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In Vitro Sumoylation of Recombinant Proteins and Subsequent Purification for Use in Enzymatic Assays

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INTRODUCTION

The sumoylation reaction is mechanistically similar to ubiquitination. It is ATP-dependent and in vitro can be completed in two steps using purified E1 (SAE1/SAE2), E2 (ubc9), and SUMO. Even without the inclusion of an E3 ligase, many substrates can be modified at the same lysines in vitro as in vivo. Here we describe a simplified in vitro sumoylation protocol using recombinant sumoylation substrate, E1, E2, SUMO, and an ATP-regenerating system. The modified substrate can then be repurified from the reaction mixture in a single step to be used in assays to assess the impact of sumoylation on enzymatic and/or other activities.

RELATED INFORMATION

This protocol is adapted from the original procedure in Desterro et al. (1998), and has been used in Vethantham et al. (2008). For further details about protein detection methods, see Immunoblotting: Antigen Detection Using Chemiluminescence (Harlow and Lane 2006) and Staining Proteins in Gels with Silver Nitrate (Simpson 2007). More information about protein purification methods can be found in Preparation of GST Fusion Proteins (Einarson et al. 2007) and Purification of Histidine-Tagged Proteins by Immobilized Ni²⁺ Absorption Chromatography (Sambrook and Russell 2006).

MATERIALS

Reagents

Antibodies, anti-His (for immunoblot analysis; see Steps 13.ii and 23.ii)

ATP regeneration mix (10X)

Make the 10X ATP regeneration mix before setting up reaction mixtures in Step 11.

Bacterial cultures harboring plasmids encoding the following proteins:

GST (1–2 µg/µL) (see Steps 1–7 and 14)

GST-SAE2/SAE1 (1–2 µg/µL) (see Steps 1–7)

GST-Ubc9 (1–2 µg/µL) (see Steps 1–7)

Protein of interest, His-tagged (1 µg/µL) (e.g., see Purification of Histidine-Tagged Proteins by Immobilized Ni²⁺ Absorption Chromatography [Sambrook and Russell 2006])

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SUMO proteins (SUMO-1GG, SUMO-2GG, or SUMO-3GG) (1–2 $\mu\text{g}/\mu\text{L}$) (see Steps 8 and 9)

METHOD

Preparation of Recombinant Proteins

GST Proteins (GST-SAE2/SAE, GST-Ubc9, and GST)

These proteins are purified according to standard procedures such as Preparation of GST Fusion Proteins (Einarson et al. 2007). The following steps are a brief outline of the purification procedure; additional details can be obtained from Desterro et al. (1997, 1999) and Tatham et al. (2001).

- 1 Induce bacterial cultures harboring plasmids encoding the GST proteins with 1 mM IPTG for 3 h at 30°C.
- 2 Harvest the bacteria, and prepare lysates using NETN buffer.
- 3 Agitate the lysates with glutathione Sepharose beads.
- 4 Wash the beads with NETN buffer, and elute with Tris-Cl (50 mM, pH 8.0) containing 20 mM reduced glutathione.
- 5 Dialyze the eluted proteins against the following buffers:
 - i. Dialyze GST-SAE2/SAE1 against dialysis buffer S1.
 - ii. Dialyze GST-Ubc9 against dialysis buffer S2.

Some protocols recommend that the GST fusion tag be removed from Ubc9 before its use in sumoylation reactions in vitro. Proteases such as thrombin may be used for removal of the GST tag.

