Regulation of actin oxidation at Met44/Met47 has been shown to destabilize F-actin depolymerization and interfere with actin monomer interaction and promotes F-actin severing and depolymerization. Functionally, oxidation of Met44 has a profound effect on actin polymerization because this residue resides in the D-loop of subdomain 2 of the protein, which is critical for actin subunit contacts; thus, upon oxidation, Met44 becomes negatively charged and interferes with actin monomer-monomer interaction and promotes F-actin severing and depolymerization. Regulation of actin oxidation at Met44/Met47 has been shown to destabilize F-actin in vivo and to play a key role in a growing number of cellular processes, including, cytokinesis, axonal guidance, dendritic organization, synaptic development, heart and muscle development and cell viability.

Material
Purified rabbit muscle actin (Cat. # AP05) has been modified to contain covalently linked pyrene at the cysteine 374 residue. An N-(1-pyrene) iodoacetamide is used to label the actin protein. Pyrene labeling stoichiometry has been determined to be between 0.4-0.6 dyes per actin monomer.

Pyrene labeled rabbit skeletal muscle actin protein (MICAL-oxidized) (Cat. # MXAP95) has been enzymatically oxidized at methionines 44 and 47 (β-actin nomenclature) with the MICAL flavoprotein monooxygenase protein (Cat. # MIC01). MICAL-oxidized (pyrene labeled) rabbit muscle actin has an approximate molecular weight of 43 kDa and is supplied as a white lyophilized powder.

Storage and Reconstitution
Shipped at ambient temperature. The lyophilized protein can be stored desiccated at 4°C for 6 months. For reconstitution, the product tube should be briefly centrifuged to collect the powder to the bottom of the tube. Each tube of protein should be reconstituted to 10 mg/ml with 25 µl of cold distilled water; it will then be in the following buffer: 5 mM Tris-HCl pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 5% (w/v) sucrose and 1% (w/v) dextran. The concentrated protein can then be snap frozen in liquid nitrogen and stored at -70°C where it is stable for 6 months.

For working concentrations, further dilution of the protein should be made with General Actin Buffer (Cat. # BSA01) supplemented with 0.2 mM ATP (Cat. # BSA04) and 0.5 mM DTT. MICAL-oxidized (pyrene labeled) actin is a labile protein and should be handled with care. Diluted pyrene actin is stable for a maximum of 4 h at 4°C and should not be frozen. Avoid repeated freeze-thaw cycles and do not freeze below 10 mg/ml.

When stored and reconstituted as described, MXAP95 is stable for 6 months at -70°C.

Purity
Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 4-20% gradient tris-glycine gel. Actin protein (MICAL-oxidized) was determined to be ≥ 95% pure (Figure 2).
Evaluating Methionine Oxidation of Actin: Subtilisin A Limited Proteolysis Application

Rationale:
Subtilisin A is a protease that has been shown to cleave actin between M47 and G48 \[\text{[9,10]}\]. When actin is oxidized at M47 the efficiency of subtilisin A cleavage is significantly reduced \[\text{[9]}\]. MICAL-1 is a protein that can specifically oxidize actin at amino acids M44 and M47 (\(\text{fi-actin nomenclature}\)). It has been reported that M44 oxidation by MICAL is concomitant with M47 oxidation \[\text{[3]}\]. Therefore, subtilisin A can be used in limited proteolysis assays to evaluate MICAL-oxidized actin versus native actin by measuring cleaved versus uncleaved actin.

Expected Results:
Purified MICAL-oxidized (pyrene labeled) actin has reduced susceptibility to subtilisin A cleavage at M47/G48 by > 90% under the conditions described below.

Reagents Needed:
MICAL-oxidized (pyrene labeled) rabbit skeletal muscle actin (Cat. # MXAP95): 250 \(\mu\)g is sufficient for roughly 113 subtilisin reactions when used at 2 \(\mu\)g / rxn
Pyrene labeled rabbit skeletal muscle actin (Cat. # AP05)
G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl\(_2\), 0.2 mM ATP, 0.5 mM DTT)
Precision red advanced protein assay (Cat. # ADV02)
Phenylmethylsulfonyl fluoride (PMSF)
Subtilisin A (Cat. # P5380; Sigma)

Method:

Day 1
1. Resuspend MICAL-oxidized (pyrene labeled) rabbit skeletal muscle actin (Cat. # MXAP95) and pyrene labeled rabbit skeletal muscle actin (Cat. # AP05) to 0.2 mg/ml with G-buffer (5 mM Tris pH 8.0, 0.2 mM CaCl\(_2\), 0.2 mM ATP, 0.5 mM DTT)

2. Incubate both MXAP95 and AP05 actin on ice for 2 hours
   **Note:** Longer incubation is necessary for AP05 to disperse nucleation bodies

3. Centrifuge the MXAP95 and AP05 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C

4. Transfer the MXAP95 and AP05 supernatants to new, labeled tubes on ice

5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)

6. Dilute MXAP95 and AP05 actin samples to 0.1 mg/ml with G-buffer

7. Aliquot 20 \(\mu\)l (2 \(\mu\)g actin) of each sample into individual 1.5 ml tubes. 2 aliquots per sample are needed for untreated versus subtilisin treated conditions. You should have the following 4 conditions as shown in Table 1

8. Before beginning subtilisin treatment boil a beaker of water. The water should be boiling when the subtilisin reaction is complete to inactivate the protease

9. Make a stock of subtilisin: 1 mg of subtilisin can be dissolved in 32 ml of 2 mM Tris pH 8.0/0.2 mM CaCl\(_2\) to make a 31.25 \(\mu\)g/ml stock. Aliquot and store at -70°C.

