

[37] Preparation and Characterization of Yeast and Human Desumoylating Enzymes

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Introduction

As is true for ubiquitin, most ubiquitin-like proteins (Ubls) are processed from C-terminally extended precursor forms and are reversibly linked to substrate proteins. Ubl-specific proteases (ULPs) are proteases that cleave off the C-terminal peptides from Ubl precursors or cleave Ubls from molecules to which they had been added posttranslationally (or both). The first ULPs identified were a family of cysteine proteases that are specific for the Ubl called SUMO (small ubiquitin-like modifier) (Li and Hochstrasser, 1999, 2000). These proteases were shown to specifically cleave the peptide (α -amino) or isopeptide (ϵ -amino) linkages after the C terminus of SUMO. The remainder of this chapter focuses almost exclusively on SUMO-specific ULPs.

SUMO-specific ULPs are responsible both for the processing of SUMO precursors to the active form and for selectively deconjugating SUMO from proteins (and presumably from other molecules, such as abundant cellular nucleophiles) (Hochstrasser, 2000; Johnson, 2004; Melchior *et al.*, 2003). Analogous to deubiquitinating enzymes (DUBs), the ability of ULPs to cleave isopeptide linkages between the C-terminal glycine of SUMO and the lysine side chains of modified substrate proteins allows the SUMO modification of proteins to be highly dynamic. For example, protein sumoylation profiles fluctuate dramatically as a function of cell cycle phase or growth condition (Li and Hochstrasser, 1999).

Despite the similarities shared by the ubiquitin and SUMO pathways, both in their biochemistry and in the sequences of many key enzymes (Schwartz and Hochstrasser, 2003), SUMO-specific ULPs and DUBs exhibit little obvious sequence similarity to one another. These ULPs are characterized by a \sim 200 residue segment called the ULP domain (UD); the core of this region bears distant similarity to the active site sequences of certain viral and bacterial cysteine proteases, including the adenovirus protease Avp, whose three-dimensional structure had been known (Li and Hochstrasser, 1999). A cocrystal structure of the C-terminal domain—the Ulp—of the yeast *Saccharomyces cerevisiae* Ulp1 protein linked covalently through its active site cysteine to yeast SUMO (called Smt3) has been determined (Mossessova and Lima, 2000). It revealed that the predicted

His, Cys, and Asp catalytic triad residues were in fact arranged in the classical cysteine protease arrangement, with a conserved Gln residue serving as the oxyanion hole. The overall fold of the UD was similar to that of Avp.

Saccharomyces cerevisiae have two SUMO proteases, Ulp1 and Ulp2, which have distinct substrate specificities and participate in different cellular processes (Li and Hochstrasser, 1999, 2000). Ulp1 accounts for most of the SUMO precursor processing *in vivo* and desumoylates a subset of isopeptide-linked SUMO–protein conjugates. Deletion of *ULP1* is lethal. Conditional *ulp1-ts* mutants arrest as large-budded cells with short spindles and undivided nuclei at the nonpermissive temperature, indicating that Ulp1 function is required in G2/M prior to the metaphase-to-anaphase transition.

Ulp2 is less active than Ulp1 against the SUMO precursor *in vivo*, and *in vitro* Ulp2 has shown much lower activity against both recombinant SUMO substrates bearing peptide bond-linked C-terminal peptides and isopeptide-linked conjugates isolated from yeast (Li and Hochstrasser, 2000). The *ulp2* Δ mutant is viable, but exhibits a pleiotropic phenotype that includes slow growth, a severe sporulation defect, and hypersensitivity to UV radiation, elevated temperature, and chemicals that damage the mitotic spindle or DNA. Some *ulp2* Δ defects are apparently caused by the accumulation of isopeptide-linked SUMO homopolymers, suggesting that Ulp2 also functions in limiting the levels of free SUMO chains *in vivo* (Bylebyl *et al.*, 2003). Ulp1- and Ulp2-deficient strains accumulate different sumoylated proteins, based on anti-SUMO Western immunoblot analysis (Li and Hochstrasser, 2000). This observation and the distinctive phenotypes of these mutants indicate that Ulp1 and Ulp2 act on distinct substrates in the cell. This is at least partially attributable to their different subcellular localization. Ulp1 concentrates at nuclear pore complexes, and Ulp2 localizes to the nucleus (Li and Hochstrasser, 2000; Panse *et al.*, 2003; Schwienhorst *et al.*, 2000). The N-terminal domain of Ulp1 binds to specific karyopherins, tethering the enzyme to the NPC (Panse *et al.*, 2003), which appears to limit the access of Ulp1 to Ulp2 substrates inside the nucleus (Li and Hochstrasser, 2003).

