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Partial purification and substrate specificity of a ubiquitin hydrolase from *Saccharomyces cerevisiae*

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A ubiquitin hydrolase that removes ubiquitin from a multi-ubiquitinated protein has been purified 600-fold from *Saccharomyces cerevisiae*. Four different ubiquitin–protein conjugates were assayed as substrates during the purification procedure. Enzymic activities that removed ubiquitin from ubiquitinated histone H2A, a ubiquitin–ubiquitin dimer and a ubiquitin–ribosomal fusion protein were separated during the purification from an activity that removed a single ubiquitin molecule linked by an isopeptide bond to a ubiquitinated protein. The size of the native enzyme was 160 kDa, based on its sedimentation in a sucrose gradient, and the subunit molecular mass was estimated to be 160 kDa, based on a profile of proteins eluted in different fractions by thiol-affinity chromatography. The partially purified hydrolase was not inhibited by a variety of protease inhibitors, except for thiol-blocking reagents. The natural substrate for this enzyme may be the polyubiquitin chain containing ubiquitin molecules bound to each other in isopeptide bonds, with one of them linked to a lysine residue of a protein targeted for intracellular proteolysis.

INTRODUCTION

Ubiquitin is a 76-amino-acid polypeptide found in high levels in most eukaryotic cells. It plays a critical role in intracellular protein turnover, chromatin structure and function, and ribosome assembly [1–3]. About half of the total ubiquitin in a cell is detected as the free 76-amino-acid molecule, with the balance bound in isopeptide linkage to the ϵ-amino group of a lysine residue in a variety of proteins [4]. Among the latter are the histones H2A and H2B. Monoubiquitinated conjugates are stable proteins whose functions are still obscure; however, polyubiquitinated proteins are destined for proteolytic degradation. Recent data show that these latter polyubiquitin structures consist of many ubiquitin molecules linked to each other in isopeptide bonds [5,6]. Three enzymic activities are required to activate ubiquitin and conjugate it to a protein [7]. Another complex of enzymes is used to degrade polyubiquitinated proteins [8,9]. Both systems require ATP, and coupling of the two is postulated to account for the major pathway of intracellular protein turnover that removes both short-lived cellular proteins and abnormal proteins formed under various conditions, such as heat shock and oxidative stress [10–12].

Ubiquitin is initially synthesized from mRNAs that encode either polyubiquitin sequences ranging from 3 to > 50 ubiquitin molecules connected together without any intervening stop and start sequences or a single ubiquitin molecule fused in frame to sequences coding for ribosomal subunits (reviewed in [13]). Thus formation of monoubiquitin *de novo* requires hydrolases recognizing a polyubiquitin and a ubiquitin–ribosomal fusion protein. Monoubiquitin also is recycled from ubiquitinated histones and from the polyisoubiquitinated protein conjugates noted above. Several ubiquitin hydrolases have been described [14–17], and some of these have recently been shown to be separable chromatographically [18,19]. All of these are sensitive to thiol-blocking agents, but they differ in substrate specificity and other properties. In the most thorough comparative study, Mayer & Wilkinson [18] isolated four ubiquitin *C*-terminal esterases in highly purified states from extracts of bovine thymus. All four enzymes were purified with the use of a ubiquitin ethyl ester as substrate, but only one of these, a high-molecular-mass protein of 100–200 kDa, could remove ubiquitin from putative ubiquitin–protein conjugates that had been prepared by conjugation *in vitro* of ^{125}I-ubiquitin to proteins in a reticulocyte lysate. The other three enzymes had molecular masses in the 20–30 kDa range, and one of these, termed L1, was found to be closely related to a major human neuronal protein [20]. On the basis of sequences derived from cloning of the cDNA [20], L1 is similar to a cloned yeast ubiquitin hydrolase that was purified with a ubiquitin fusion protein as substrate [21]. This yeast ubiquitin hydrolase removed ubiquitin from synthetic substrates that had sequences from one to 72 amino acids bound in a normal peptide bond to the *C*-terminal glycine residue of ubiquitin. Another isoenzyme, noted L3, had properties resembling those of a ubiquitin *C*-terminal hydrolase purified from rabbit reticulocytes [16]. This latter isoenzyme does not hydrolyse ubiquitin–protein conjugates and is postulated to cleave ubiquitin from small peptide fragments. The L2 isoenzyme appears to be similar to the hydrolase that cleaves the single ubiquitin bound by isopeptide bond to histones H2A and H2B [14].

