4°C until used.

Buffer A - 25 mM tris, 5 mM GSH, 1 mM DTT, 250 mM sucrose, pH 8.3

Prepare 25 mM tris stock by mixing 0.92 g trizma HCl and 2.33 g trizma base in ~ 900 ml deionized water and stirring to dissolve. Then adjust to final volume of 1000 ml. To 900 ml of 25 mM tris stock, add 87.6 g sucrose, 1.54 g GSH, and 154.3 mg DTT. Stir mixture to dissolve. Adjust to final volume of 1000 ml by addition of 25 mM tris stock. Check pH of final mix. At room temperature (~ 25°C), the pH should be 8.6. If necessary, adjust to this final value. Aliquot Buffer

A into 25 or 50 ml volumes and store at -20°C . This buffer is stable for several months with low temperature storage.

Buffer B – 1/9 diluted (v/v) Polybuffer 96 with 5 mM GSH and 1 mM DTT

Add 25 ml of Polybuffer 96 to ~ 200 ml of deionized water. To diluted Polybuffer 96, add 385 mg GSH and 38.6 mg DTT. Stir mixture to dissolve additives. Check pH of mixture and adjust to pH 6 with dropwise addition of acetic acid. Adjust to final volume of 250 ml with deionized water. Use immediately after preparation. Because the pH of the mixture can change on exposure to air, it should be prepared and used immediately.

Buffer C – 50 mM Na phosphate, 5 mM GSH, 1 mM DTT, pH 7.4, with 5% (v/v) glycerol

Prepare 50 mM Na phosphate by dissolving 1.56 g monosodium phosphate, monohydrate with 10.37 g disodium phosphate, heptahydrate in ~ 900 ml of deionized water. Then adjust to final volume of 1000 ml with deionized water. To 900 ml of 50 mM sodium phosphate stock, add 1.54 g GSH and 154.3 mg DTT. Stir mixture to dissolve. Adjust to final volume of 1000 ml by addition of 50 mM sodium phosphate stock. Check pH of final mix. Mix 950 ml of 50 mM Na phosphate, 5 mM GSH, 1 mM DTT, pH 7.4, with 50 ml glycerol. Aliquot 50 mM Na phosphate, 5 mM GSH, 1 mM DTT, pH 7.4, with 5% glycerol into 50 ml volumes and store at -20°C . This buffer is stable for several months with low temperature storage.

A useful phosphate buffer calculator can be found at <http://home.fuse.net/clymer/buffers/phos2.html>. Premixed phosphate buffers are available from many commercial vendors. Using commercially prepared phosphate buffers probably provides greater consistency in composition than can be obtained in buffer solutions made at infrequent intervals in a laboratory.

Buffer D – 50 mM Na phosphate, 5 mM GSH, 1 mM DTT, 1 mM AdoMet, 5% (v/v) glycerol, pH 7.4

To 250 ml of Buffer C, add 99.6 mg of AdoMet and mix to dissolve. Store on ice until used. Because AdoMet is unstable in aqueous solution, prepare Buffer D only on the day of use and store on ice until used. In our experience, AdoMet-containing buffers cannot be frozen for later use.

Assay Buffer 1 – 50 mM Na phosphate, 5 mM GSH, 1 mM DTT, pH 7.4

Prepare 50 mM Na phosphate by dissolving 1.56 g monosodium phosphate, monohydrate with 10.37 g disodium phosphate, heptahydrate in ~ 900 ml of deionized water. Then adjust to final volume of 1000 ml with deionized water. To 900 ml of 50 mM sodium phosphate stock, add 1.54 g GSH and 154.3 mg DTT. Stir mixture to dissolve. Adjust to final volume of 1000 ml by addition of 50 mM sodium phosphate stock. Check pH of final mix. Aliquot Assay Buffer 1 into 50 ml volumes and store at -20°C . This buffer is stable for several months with low temperature storage.

Assay Buffer 2 – 50 mM Na phosphate, 5 mM GSH, 1 mM DTT, 1 mM AdoMet, pH 7.4

To 50 ml of Buffer C, add 19.9 mg of AdoMet and mix to dissolve. Store on ice until used. Because AdoMet is unstable in aqueous solution, prepare Assay Buffer 2 only on the day of use and store on ice until used. In our experience, AdoMet-containing buffers cannot be frozen for later use.