Sequence database searches for potential orthologs of the yeast ULPs yielded seven putative hULPs (or SENPs, for sentrin-specific proteases; sentrin is an alternative name for SUMO), based on the presence of the UD domain (Li and Hochstrasser, 1999; Yeh *et al.*, 2000). Most of these have been shown to have desumoylating activity. Interestingly, one of them, hULP8/SEN8/Den1/NEDP1, was shown to process a distinct Ubl, called NEDD8 or Rub1, rather than SUMO (Mendoza *et al.*, 2003; Wu *et al.*, 2003). The human ULPs and their splice variants are localized to

different cellular compartments and are anticipated to have different *in vivo* substrate specificities as well (Seeler and Dejean, 2003).

In addition to the large number of different SUMO targets in humans (over 50 substrates have been identified thus far), SUMO is actually encoded by a small gene family (*SUMO1* through *SUMO4*) (Bohren *et al.*, 2004; Guo *et al.*, 2004). Thus, human ULPs, and those from many other organisms, may distinguish not only between different SUMO-linked substrates, but also between variant SUMO precursors and SUMO variant-specific protein conjugates. The availability of purified ULPs and assays for their activity provide a starting point from which to approach questions of ULP substrate specificity and cellular function.

Preparation of Yeast Desumoylating Enzymes Ulp1 and Ulp2

One of the advantages to using a bacterial system of expression for ULP purification is that *Escherichia coli* contains no endogenous desumoylating activity (Li and Hochstrasser, 1999). Active, full-length glutathione *S*-transferase (GST) fusions of yeast Ulp1 and Ulp2 are purified readily by one-step glutathione affinity chromatography. One or more subsequent column chromatography steps can be used in conjunction with this initial purification to yield preparations of higher purity. Plasmids for the expression of full-length recombinant GST-Ulp1 and GST-Ulp2 have been constructed using the pGEX-KG vector (Li and Hochstrasser, 1999, 2000). *E. coli* JM101 transformed with pGEX-Ulp1 or pGEX-Ulp2 are maintained on an M9 minimal plate containing 50 $\mu\text{g/ml}$ ampicillin. Expression levels have been observed to decrease if cultures are inoculated from old plates rather than freshly transformed cells.

An overnight culture of JM101 carrying either pGEX-Ulp1 or pGEX-Ulp2 is diluted 1:100 into 4 liters of LB containing 100 $\mu\text{g/ml}$ ampicillin. GST-Ulp expression is then induced with 0.4 mM isopropyl- β -D-thiogalactoside (IPTG), which is added during midlog phase ($\text{OD}_{600} = 0.8$) for 3 h at 30°. The cells are pelleted (5000g for 10 min), washed with ice-cold TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), and resuspended in 2–5 cell pellet volumes of lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol (DTT), 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 20 $\mu\text{g/ml}$ each of the protease inhibitors antipain, aprotinin, chymostatin, leupeptin, and pepstatin]. It is important to include 2 mM DTT in the buffer to preserve cysteine protease activity. The cell suspension is supplemented with lysozyme to a final concentration of 100 $\mu\text{g/ml}$ and is kept on ice for 30 min. Cells are lysed with 0.1% Triton X-100 by gentle mixing and sonication with a Misonix ultrasonic processor (setting 4; 1-s pulse cycle for 5 min) to improve cell lysis and to reduce lysate viscosity.

After clearing of cell debris by centrifugation at 10,000g in a Beckman JA-20 rotor for 30 min at 4°, the supernatant is ready for fractionation by glutathione affinity chromatography. For GST-Ulp1, ~60% of the protein is found in the soluble fraction.