We were interested in studying the hydrolase that produces monoubiquitins from the polyprotein translated from an mRNA encoding multiple ubiquitins. In order to identify this type of ubiquitin hydrolase, we attempted to prepare, by a coupled transcription–translation system *in vitro*, a ubiquitin–ubiquitin dimer encoded in a cDNA that we had previously isolated and sequenced [22]. However, in a cell-free translation system primed with this diubiquitin mRNA, we found only ubiquitin and could not detect the diubiquitin precursor. Ubiquitin has a methionine residue at its *N*-terminus, and, to rule out initiation at the internal AUG site of the diubiquitin mRNA, we modified the cDNA by restriction endonucleases such that the mRNA would code for a truncated diubiquitin in which the second ubiquitin

Abbreviations used: DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; Ub-Rp$_L$, ubiquitin–ribosomal fusion protein; uH2A, ubiquitin–histone H2A; Ub-Ub, ubiquitin dimer.

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had 40% of its *C*-terminal sequence removed. Surprisingly, the major product of translation *in vitro* of this mRNA was a multi-ubiquitinated protein of 30 kDa that appeared to be formed by endogenous ubiquitination of the predicted truncated diubiquitin translation product [23]. This 30 kDa ubiquitin conjugate was unstable in the reticulocyte extract and was converted initially into a 24 kDa protein plus ubiquitin by an enzymic activity that resembled a ubiquitin hydrolase in that the conversion was insensitive to all protease inhibitors except thiol-blocking agents, but was inhibited by ubiquitin aldehyde, a synthetic molecule known to block ubiquitin-processing enzymes [24]. This putative ubiquitin hydrolase was detected also in extracts of wheat germ, human placenta, *Dictyostelium discoideum* and *Saccharomyces cerevisiae*. We elected to use the last of these for purifying the hydrolase, and describe here its purification and properties.

EXPERIMENTAL

Materials

Bulk baker's yeast was obtained from a local bakery. The reticulocyte lysate and reagents for translation *in vitro* were from Promega, radioactive amino acids from Amersham, DE 52 DEAE-cellulose ion-exchange resin was from Whatman and activated-Thiol–Sepharose 4B from Sigma. Proteinase inhibitors were from Sigma, except for leupeptin, which was from Boehringer–Mannheim.

Formation of substrates

The substrate for hydrolase purification was a multi-ubiquitinated 30 kDa product (Ub1.6) of translation *in vitro* from a mRNA containing a ubiquitin–0.6-ubiquitin sequence, described previously [23]. After a 15 min translation, samples were heated 3 min at 60 °C in the presence of 1 mM-dithiothreitol (DTT) and proteinase inhibitors, including 1 mM-phenylmethanesulphonyl fluoride (PMSF), 0.1 mM-N^{α}-*p*-tosyl-L-lysylchloromethane (TLCK), 0.1 mM-tosyl-L-phenylalanylchloromethane (TPCK), leupeptin (0.1 μg/μl) and aprotinin (20 μg/μl). Cycloheximide was added to a final concentration of 0.28 μg/μl.