Bradford Assay, including Bradford dye and BSA (Bio-Rad 500-0201)

Dialysis buffer S1

Dialysis buffer S2

Dialysis buffer S3

Glutathione Sepharose beads

IPTG (Isopropyl- β -D-thio-galactoside) (1 mM)

Laemmli sample buffer (2X) with DTT

NETN lysis buffer

Ni-NTA agarose (QIAGEN)

Protease (optional; see Step 5.ii)

Reagents for SDS gel electrophoresis

SUMO buffer (10X)

Sumoylation buffer A

Sumoylation buffer B

Sumoylation buffer C

Tris-Cl (50 mM, pH 8.0) containing 20 mM reduced glutathione

Equipment

Equipment for SDS gel electrophoresis

Freezer pre-set to -80°C

Ice

Incubator pre-set to 37°C

Incubator (shaking), pre-set to 30°C

Microcentrifuge

Minicentrifuge

Mini-columns for chromatography (Bio-Rad PolyPrep chromatography columns)

Protein concentrator (Centricon)

Rotator

Tubes, microcentrifuge (1.5-mL)

Water bath, boiling

6 Concentrate the protein to 1 mg/mL using a Centricon concentrator.

7 Store the purified protein at -80°C .

SUMO Proteins

8 Use untagged SUMO proteins in their active form (mature SUMO proteins or SUMO-GG). SUMO may be cloned downstream from a tag such as GST or 6-His containing a protease cleavage site (see Preparation of GST Fusion Proteins [Einarson et al. 2007] and Purification of Histidine-Tagged Proteins by Immobilized Ni^{2+} Absorption Chromatography [Sambrook and Russell 2006]). Include a protease cleavage step in the purification procedure to yield untagged protein.

9 Dialyze the SUMO proteins against dialysis buffer S3.

10 Store the purified protein at -80°C .

Assembling the In Vitro Sumoylation Reaction

11 Assemble 20- μL reactions by mixing the following components in the order indicated. Keep the reagents and reactions on ice.

i. Assemble the SUMO master mix:

Reaction component -Amount per reaction

10X SUMO buffer- 2 μL

GST-SAE2/SAE1 (1–2 $\mu\text{g}/\mu\text{L}$)- 500 ng

GST-Ubc9 2 μg

Alternatively, use Ubc9 (1–2 $\mu\text{g}/\mu\text{L}$) - 1 μg

SUMO proteins (mature, untagged SUMO-1GG,

SUMO-2GG, or SUMO -3GG) (1–2 $\mu\text{g}/\mu\text{L}$)-2 μg

When assembling these reactions, include control reactions omitting the various components of the SUMO master mix. The different SUMO isoforms should be used separately first to assess the efficiency of each of the isoforms in conjugating to the substrate.

- ii. Add 1–2 μg of the recombinant His-tagged protein of interest (1 $\mu\text{g}/\mu\text{L}$) and H₂O to bring the volume of each reaction to 18 μL .
 - iii. Initiate the reactions by adding 2 μL of 10X ATP regeneration mix.
- 12 Incubate the reactions for 2 h at 37°C.
 Incubation time and temperature depend on the substrate and experiment, so optimize as necessary. Times can vary from 1 to 3 h, and some in vitro sumoylation protocols recommend incubations at 30°C.
- 13 Assess the efficiency of sumoylation by performing a Western blot analysis:
 - i. Mix 20 μL of 2X Laemmli buffer with 20 μL of each reaction.
 - ii. Boil for 5 min and analyze by SDS gel electrophoresis and detection with anti-His antibodies (see Immunoblotting: Antigen Detection Using Chemiluminescence [Harlow and Lane 2006]).
 See Troubleshooting.