Ten milliliters (settled volume) of glutathione-Sepharose resin (Amersham) preequilibrated in lysis buffer is bound to the GST-Ulp fusion protein in the bacterial lysate during 60 min of gentle mixing on a rotating platform at 4°. The resin is then packed into a disposable column (Bio-Rad) by gravity and is washed with 20 column volumes of ice-cold lysis buffer with 0.1% Triton X-100 until the OD₂₈₀ is less than 0.1. The GST-Ulp fusion protein is eluted with 2 column volumes of lysis buffer containing 20 mM reduced glutathione (Sigma, St. Louis, MO). Most of the fusion protein is eluted in the breakthrough fraction. The presence and purity of the GST-Ulp fusion can be tested by an activity assay (see later) and by SDS-PAGE. Fractions containing active protein are then pooled and dialyzed overnight against 1000 volumes of dialysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM DTT, 1 mM PMSF, and 20% glycerol) at 4°. Both GST-Ulp1 and GST-Ulp2 eluates from the glutathione-Sepharose column contain proteolytic fragments derived from the GST-fusion proteins. Roughly 25% of GST-Ulp1 is full-length, but proteolysis is more severe for the ~140-kDa GST-Ulp2 fusion protein. The glutathione-Sepharose eluates can be fractionated further on an S-200 gel filtration column (16 × 60 cm, Amersham). The column is equilibrated in 50 mM Tris-HCl, 150 mM NaCl, and 2 mM DTT and is developed in the same buffer. This produces satisfactory purifications of the full-length GST-Ulps: ~85% pure in the case of GST-Ulp1 and ~50% pure for GST-Ulp2, which are suitable for many applications. Additional chromatography procedures can be used if higher purity is required. The yield of purified GST-Ulp1 is approximately 50 µg per liter of culture, whereas that of GST-Ulp2 is roughly 10 µg per liter.

As an alternative to using full-length GST-Ulp1, several N-terminal truncations of Ulp1 that retain SUMO protease activity have been generated and characterized *in vivo* and *in vitro* (Li and Hochstrasser, 2003; Mossesso and Lima, 2000). GST-Ulp1C275 contains only the C-terminal 275 amino acids of Ulp1 and is both expressed at a higher level and subject to less degradation in *E. coli* than full-length GST-Ulp1. The affinity purification scheme for GST-Ulp1C275 is identical to that of the full-length protein. Because GST-Ulp1C275 has a basic isoelectric point, a CM-Sephadex (Amersham) purification step can be used to purify the affinity-purified enzyme further. The pooled GST-Ulp1C275 fractions collected from the glutathione-Sepharose column are dialyzed into a low-salt buffer containing 50 mM Tris-HCl, pH 7.4, and 2 mM DTT and are then loaded onto a

preequilibrated 10-ml Hi-Trap CM-Sephadex cartridge (Amersham). The column is washed with 2 volumes of loading buffer and eluted with an NaCl gradient (from 0 to 0.5 M). The GST-Ulp1C275 protein elutes at 0.25 M NaCl, separate from the degradation products, which come off in lower salt fractions. The approximate yield of purified GST-Ulp1C275 is 600 μ g per liter of culture.

Attempts to use a GST fusion of the catalytic domain of Ulp2 alone (GST-UD2) have been less successful. Although the protein is produced at much higher levels in *E. coli* than the full-length protein and is purified readily by affinity chromatography, very little activity against model SUMO fusion substrates has been detected. As an alternative, we have also attempted to express full-length Ulp2 in *E. coli* with a different purification tag, specifically an N-terminal His₆ peptide (pQE30-Ulp2). This is not significantly better than GST-Ulp2 with respect to expression level, yield, or activity (S.-J. Li and M. Hochstrasser, unpublished result).

Preparation of Human Desumoylating Enzymes

We have also generated *E. coli* plasmids for the expression of GST fused to different human ULPs (hULP or SENP) (Table I) (Li and Hochstrasser, 1999; Yeh *et al.*, 2000). We have found that full-length GST-hULP3 (SENP3) and GST-hULP8 (SENP8) are readily expressed in and purified from bacteria. However, deletion of N-terminal sequences from hULP1, hULP2, and hULP5 is necessary to facilitate their expression in *E. coli*. After a series of trials, we have found that deletion of the first 300 amino acids from hULP1, the first 100 amino acids from hULP2, and the first 60 amino acids from hULP5 are sufficient to enable reasonable

TABLE I
HUMAN ULP PROTEINS^a

Name	SENP	Synonyms/orthologs
hULP1	SENP1	SuPr-2 (mouse)
hULP2	SENP2	SSP3, Smt3ip2 (splice variant), SuPr-1 (splice variant), Axam (rat)
hULP3	SENP3	SMT3IP1, SUSP3, SENP4 (splice variant), SuPr-3 (mouse)
hULP5	SENP5	
hULP6	SENP6	SUSP1
hULP7	SENP7	SUSP2
hULP8	SENP8	DEN1, NEDP1