Three additional ubiquitin conjugates were tested as substrates during enzyme purification. (1) A normal form of the ubiquitin–ribosomal fusion protein (Ub-Rp_L) was prepared from an *Escherichia coli* strain carrying a plasmid with an insert of sequences of a human ubiquitin–ribosomal fusion protein [19]; kindly provided by D. Ecker, Smith Kline and French, Philadelphia, PA, U.S.A. Extracts were prepared by sonication of bacteria harvested from a 5 ml culture that had been labelled with [^{35}S]methionine immediately after a temperature shift. The extraction buffer contained 50 mM-Tris/HCl, pH 7.2, 2 mM-EDTA, 10% (v/v) glycerol, 1 mM-DTT and the proteinase inhibitors noted above. Extracts were heated at 60 °C for 3 min to block endogenous proteinases, and a 5 μl sample was used in the enzyme assay. (2) Ubiquitin–histone H2A (uH2A) was isolated from nuclei of chick-embryo fibroblast cells. Nuclei were purified by washing pelleted cells (approx. 8×10^7 cells) twice with a buffer consisting of 70 mM-KCl, 5 mM-$MgCl_2$, 3 mM-Hepes, pH 7.4, 3 mM-EGTA, 0.5% Triton X-100 and 1 mM-PMSF, and once with the same buffer without Triton X-100. Purified nuclei were extracted twice with 100 μl of 0.2 M-H_2SO_4 for 30 min at 4 °C. Protein was precipitated with 6 vol. of ethanol at −20 °C, and precipitates were washed sequentially with acetone/0.1 M-HCl (6:1, v/v), acetone and ethanol. The dried pellet was dissolved in 200 μl of 50 mM-Tris/HCl (pH 7.2)/10% glycerol/2 mM-EDTA. (3) A purified ubiquitin dimer (Ub-Ub) expressed in *E. coli* and labelled with [^{35}S]methionine was given by C. Liu (Genentech, South San Francisco, CA, U.S.A.). A 5 μl sample containing 30000 c.p.m. was used in enzyme assays.

Detection and determination of the different substrates

The Ub1.6 mRNA products of translation *in vitro*, the Ub-Rp_L and the Ub-Ub dimer proteins expressed in *E. coli* were separated by SDS/PAGE in a phosphate/urea/15%-acrylamide gel system [25] or a Laemmli 12.5%-acrylamide gel system [26] and analysed by fluorography [27]. The amounts of substrate and products were determined by excising the specific bands from the gel and counting them for radioactivity in a liquid-scintillation counter. uH2A was separated by SDS/PAGE in a 12.5%-acrylamide gel system [26] and electrophoretically transferred to nitrocellulose (0.22 μm pore size). Anti-ubiquitin antibodies were used to detect the uH2A by published procedures [28].

Enzyme assay

To assay for hydrolase activity, a 5 μl sample of substrate was added to a 5 μl sample of enzyme in 50 mM-Tris/HCl (pH 7.2)/10% glycerol/2 mM-EDTA, and the mixture was incubated at 37 °C in the presence of 1 mM-DTT and protease inhibitors, including 1 mM-PMSF, 0.1 mM-TPCK, 0.1 mM-TLCK, leupeptin (0.1 μg/μl) and aprotinin (20 μg/μl). From the radioactivity in the 30 kDa substrate (Ub1.6) and the specific radioactivity of the [^{35}S]methionine used, this substrate was estimated to be present at about 0.1 μM concentration in the enzyme assay. The concentration of Ub-Rp_L was estimated to be 10 μM. For assays containing Ub-Rp_L and uH2A, DTT and the inhibitors were used at double the concentrations above. The incubation times were 10 min for Ub1.6 and Ub-Ub, 30 min for uH2A and 45 min for Ub-Rp_L processing. The amount of hydrolase added to a substrate was adjusted to ensure that measurements were carried out in the linear range of enzyme activity. One unit is defined here as the amount of enzyme that releases 20% of labelled ubiquitin from the ubiquitin conjugate in 10 min at 37 °C.