10. Dilute a tube of subtilisin stock to 2 \(\mu\)g/ml with G-buffer.
   **(Subtilisin analysis: 1:200 w/w subtilisin to actin = 10 ng subtilisin / 2\(\mu\)g actin)**

11. Add 10 ng of subtilisin to treated condition for all 4 samples
   **(Subtilisin should be diluted and added to sample in less than 10 min)**

12. Treat actin samples with subtilisin for 15 min

13. During treatment make PMSF (10 mM: 1.74 mg PMSF per ml of isopropanol)

14. After 15 min of subtilisin treatment, stop the reactions by adding 1 \(\mu\)l of PMSF to each sample (1-4)

15. Immediately place samples into boiling water. Leave samples for 5 min

16. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes

Expected Results:

Table 1: Composition of Experimental Samples for Subtilisin Digestion

<table>
<thead>
<tr>
<th>Sample</th>
<th>Name</th>
<th>Add Subtilisin</th>
<th>Add PMSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AP05</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>AP05</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>MXAP95</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>MXAP95</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

17. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel

18. Visualize with Coomassie staining. See Figure 3

Figure 3. Subtilisin Assay on MICAL-Oxidized Pyrene Actin vs Native Pyrene Actin. Pyrene labeled actin (Cat. # AP05) and MICAL-oxidized (pyrene labeled) actin (Cat. # MXAP95) was diluted to 0.1 mg/ml (2.3 \(\mu\)M). 2 \(\mu\)g of each sample was then left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.
Evaluating Methionine Oxidation of Actin: Polymerization Application

Rationale:
When actin is oxidized at M44 its ability to polymerize is significantly diminished[3]. However, with increasing concentrations, oxidized actin can form augmented polymers[5]. Sedimentation assays can be used to measure the polymerization capabilities of native actin versus MICAl-oxidized actin.

Expected Results:
Purified MICAl-oxidized (pyrene labeled) actin will polymerize less efficiently relative to native pyrene labeled actin in an actin concentration dependent manner as shown in Table 2.

Table 2: Expected % actin polymer formed with native versus oxidized actin

<table>
<thead>
<tr>
<th>% polymer</th>
<th>Unoxidized actin</th>
<th>MICAl oxidized actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 mg/ml actin</td>
<td>100%</td>
<td>&lt;10% of AP05</td>
</tr>
<tr>
<td>0.1 mg/ml actin</td>
<td>100%</td>
<td>40-55% of AP05</td>
</tr>
</tbody>
</table>

Reagents Needed:
MICAl-oxidized (pyrene-labeled) rabbit skeletal muscle actin (Cat. # MXAP95): 250 µg is sufficient for roughly 22 polymerization reactions when used at 0.05 mg/ml and 11 reactions when used at 0.1 mg/ml final.

Pyrene labeled rabbit skeletal muscle actin (Cat. # AP05) G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)

Precision red advanced protein assay (Cat. # ADV02)

Equipment Needed:
1. Fluorescence spectrophotometer with an excitation wavelength of 360 +/- 20 nm and an emission wavelength of 405 +/- 10 nm or 420 +/- 20 nm. Cytoskeleton recommends the SPECTRAFluor Plus from TECAN Austria GmbH or Gemini from Molecular Devices Inc.
2. Black polystyrene 96 well assay plate (Costar, Cat. # 3915).

Method:
1. Resuspend MICAl-oxidized (pyrene labeled) rabbit skeletal muscle actin (Cat. # MXAP95) and pyrene labeled rabbit skeletal muscle actin (Cat. # AP05) to 1.0 mg/ml with G-buffer (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)
2. Incubate both MXAP95 and AP05 actin on ice for 1 h
3. Centrifuge the MXAP95 and AP05 actin samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the MXAP95 and AP05 supernatants to new, labeled tubes on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Dilute MXAP95 and AP05 actin samples to the following conc. 0.1 mg/ml and 0.2 mg/ml with G-buffer
7. Prepare 2x polymerization buffer
8. Pipet 100 µl of G-buffer into two wells (A1-2) (control samples)
9. Pipet 100 µl of 0.1 mg/ml AP05 into two wells (A3-4) of a black 96 well plate
10. Pipet 100 µl of 0.1 mg/ml MXAP95 into two wells (B3-4) of a black 96 well plate
11. Pipet 100 µl of 0.2 mg/ml AP05 into two wells (A5-6) of a black 96 well plate
12. Pipet 100 µl of 0.2 mg/ml MXAP95 into two wells (B5-6) of a black 96 well plate
13. Place the 96 well plate into the fluorescent spectrophotometer and read the samples for 3 min to establish a baseline fluorescence measurement.
14. After 3 min add 100 µl of 2x polymerization buffer to all wells with samples or blanks. (A1-6, B3-6)
15. Return the plate to the spectrophotometer and read the fluorescence every 30 s for 2 h

Expected Results:

Results from Table 2 show that MICAl-oxidized actin polymerizes less efficiently than native actin. The table indicates that at 0.05 mg/ml actin, MICAl-oxidized actin polymerizes at <10% of the rate of AP05, while at 0.1 mg/ml actin, it polymerizes at 40-55% of AP05.

References:

Product Citations/Related Products
For the latest citations and related products please visit www.cytoskeleton.com