^a We use the original ULP nomenclature here (Li and Hochstrasser, 1999) but have carried over the numbering from SENP names (Yeh *et al.*, 2000) to avoid future confusion.

expression and facilitate purification of these proteins. We have so far been unable to express either full-length or truncated forms of hULP6 and hULP7 as GST fusions in *E. coli*. The conditions for the growth and induction of bacterial cultures and for affinity purification of the human GST-ULP fusions are essentially the same as those described for yeast GST-Ulp1 and GST-Ulp2. We have purified GST-hULP1(Δ N) and GST-hULP2(Δ N) and have shown peptidase and isopeptidase activity against human SUMO substrates for both proteins (S.-J. Li, unpublished results).

Desumoylating Enzyme Activity Assays

This section describes enzyme assays that use a chimeric recombinant SUMO fusion protein as substrate and also describes a method of generating crude preparations of isopeptide-linked sumoylated substrates that can be used for *in vitro* analysis of ULP activity by anti-SUMO immunoblotting. Intein-based peptide ligation has been used to synthesize fluorogenic SUMO-AMC (7-amido-4-methyl coumarin) substrates (Gan-Erdene *et al.*, 2003). The availability of fluorogenic substrates simplifies kinetic analyses of desumoylating enzymes, although the use of SUMO-AMC by itself cannot address questions of ULP specificity for different SUMO-protein conjugates. We have also tried, so far unsuccessfully, to develop an AMC-labeled peptide substrate for yeast Ulp1 based on the C-terminal sequence of SUMO. This approach may have failed because a larger contact area between Ulp1 and SUMO might be needed for stable binding (Mossessova and Lima, 2000).

Peptidase Activity Assays

Recombinant Model Substrate Protein. A chimeric substrate protein containing an N-terminal His₆-tag fused to yeast SUMO (Smt3) with a C-terminal HA tag has been used successfully to characterize yeast Ulp activities (Li and Hochstrasser, 1999, 2000). Cleavage by Ulp1 of the nine-amino acid HA peptide that follows the SUMO C terminus produces a size shift detected readily by SDS-PAGE. This cleavage reaction can be monitored either by immunoblotting with antibodies against His₆ or Smt3 or, if a radiolabeled substrate is used, by phosphorimager measurement or autoradiography. The use of radiolabeled substrate facilitates quantitation.

A plasmid expressing the chimeric His₆-SUMO-HA substrate construct from a *lac* promoter is transformed into an *E. coli* lacI^Q strain such as JM101 (Li and Hochstrasser, 1999). Genes encoding yeast Smt3, human SUMO1, or human SUMO2 have been cloned into pQE30 (Qiagen) for this purpose. For purifying the radiolabeled SUMO chimeras, expression

of the recombinant substrate is induced with 1 mM IPTG in a 50-ml LB + ampicillin culture of log phase JM101 cells and incubated for 1 h at 37°. The cells are then pelleted, washed twice in M9 minimal medium (1 mM MgSO₄, 0.2% glucose, 10 µg/ml thiamine, 1 mM CaCl₂), and resuspended in 25 ml of M9 minimal medium with 25 µg/ml ampicillin and 1 mM IPTG. ³⁵S-Translabel (1 mCi; Amersham) is then added, and the culture is placed on a shaker platform for an additional hour at 37°. Cells are harvested and washed once with M9 minimal medium and are then resuspended in 0.5 ml spheroplasting buffer (25% sucrose, 50 mM Tris-HCl, pH 8.0). Lysozyme (100 µg) is added at this point, and the suspension is left on ice for 5 min. The cell suspension is diluted to 1 ml with 50 mM Tris-HCl, pH 8.0, buffer, and 10 µl of 10% Triton X-100 is added to lyse the cells. The resulting lysate is sonicated briefly if necessary and is centrifuged at 14,000g in a microcentrifuge at 4° for 10 min. The NaCl concentration in the resulting supernatant is adjusted to 150 mM with 5 M NaCl. These Triton-based lysis conditions do not require intensive sonication (especially if used in conjunction with DNase I treatment to reduce lysate viscosity); prolonged sonication should be conducted with caution to minimize the risk of producing radioactive aerosol.