Purification of the enzyme

A baker's yeast preparation (300 g wet wt.) was resuspended in 350 ml of 100 mM-Tris/HCl, pH 8.1, containing 1 mM-EDTA, 5% glycerol, 0.05% Brij 58, 0.5 μM-pepstatin, 1.25 mM-$NaHSO_3$, 125 μM-PMSF, 0.5 mM-benzamidine and 1 mM-DTT (Buffer A) and disrupted with glass beads by using a bead beater (Biospec. Products) [29]. Solid $(NH_4)_2SO_4$ was added to obtain a fraction that was soluble at 16%- and insoluble at 60%-satd.-$(NH_4)_2SO_4$. Polymin P was added to a final concentration of 0.4% during this fractionation step [29]. The precipitate was resuspended in a buffer consisting of 50 mM-KH_2PO_4, pH 7.2, 2 mM-EDTA, 5 mM-DTT, 2 mM-benzamidine, 2 μM-pepstatin A, 10 mM-$NaHSO_3$, 1 mM-PMSF and 10% glycerol (Buffer B) and dialysed against Buffer B. During dialysis, some protein precipitated and was collected by centrifugation at 12000 *g* for 60 min. The precipitate was re-solubilized by homogenization with 150 ml of Buffer B containing 50 mM-Tris/HCl, pH 7.2, in place of the phosphate, and dialysed against the same buffer. This fraction was greatly enriched in the Ub1.6 hydrolase activity. The ratio of activity between the phosphate-soluble and Tris-soluble fractions was approx. 1:4. The Tris-soluble fraction (900 mg) was applied to a DE 52 column (23 cm × 2.5 cm) equilibrated with 50 mM-Tris/HCl, pH 7.2, containing 10% glycerol, 2 mM-EDTA, 1 mM-PMSF, 1 mM-DTT and 1 mM-benzamidine (Buffer C) and washed with 5 column vol. of Buffer C containing 100 mM-KCl. A 1400 ml linear gradient from 100 to 325 mM-KCl in Buffer C was used to elute the protein from the column, and 20 ml fractions were collected. The peak of enzyme activity was recovered in the fractions eluted between 200 mM- and 225 mM-KCl. The active fractions were pooled, dialysed against 50 mM-Tris/HCl (pH 7.2)/2 mM-EDTA/10% glycerol, concentrated with CF25 membrane cones (Amicon) and applied to a gradient of 10–25%-

sucrose in 50 mM-Tris/HCl (pH 7.5)/100 mM-NaCl/1 mM-EDTA. After centrifugation at 4 °C for 24 h at 37000 rev./min in a Beckman SW41 Ti rotor, fractions (0.5 ml) were collected and assayed for hydrolase activity. The active fractions (14–16 % sucrose) were pooled, dialysed, concentrated and applied to an activated-Thiol–Sepharose column. Details are described in the Results section. Protein concentrations were determined with the BCA reagent (Pierce) or by using the A_{280} and assuming $A^{0.1\%}_{1\,cm,\,280} = 1$.

RESULTS

Partial purification of a ubiquitin hydrolase

To purify the ubiquitin hydrolase studied here, we assayed the conversion of the 30 kDa ubiquitin conjugate generated *in vitro* (Ub1.6) into free ubiquitin and a 24 kDa protein (Fig. 1*a*).

