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Purification Strategy for Recombinant Forms of the Human Mitochondrial DNA Helicase

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Abstract

In this chapter, we present a streamlined purification for the production of near-homogeneous and high yield recombinant forms of the human mitochondrial DNA helicase. Minimizing the number of steps and the time elapsed for purification of this enzyme facilitates studies of its structure and mechanism and allows elucidation of native features of both wild-type- and human disease-related forms.

Keywords

mitochondrial DNA replication; DNA helicase; human; DNA unwinding

1. Introduction

The human mtDNA helicase is a member of the *Escherichia coli* DnaB-like family of replicative helicases, also known as Superfamily 4(1). These family members contain five conserved sequence motifs, including the classic Walker A and Walker B motifs that participate directly in the ATPase binding and hydrolysis activity required for DNA unwinding (2, 3). The human enzyme transduces the hydrolysis energy for replication fork advancement and translocates along ssDNA in a 5′–3′ direction(4). Its native conformation is that of a hexamer, with an overall modular architecture comprising distinct N- and C-terminal domains(1). A unique feature of the enzyme is a regulatory role in ATP hydrolysis for its extreme N- and C-termini(1). In addition, amino acid residue R609 has been identified as the arginine finger that serves to ligate the γ phosphate of the incoming ATP molecule during the catalytic cycle(1).

To date, biochemical and physical data are limited for this novel enzyme, and further analysis is mandated by its direct link to a human disease associated with multiple mtDNA deletions(5). This finding, in conjunction with studies revealing the role for the mtDNA helicase in mtDNA maintenance and regulation of mtDNA copy number, reveals its importance for proper mitochondrial physiology and in turn, overall cellular processes(6). In this chapter, we present a streamlined purification for recombinant forms.

2. Materials

2.1. Recombinant Human Mitochondrial DNA Helicase Purification from *Spodoptera frugiperda* Cells

1. 1M Tris-HCl, pH 7.5, pH 8.0, stored at 24°C.

2. 0.5M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
3. 5M Sodium chloride (NaCl).
4. 2M Tris-HCl, pH 6.8, pH 8.8.
5. 0.2M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Store aliquots at –20°C.
6. 1M Sodium metabisulfite, prepared as a 1.0M stock solution at pH 7.5 and stored at –20°C.
7. Leupeptin is prepared as a 1 mg/ml stock solution in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and stored at –20°C.
8. 1M Dithiothreitol (DTT). Store aliquots at –20°C.
9. 14M 2-Mercaptoethanol (β -Me). Store at 4°C.
10. 30% Polyacrylamide (29:1; acrylamide:bisacrylamide). Store at 4°C.
11. 10% Sodium dodecyl sulfate (SDS).
12. 4× Resolving gel buffer: 1.5M Tris-HCl, pH 8.8, 0.4% SDS.
13. 4× Stacking gel buffer: 0.5M Tris-HCl, pH 6.8, 0.4% SDS.
14. 5× SDS-PAGE running buffer: 0.125M Tris base, 0.95M glycine, 0.5% SDS.
15. 5× SDS-PAGE loading buffer: 50% glycerol, 2M Tris base, 0.25M DTT, 5% SDS, 0.1% bromophenol blue. Aliquots are stored at –20°C.
16. 10% Ammonium persulfate (APS).
17. TC-100 Insect cell culture medium and fetal bovine serum (Life Technologies).
18. Amphotericin, penicillin-G, and streptomycin (Sigma).
19. Insect cell transfection buffer and Grace's medium (PharMingen).
20. *S. frugiperda* (Sf9) cells.
21. Baculoviruses encoding N- and C-terminally His-tagged human mitochondrial helicase, obtained from Dr. Maria Falkenberg (Karolinska Institute).
22. Phosphate-buffered saline, stored at 4°C.
23. Buffer A: 50 mM Tris-HCl, pH 8.0, 0.6M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol.
24. Nickel-nitrilotriacetic acid agarose resin (Qiagen).
25. 1M Imidazole, stored at 24°C.
26. Heparin Sepharose agarose resin (Amersham Pharmacia Biotech).
27. Polyallomer tubes (14 × 89 mm, Beckman)

2.2. Human Mitochondrial DNA ATPase Assay

1. Recombinant human mitochondrial DNA helicase at approx. 200 µg/ml.
2. 1M Tris-HCl, pH 7.5, stored at 24°C.
3. 1M MgCl₂, stored at 24°C.
4. 0.29 mg/ml Bovine serum albumin (BSA, Sigma).
5. 82 mM ATP, store aliquots at 4°C.
6. 1M Dithiothreitol (DTT). Store aliquots at -20°C.
7. [γ-³²P]ATP.
8. 2.0 mg/ml DNase I-activated calf thymus DNA.
9. 0.5M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
10. Polygram polyethyleneimine cellulose paper (Brinkmann Instruments, Inc.)
11. 50 mM ADP/ATP, store aliquots at 4°C.
12. 1M Formic acid.
13. 0.5M Lithium chloride (LiCl).

3. Methods

3.1. Recombinant Human Mitochondrial DNA Helicase Purification from *S. frugiperda* Cells

3.1.1. sf9 Cell Growth and Protein Over-expression

1. Grow *S. frugiperda* cells (2.01) in TC-100 insect cell culture media containing 10% (v/v) fetal bovine serum at 27°C to a cell density of 2×10^6 , dilute to a cell density of 1×10^6 with TC100 containing 10% fetal bovine serum.
2. Infect with baculovirus encoding N- or C-terminally His-tagged helicase (complete cDNA sequence lacking the mitochondrial presequence) at a multiplicity of infection of 5 at 27°C.
3. Harvest cells at 72 h postinfection by centrifugation and wash with an equal volume of cold phosphate-buffered saline twice.
4. Recentrifuge, discard supernatant, and freeze pellet in liquid nitrogen. Store at -80°C.