1 M acetic acid

Based on a formula weight of 60.05 and a density of 1.049 g/ml, dilute 57.25 ml of glacial acetic acid to 1000 ml with deionized water. Mix and store at room temperature.

1 M ammonium hydroxide

Based on a formula weight of 35.05 and a density of 0.9 g/ml for a 30% solution, dilute 38.94 ml of 30% solution to 1000 ml with deionized water. Mix and store at room temperature.

1 N hydrochloric acid

Using reagent grade concentrated hydrochloric acid (38% HCl), dilute 8.3 ml of concentrated HCl to a final volume of 100 ml with deionized water. Store at room temperature.

1 M sodium chloride

Dissolve 29.23 g of sodium chloride to a final volume of 500 ml using deionized water. Store at room temperature.

Sodium bicarbonate, saturated solution

At room temperature, sodium carbonate, monohydrate, is soluble in deionized water at a 1:10 (w/v) ratio. For a saturated solution, add 2 g of sodium bicarbonate to 10 ml of deionized water. Store at room temperature.

0.2 M cuprous chloride in 0.2 N hydrochloric acid

Prepare 0.2 N HCl by mixing 20 ml of 1 N HCl with 80 ml of deionized water. Dissolve 198 mg of CuCl in 100 ml of 0.2 N HCl. Store at room temperature.

30% hydrogen peroxide

Use commercially prepared 30% hydrogen peroxide. This is a strong oxidizer. Use due caution in handling.

Commentary

Background Information

Purification of an enzyme that catalyzes the methylation of iAs was first reported by Aposhian and associates (Zakharyan et al., 1995) using rabbit liver cytosol as the starting material. These investigators reported the protein to be about 60 kDa and to require a dithiol (DTT) for activity. These investigators subsequently reported the presence of a similar protein in tissues of other mammals, (Zakharyan et al., 1996, Wildfang et al., 1998).

However, the primary sequence of this protein has not been reported and the gene encoding it has not been cloned from any species.

Using rat As3mt as the prototype of a group of proteins with related function, the genomes of many species can be searched for genes that encode proteins with a similar function (Thomas et al., 2007). To date, orthologous genes have been identified in organisms ranging from sea urchins to humans. Rat, mouse, and human genomes have orthologous *AS3MT* genes in syntenic regions of chromosomes 1, 19, and 10, respectively (<http://www.informatics.jax.org/orthology.shtml>). Among these orthologs, sequence motifs characteristic of AdoMet-dependent methyltransferase activity are highly conserved (Kagan and Clarke, 1994). Although structure-activity relations for these proteins have not been fully explored, the predicted sequences of these proteins are distinguished by conservation of critical cysteine residues. The replacement of these cysteines with serine residues eliminates the catalytic activity of the protein (Fomenko et al., 2007).

The nomenclature for this protein has evolved since its purification. The protein and its gene were initially termed cyt19, following its original identification in GenBank with a putative methyltransferase of unknown function (NCB accession NP065602 and NP 05133). More recently, application of the rules of the Human Genome Nomenclature Committee (<http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl>) has led to designation of gene and protein by the systematic name arsenic (+3 oxidation state) methyltransferase (AS3MT) for the human gene and protein. (The rules for nomenclature in non-human species are summarized in Wright and Bruford (2006)).

Critical Parameters

Purification process—The rationale for purification of enzymes has been succinctly presented by Kornberg (1990). In a post-genomic world, purification of an enzyme and the determination of its partial amino acid sequence has become a route to cloning and expressing the gene that encodes the protein. Hence, purification of the protein is not the goal of research but rather a step in a larger process understanding structure and function. Adapting available techniques and developing reliable methods for assays of enzymatic activity were vital to the success of this purification strategy.

In vitro assays—The availability of a reliable and adaptable assay for As methyltransferase activity is a vital tool in the purification process. The assay described here is based on a method developed in the earliest phases of our study of As methylation. This assay is characterized by a flexibility that allows its adaptation to the purification process. Although this Protocol describes a relatively short incubation time, it is possible to extend this period to increase the yield of methylated products. Longer incubation times might be needed if a tissue with relatively lower As methyltransferase activity were to be used as the starting material for enzyme purification.