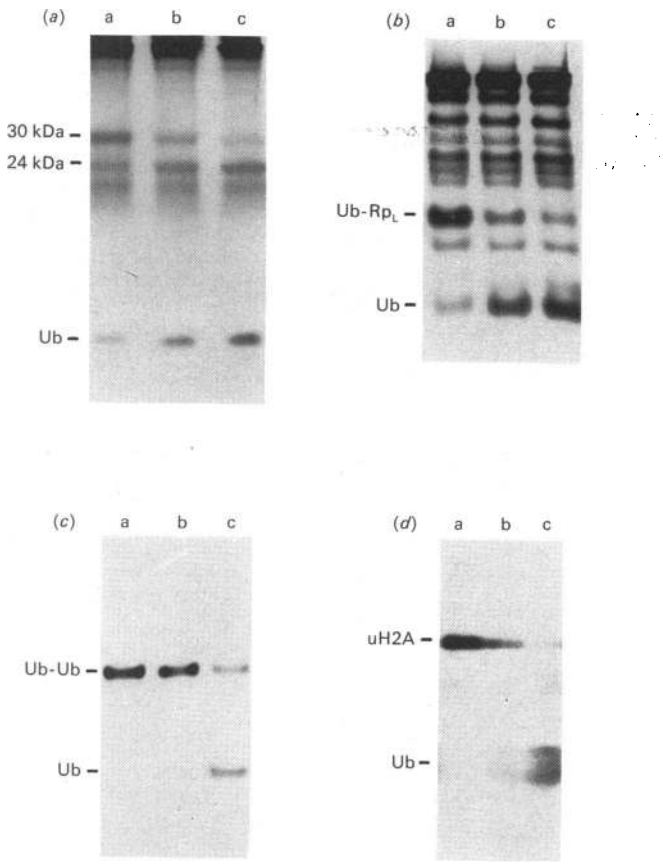

Fig. 1. Ubiquitin hydrolase assays with different ubiquitin conjugates

(*a*) The 30 kDa ubiquitin conjugate was produced by translation *in vitro* of the Ub1.6 mRNA and treated as described in the Experimental section. SDS/PAGE analysis: a 5 μl sample was incubated for 10 min at 37 °C with 5 μl of buffer (lane a) or with 5 μl of buffer containing 0.02 μg (lane b) or 0.05 μg (lane c) of the thiol-column-purified enzyme (10 mM-DTT eluate). (*b*) SDS/PAGE analysis: a 5 μl sample of Ub-Rp$_L$ expressed in *E. coli* was incubated for 45 min at 37 °C with 5 μl of buffer (lane a) or with 5 μl of buffer containing 0.4 μg (lane b) or 0.6 μg (lane c) of the thiol-column-purified enzyme (10 mM-DTT eluate). (*c*) SDS/PAGE analysis: a 5 μl sample of Ub-Ub expressed in *E. coli* was incubated for 10 min at 37 °C with 5 μl of buffer (lane a) or with 5 μl of buffer containing 0.2 μg (lane b) or 0.5 μg (lane c) of the 16–60 %-$(NH_4)_2SO_4$ fraction soluble in Tris. (*d*) Immunoblot analysis: a 5 μl sample of uH2A was incubated for 30 min at 37 °C with 5 μl of buffer (lane a) or with 5 μl of buffer containing 1 μg (lane b) or 2 μg (lane c) of the DE 52-purified enzyme.

Throughout the purification we also tested for activities that removed ubiquitin from a human ubiquitin–ribosomal fusion protein (Ub-Rp$_L$), from a ubiquitin dimer (Ub-Ub) and from the ubiquitin–histone conjugate uH2A (Figs. 1*b*–1*d*).

Yeast extract was a good source for the Ub1.6 hydrolase, and because of its availability and ease of genetic analysis, it was chosen for purification of the hydrolase. Initially, we used a laboratory strain of *S. cerevisiae* carrying deletions in several yeast vacuolar protease genes [29], but found that common baker's yeast treated with protease inhibitors was equally suitable. Details of the initial steps of purification of the yeast ubiquitin hydrolase are given in the Experimental section. From the sucrose-gradient-centrifugation step, the hydrolase was recovered in fractions that sedimented with an *s* value corresponding to a native molecular mass of 160 kDa, assuming that the protein is globular (Fig. 2).