One milliliter of cobalt-affinity matrix (BD Talon-Clontech) preequilibrated in TBST (150 mM NaCl, Tris-HCl, pH 7.5, 0.05% Triton X-100) is incubated with the cell lysate using gentle mixing for 1 h on a rotator at 4° to bind the His₆-tagged protein to the resin. The beads are poured into a disposable column (Bio-Rad, Polyprep) and washed with 50 ml TBST. The bound proteins are eluted with 100 mM imidazole in TBS and dialyzed overnight into TBS at 4° against 1000 volumes of dialysis buffer. The purity of the substrate is checked by SDS-PAGE on a 12.5% gel. The expected yield of the purified substrate is approximately 1 mg/liter. Specific radioactivity can be measured by radioactive counting of an aliquot of the purified protein and comparing it to the protein concentration. The specific activity of GST-Ulp1 is 120 nmol of substrate cleaved per milligram of enzyme per minute, whereas the activity of the purified GST-Ulp2 is too low to provide a meaningful measurement of specific activity.

Substrate cleavage assays are performed at 30° with varying concentrations of substrate and GST-Ulp1 or GST-Ulp2 in a reaction buffer containing 150 mM NaCl, 1 mM DTT, 50 mM Tris-HCl, pH 7.5, and 0.2% Triton X-100 (the latter for GST-Ulp2 only). Typical substrate concentrations are 1–10 µM, and enzyme concentrations are usually in the range of 0.3–3 nM. At each time point, an aliquot of the reaction mix is added to an equal volume of 2× SDS gel-loading buffer and heated to 95° for 5 min to stop the reaction. For assaying GST-Ulp1 activity, 5-min time points are usually taken over a 30-min period, but for GST-Ulp2, the reaction times need to

be extended to 3 h and longer. Samples from each time point can then be separated on a 12.5% SDS–polyacrylamide gel and analyzed by phosphor-imaging. The percentage of cleavage at each time point is calculated from the ratio of pixels in the cleavage product (the fast-migrating His6-SUMO band) to the combined pixels of the cleavage product plus remaining substrate (the slow-migrating His6-SUMO-HA band) in the same lane. (The HA tag does not contain any methionine or cysteine, so no radioactivity is lost due to its cleavage.) When we substitute human SUMO1 or SUMO2 for the yeast Smt3 sequence in the chimeric substrate, they are readily cleaved in assays of human ULPs.

SUMO-AMC Substrate

To obtain a model substrate for SUMO-specific proteases that can be monitored by fluorescence, we generated a fusion of human SUMO1 (Gly96) and intein-CBD (*Bacillus circulans* chitin-binding domain) in a pTYB vector backbone (New England Biolab). The fusion protein product can be expressed in *E. coli* and purified using a chitin affinity column as described by the manufacturer (Impact system, New England Biolab). SUMO1-MESNa (β -mercaptoethanesulfonic acid) is generated by cleavage of the fusion protein with MESNa. SUMO-MESNa is then converted to SUMO-AMC (7-amido-4-methyl coumarin) by a chemical ligation reaction performed with a large excess of glycine-AMC. Details of the fusion protein purification and chemical ligation reaction have been published (Gan-Erdene *et al.*, 2003). In addition, human SUMO1-AMC is now available from a commercial source (Boston Biochem).

Hydrolysis of the SUMO-AMC substrate is determined spectrofluorometrically in a final volume of 100 μ l. Typical assays contain 10 to 100 pM concentrations of enzyme, and the substrate is present in 10^3 to 10^4 molar excess over enzyme in the reaction buffer (50 mM Tris HCl, pH 7.5, at 25°). The estimated K_m of human GST-hUlp1 for this substrate is 300 nM, measured at 10 pM concentration of the purified enzyme (Li and Craig Hill, unpublished result).