Repurification of Substrate Protein from the In Vitro Sumoylation Reaction

- 14 If the protein is to be purified further after the in vitro sumoylation experiment, scale up the reaction 10-fold in Steps 1 and 2 (Set I). Assemble a second large-scale reaction (Set II) with GST substituted for the SUMO proteins in the SUMO master mix.
- 15 Transfer 80 μL of Ni-NTA agarose bead suspension (40 μL beads volume) into two microcentrifuge tubes each. Centrifuge at 5000 rpm for 1 min, and aspirate the liquid from the beads. Wash the beads three times with 1 mL of sumoylation buffer A. Centrifuge again, and carefully aspirate the liquid from the beads.
- 16 After the large-scale reactions have finished incubating (see Step 2), transfer 20 μL of the reaction mix from Sets I and II into microcentrifuge tubes containing 20 μL of 2X Laemmli buffer. Store at -80°C until electrophoresis in Step 23.
- 17 Dilute the remainder of each reaction fourfold in sumoylation buffer A. Add these dilutions to the two tubes containing the Ni-NTA beads prepared in Step 15.
- 18 Rotate the mixtures for 3 h at 4°C.
- 19 Centrifuge the mixtures at 5000 rpm for 1 min. Carefully aspirate the liquid from the beads and transfer to another microcentrifuge tube.
- 20 Carefully wash the beads three times with 1 mL of sumoylation buffer A and then three times with 1 mL of sumoylation buffer B. Centrifuge again, and carefully aspirate the liquid from the beads.
- 21 Add 200 μL of sumoylation buffer C to the beads and place on a rotator for 30 min at 4°C.

- 22 Load the beads on a mini-column for chromatography (Bio-Rad PolyPrep) and collect the flow-through.
- 23 Characterize the protein preparation:
 - i. Carefully estimate the concentration of the protein eluted from the column for the two sets of reactions using the Bio-Rad Bradford assay as per the manufacturer's instructions.
 - ii. Estimate the efficiency of the sumoylation reaction by performing immunoblot analysis with anti-His antibodies, using the samples collected in Step 16 (see Immunoblotting: Antigen Detection Using Chemiluminescence [Harlow and Lane 2006]).
 - iii. Test the purity of the protein by SDS gel electrophoresis and silver staining (see Staining Proteins in Gels with Silver Nitrate [Simpson 2007]).

See Troubleshooting.
- 24 Use equal quantities of protein from Sets I and II in enzymatic assays.

TROUBLESHOOTING

Problem

Sumoylated species are very faint; efficiency of the sumoylation reaction is very poor.

[Steps 13 and 23]

Solution

Although this can be a property of the substrate per se, the efficiency of the reaction may be increased in the following ways:

1. Recalibrate the reaction and increase the amount of SUMO-GG proteins to as much as 5 μ g per reaction. Appropriately increase the amount of substrate used to at least 3 μ g.
2. Assess the quality of the E1 and E2 enzymes used in the reaction by using a standard substrate such as RanGAP1/PML (Matunis et al. 1996; Müller et al. 1998).
3. If the efficiency of the reaction is too poor to be able to use the product in enzymatic assays after purification in a single step, introduce a second purification step. A short N-terminal tag such as Flag may be introduced in the cloning of SUMO protein-encoding constructs so that, after purification with the Ni-NTA beads, a second step with anti-Flag antibody beads may be used to purify the sumoylated species away from non-sumoylated substrate.
4. If the efficiency of the reaction is not improved by the above suggestions, add the RanBP2 IR1+M E3 ligase protein (Pichler et al. 2004) to the reaction. This is a truncated version of the RanBP2 SUMO ligase that has high SUMO ligase activity and low substrate specificity. Prepare the protein using GST or an appropriate tag, and add it to the reaction after adjusting for the amount of ubc9 (for details, see Pichler et al. 2004). This is particularly useful when efficiency is low and the authentic SUMO E3 ligase is unknown.

DISCUSSION

SUMO substrates are now known to function in a large variety of biological processes, such as transcription, RNA processing, DNA repair, and chromatin organization, to name a few. The *in vitro* sumoylation reaction described in this protocol can help to confirm whether a particular protein is a SUMO substrate. This method also allows for scaling up and purification of the sumoylated protein to be used in assays to study the effects sumoylation may have on the enzymatic activity of the protein (e.g., Vethantham et al. 2008). Even though previous experiments using similar assays have shown that the same lysines that are modified *in vivo* are commonly modified *in vitro* (e.g., Desterro et al. 1998; Salinas et al. 2004), those wishing to accurately mimic an *in vivo* scenario may include an E3 ligase (if known) in the system (Pichler 2008).

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