3.1.2. Soluble Cytoplasmic Fraction Preparation—All buffers contain 1 mM PMSF, 10 mM sodium metabisulfite, 2 µg/ml leupeptin, and 10 mM 2-mercaptoethanol. Perform all steps at 0–4°C.

1. Thaw cells and resuspend in 1/45 volume of original cell culture in 25 mM Tris-HCl, pH 8.0. Allow cells to sit for 20 min.
2. Homogenize cells in a Dounce homogenizer using 20 strokes with a tight pestle.

3. Adjust homogenate to 1M NaCl followed by centrifugation at 45,000 rpm for 27 min in a 60 Ti rotor. Use Buffer A to balance.
4. Extract supernatant (fraction I)

3.1.3. Nickel-Nitrilotriacetic Acid Chromatography

1. Dilute fraction I with an equal volume of Buffer A and load onto a nickel-nitrilotriacetic acid column (2.5 ml resin/l of cells) equilibrated with Buffer A containing 10 mM imidazole at a flow rate of 12 ml/h.
2. Wash the column with equilibration buffer at a flow rate of 20 ml/h.
3. Elute the column with Buffer A containing steps of 25 mM, 250 mM, and 500 mM imidazole. Protein will elute with buffer containing 250 mM imidazole.
4. Analyze fractions by SDS-PAGE on 10% minigels followed by silver staining, and pool fractions accordingly (fraction II)

3.1.4. Heparin Sepharose Chromatography

1. Dilute fraction II to an ionic equivalent of 150 mM NaCl and load onto a Heparin Sepharose column (2.7 mg/ml resin) equilibrated with buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA at a flow rate of 3–5 ml/h (*see Note 1*).
2. Wash the column with equilibration buffer containing 200 mM NaCl at a flow rate of 9 ml/h.
3. Elute the column with equilibration buffer containing salt steps of 0.6 and 1M NaCl. Protein will elute at 350–700 mM NaCl (*see Note 2*).
4. Adjust eluted fractions to 1.0M NaCl.
5. Analyze fractions by SDS-PAGE on 10% minigels followed by silver staining, and pool fractions accordingly (fraction III).

3.1.5. Glycerol Gradient Sedimentation

1. Layer fraction III onto pre-formed 12–30% glycerol gradients containing 35 mM Tris-HCl, pH 7.5, 330 mM NaCl, 1 mM EDTA, prepared in polyallomer tubes for use in a Beckman SW 41 rotor.
2. Centrifuge at $140,000 \times g$ for 37 h at 3°C, then fractionate by collecting four-drop (200 μ l) fractions.
3. Analyze fractions by SDS-PAGE on 10% minigels followed by silver staining, and pool fractions accordingly (fraction IV).
4. Freeze fraction IV in liquid nitrogen and store at –80°C. Samples are analyzed by SDS-PAGE on 10% gels to evaluate purity and yield (*see Note 3*).

3.2. ATPase Assay

This assay measures the amount of ATP hydrolyzed to ADP + inorganic phosphate by the human mtDNA helicase in a DNA-independent and dependent manner.

1. Each reaction (0.02 ml) contains 20 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 10% glycerol, 0.5 mM ATP, 10 mM dithiothreitol, 4 µCi of [γ -³²P]ATP, 100 µM DNase I-activated calf thymus DNA, and 0.05 µg N- and C- terminally His-tagged human mtDNA helicase. Prepare the reaction mix with and without DNA in a microcentrifuge tube on ice. Add the radioactivity last. Vortex and centrifuge briefly in the microcentrifuge.
2. Dispense the mix, 20 µl, to pre-chilled and numbered microcentrifuge tubes on ice.
3. Add the enzyme, 0.25 µl, to each tube avoiding bubbles and mix gently by flicking the tube three times.
4. Incubate the tubes for 15 min at 37°C.
5. Stop the reactions with addition of EDTA to 20 mM and transfer to ice.
6. Divide Polygram polyethyleneimine cellulose paper into 1 × 1 cm lanes.
7. Spot 0.5 µl ADP/ATP marker in each 1-cm lane. Allow paper to dry.
8. Spot 2.0 µl reaction sample slowly while drying.
9. Develop Polygram CEL 300 PEI/UV paper in 1M formic acid and 0.5M lithium chloride for 30 min.
10. Allow paper to dry.
11. Visualize ADP and ATP spots under UV light and cut out each band.
12. Count radioactivity in each band in scintillation fluid.

4. Notes

1. Precipitate will form as ionic strength decreases to 150 mM NaCl. As the sample loads onto the column, this precipitate will accumulate on the resin bed.
2. As the ionic strength increases during the elution, the precipitate will go back into solution.
3. The purity and yield of the final preparations are determined by SDS-PAGE followed by silver staining. Figure 8.1 shows a typical purification of the recombinant form of mtDNA helicase.

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Fig. 8.1.

Baculovirus over-expression and purification of the human mtDNA helicase. Protein fractions were denatured and electrophoresed in a 10% SDS-polyacrylamide gel. Proteins were detected by silver staining. Lane 1: *Hs* mtDNA helicase (0.3 μ g); lane 2: whole cell fraction; lane 3: soluble extract (fraction I, 0.3 μ g); lane 4: Nickel-NTA pool (fraction II, 0.8 μ g); lane 5: Heparin Sepharose pool (fraction III, 0.4 μ g); lane 6: glycerol gradient pool (fraction IV, 0.1 μ g).