Troubleshooting

Developing a strategy for the purification of As methyltransferase activity from rat liver is a complex trial and error procedure. In particular, loss of activity during purification plagued

early attempts in this laboratory to purify the protein. However, based on results from studies of the As methylation activity of rat liver cytosol and from the reports of Aposhian and coworkers (Zakharyan et al., 1995, 1996; Wildfang et al., 1998), it became apparent that addition of monothiol and dithiol in buffers might preserve the activity during purification. In addition, a continuous process minimized loss of As methyltransferase activity during isolation and purification. Development of purification procedures using chromatofocusing and AdoHcy-agarose affinity chromatography required considerable experimentation to assure optimal separation. Based on this experience, Table 3 lists common problems that may be encountered in the purification process and usual solution to these problems.

Anticipated Results

The procedure outlined here has been used in several rounds of purification of the As methyltransferase activity from rat liver cytosol. In each case, many thousand-fold enrichment of a protein with an estimated mass of ~42 kDa has been attained. The purified enzyme in Buffer D has been stored at -80°C for at least one month without appreciable loss of activity.

Time Considerations

In our experience, the material produced by acid treatment of the cytosolic fraction and subsequent readjustment of the pH to 8.3 can be stored overnight at 4°C with little loss of activity. All subsequent steps in the purification process have been run as a continuous process. We have routinely held chromatographic fractions at 4°C for up to 16 hours while performing As methyltransferase activity assays. The entire procedure from the collection of rat livers through the final chromatographic purification of As3mt can be completed in about five days.

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Figure 1.

Flow scheme for the purification of As3mt from Fischer 344 rat liver cytosol.

Figure 2. Purification of As3mt from rat liver cytosol. Proteins from each step in the purification procedure separated by PAGE and stained with Coomassie Brilliant Blue. Lane 1- proteins present in rat liver cytosol; lane 2 - proteins in the acid-treated supernatant fraction; lane 3 - proteins in the most active fractions from chromatofocusing; lane 4 - proteins in pooled active fractions from S-adenosylhomocysteine affinity chromatography; lane 5 - protein size markers.

Table 4.34.1**Purification of Arsenic Methyltransferase from Rat Liver Cytosol**

Fraction	Total protein (mg)	Specific activity^a	Total activity^b	Purification
Cytosol	1200	0.16	192	1
Acidified cytosol	678	0.48	325	3
Chromatofocusing	22	216	4752	1347
S-Adenosyl-L-homocysteine affinity chromatography	0.76	1490	1132	9312

^aSpecific activity expressed as pmols of methylated and dimethylated arsenic formed per mg of protein in a reaction mixture containing 0.1 μ M arsenite that was incubated 45 min at 37°C.

^bTotal activity (pmols of methylated and dimethylated arsenic formed) is the product of the specific activity of a fraction and the total protein.

Table 4.34.2

Solvent Composition for Fractions Prepared in Purification of Arsenic Methyltransferase from Rat Liver Cytosol

Fraction	Sample buffer	Assay buffer
Cytosol and acidified cytosol	Buffer A (see recipe)	Assay buffer 2 (see recipe)
Chromatofocusing	Buffer B (see recipe)	Assay buffer 2 (see recipe)
<i>S</i> -Adenosyl-L-homocysteine affinity chromatography	Buffer D (see recipe)	Assay buffer 1 (see recipe)

Table 4.34.3

Troubleshooting Guide for Purification of Arsenic Methyltransferase Activity

Problem	Cause	Solution
Loss of activity during purification steps	Insufficient concentration of reductants (GSH and DTT) in buffers A–D	Prepare buffers A–D, making sure to use the correct concentrations of GSH and DTT
	Prolonged storage of chromatographic fractions	Make purification a continuous procedure. Only acid-treated supernatant can be stored overnight.
Lack of resolution of peaks from chromatofocusing or peak broadening	Failure to form pH gradient	Check pH of buffers and of individual chromatographic fractions
	Inappropriate gradient	Change gradient slope
Failure of affinity purification on AdoHcy-Agarose	Breakthrough of arsenic methylating activity during column loading	Reduce amount of material initially loaded on AdoHcy-agarose or increase volume of AdoHcy-agarose column
	Lack of capacity due to insufficient charging of agarose with selective ligand, S-adenosylhomocysteine	Prepare new AdoHcy-agarose