Processing of the multi-ubiquitinated 30 kDa protein was shown to be inhibited by thiol-binding agents [23]. On the basis of this observation, we tested a thiol-affinity column as a step in the purification. About 2.5 mg of protein from the sucrose gradient (14–16 %-sucrose fractions) was adjusted to a final concentration of 0.5 M-NaCl and applied to a 6 ml activated-Thiol–Sepharose 4B column. The column was washed with 5 column vol. of buffer containing 0.1 M-Tris/HCl, pH 7.2, 2 mM-EDTA and 0.5 M-NaCl, and bound proteins were eluted with DTT in the same buffer. In the 3 mM-DTT eluate, about five or six proteins could be detected by silver staining of an SDS/PAGE gel (Fig. 3*a*, lane c). Increasing the DTT concentration to 10 mM led to elution of additional proteins, and one protein of 160 kDa accounted for > 80 % of the protein detected by silver staining of the gel (Fig. 3*a*, lane d). Hydrolase activity for the Ub1.6 substrate was present in both DTT-eluted fractions, but there was about a 10-fold increase in the specific activity of enzyme in the 10 mM-DTT fraction compared with the 3 mM-DTT fraction (Fig. 3*b*). These data suggest that the 160 kDa protein is the hydrolase. On the basis of the sedimentation profile of the hydrolase after rate-zonal centrifugation, we postulate that the hydrolase is a monomer of 160000 kDa. A higher-molecular-mass ubiquitin hydrolase (100–200 kDa) has been reported, but most ubiquitin hydrolases have subunit molecular masses of about 30 kDa [14,18–21]. A summary of the purification procedure is presented in Table 1 and shows a 600-fold increase in specific activity.

Substrate specificity of the hydrolase

To determine the substrate specificity of the enzyme, we compared the hydrolase activity at various stages of purification with several ubiquitin conjugates as substrates (Fig. 1; Table 2). For the 30 kDa ubiquitin conjugate (Ub1.6) as substrate, the specific activity increased 410-fold between the Tris-soluble protein of the 16–60 %-satd.-$(NH_4)_2SO_4$ precipitate and the thiol-column-purified enzyme. In contrast, the hydrolase activities increased only 84-fold and 24-fold respectively when Ub-Rp$_L$ and Ub-Ub were substrates. A hydrolytic activity that removed ubiquitin from uH2A was in the DE 52 column fraction, but very low levels of this enzyme were detected in the later stages of purification (results not shown). We suspect that the uH2A hydrolase did not sediment with the other hydrolases in the sucrose gradient.

Two of these substrates, the Ub-Rp$_L$ and the Ub-Ub dimer, have a normal peptide bond linking ubiquitin to its conjugate. In contrast, ubiquitin is linked by an isopeptide bond to the ϵ-amino group of a single lysine residue in histone H2A. The ubiquitin moiety that is released from the Ub1.6 30 kDa substrate in its conversion into a 24 kDa conjugate is also in isopeptide linkage [23], but differs from the uH2A in that the linkage is to another