Isopeptidase Activity Assays

In Vitro-Translated RanGAP1

Sumoylated RanGAP1 is one of the most abundant and best-characterized SUMO1-protein conjugates in mammalian cells. RanGAP1 contains a single SUMO modification site at Lys526 (Sampson *et al.*, 2001), and approximately 20–30% of RanGAP1 translated in a rabbit reticulocyte

lysate system is conjugated to SUMO present in the reticulocyte lysate, presumably by the conjugating enzymes also contained in the lysate. A typical translation reaction consists of an aliquot of transcription/translation coupling mix (STP3 system, Novagen), [³⁵S]methionine/cysteine Translabel (Amersham), and purified supercoiled plasmid DNA (the vector pAlter-Max A contains a MYC-tagged mouse RanGAP1 cDNA controlled by a T7 promoter; kindly provided by Dr. M. Matunis of John Hopkins University). The translation reaction mix is incubated for 1 h at 30°, and the reaction is terminated by the addition of RNase A (10 µg/ml, final concentration). *N*-Ethylmaleimide (NEM) is then added to the mixture (1 mM, final concentration) and incubated for 15 min at 23° to inactivate both sumoylating and desumoylating activities in the reticulocyte lysate. β-Mercaptoethanol and L-cysteine (2 mM each, final concentration) are added to neutralize excess NEM, and the reaction mixture can be used directly as substrate in the isopeptidase reaction.

We have shown that Ulp1 from *S. cerevisiae* cleaves Lys526-linked mammalian SUMO1-RanGAP1 in a time-dependent manner (Li and Hochstrasser, 1999). The reaction mixture contains 5 µl of *in vitro*-translated RanGAP1, 1 nM purified GST-Ulp1, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM DTT in a final volume of 10 µl. The progress of the isopeptidase reaction is monitored by the disappearance of the slower-migrating ³⁵S-labeled sumoylated RanGAP1 band on a 12.5% SDS-PAGE gel and is quantified by phosphorimaging. SUMO1-RanGAP1 is processed with a *t*_{1/2} of roughly 7 min in the presence of ~1 nM GST-Ulp1 (Li and Hochstrasser, 1999).

NEM-Treated Cell Lysate

While sumoylated RanGAP1 is a well-defined isopeptide-linked substrate, some ULPs may have restricted activity toward this particular substrate (Kim *et al.*, 2000). We have therefore incubated either GST-Ulp1 or GST-Ulp2 with NEM-treated yeast cell lysate to demonstrate their abilities to cleave endogenous sumoylated proteins (Li and Hochstrasser, 1999, 2000). Wild-type or *ulp* mutant yeast cells (~10⁹ cells) are harvested from log-phase cultures in YPD, washed once with buffer A (1.2 M sorbitol, 50 mM Tris-HCl, pH 7.5), and incubated in 1 ml of buffer A containing 0.5 mg of zymolyase 100T/ml for 30 min at 30°. After cell wall digestion, the cells are washed once in cold buffer A and lysed by sonication on ice in 0.5 ml buffer B (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.2% Triton X-100, 2 mM NEM, 2 mM PMSF, and 20 µg/ml each of leupeptin, pepstatin, and antipain). The resulting lysate is centrifuged at 14,000g for 10 min to clear cell debris, and soluble protein concentrations are determined

by the bicinchoninic acid protein assay (Pierce). Prior to initiation of the cleavage reactions, L-cysteine and β -mercaptoethanol are added to 2 mM each and incubated at 23° for 15 min to consume any remaining unreacted NEM. Reactions are initiated by the addition of 50 ng of purified GST-Ulp to a 20- μ l reaction mixture containing 25 μ g of soluble yeast protein in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, and 0.1% Triton X-100 and are terminated by boiling in SDS sample buffer. Reactions are monitored by anti-SUMO immunoblot analysis. Ulp activity in yeast cell lysates has been observed to be unaffected by various protease inhibitors.

In addition to the methods described earlier, we have developed other methods to demonstrate ULP enzyme activity. For example, we immobilized the chimeric substrate His₆-ubiquitin-Smt3-HA to a 96-well plate (His sorb plate, Qiagen) and monitored the cleavage reaction with an HRP-linked anti-HA antibody in an ELISA-based colorimetric reaction (using hydrogen peroxide and the substrate 3,3',5,5'-tetramethyl benzidine). This method gives rapid results and is particularly useful for following ULP activity during enzyme purification (S.-J. Li, unpublished results). Purified SUMO E1 and E2 proteins (UBA2-AOS1 and UBC9) are now available from commercial sources (Alexis Biochemicals, San Diego, CA, or Boston Biochem, Boston, MA) so it is also possible, albeit expensive, to generate isopeptide-linked SUMO homopolymers as additional substrates for the detection of isopeptidase activity.

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