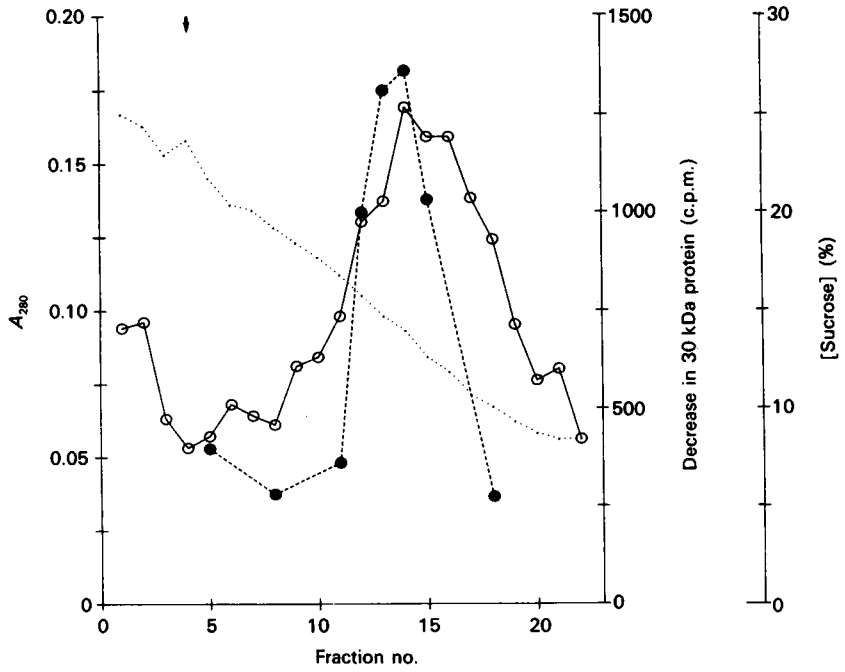

Fig. 2. Rate-zonal sedimentation of the DE 52 fractions in a 10–25%-sucrose gradient

Fractions 32–34 (200–225 mM-KCl fractions) from the DE 52 columns were pooled, dialysed and concentrated with CF25 membrane cones to approx. 10 mg/ml. Then 1 mg samples were applied to 13 ml 10–25% sucrose gradients in 50 mM-Tris/HCl (pH 7.5)/100 mM-NaCl/1 mM-EDTA and centrifuged for 24 h at 37000 rev./min in a Beckman SW41 Ti rotor at 4 °C. Fractions (0.5 ml) were collected, and protein was determined by the A_{280} (○). Those fractions enriched in protein were dialysed, concentrated with Centricon-30 micro-concentrators (Amicon) and re-assayed for protein. Samples (0.05 μg) of the concentrated fractions were used to measure hydrolase activity against the Ub1.6 substrate (●). The concentration of sucrose in the gradient is represented by (·····). A 60 μg preparation of pure β-galactosidase (Sigma) was included in the gradient fractionation. From the sedimentation rate of this enzyme and its known molecular mass, it was possible to calculate a molecular size of the hydrolase by the method of Martin & Ames [32]. The peak of β-galactosidase activity in the gradient is represented by (↓).

Table 1. Purification of the Ub1.6 ubiquitin hydrolase

Results are for a typical preparation of the hydrolase.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude yeast extract	15000	18550	1.25		
16–60%-$(NH_4)_2SO_4$: soluble in phosphate	2600	1040	0.40		
16–60%-$(NH_4)_2SO_4$: soluble in Tris	1650	3000	1.82	1.5	16
DE 52 (200–225 mM-KCl)	12.4	288	23.2	18.6	1.5
Sucrose gradient (14–16% sucrose)	2.5	83	33.3	26.6	0.45
Thiol column (10 mM-DTT)	0.01*	7.5	745	600	0.04

* Estimated from silver staining, with β-galactosidase as standard. See Fig. 3 legend for details.

Table 2. Substrate specificity of the hydrolase

Values are specific enzymic activity in units/mg (see the Experimental section) at various stages of purification.

Substrate	16–60%-$(NH_4)_2SO_4$ (Tris-soluble)	DE 52 chromatography (200–225 mM-KCl)	Thiol column (10 mM-DTT)	Total purification (fold)
UB1.6	1.8	23.2 (13)*	745 (32)†	410
UB-Rp_L	0.15	1 (7)*	12.6 (13)†	84
Ub-Ub	6	20 (3)*	143 (7)†	24

* Fold increase in activity between the DE 52 chromatography and the Tris-soluble portion of the 16–60%-$(NH_4)_2SO_4$ fractions.

† Fold increase in activity between the 10 mM-DTT eluate of the thiol column and the DE 52 fraction.

ubiquitin molecule. Thus this purification protocol appears to have selected for a polyisoubiquitin hydrolase.

DISCUSSION

The data presented here on purification of a ubiquitin hydrolase indicate that yeast contains at least three different enzymic activities that can release ubiquitin from protein conjugates. Four separable ubiquitin hydrolase activities have been detected in calf thymus [18] and three in rabbit reticulocyte extracts [19]. A ubiquitin *C*-terminal hydrolase has been purified from rabbit reticulocytes and its properties were characterized [16,17]. Enzymic activities that release ubiquitin from histone H2A have been described [14,15]. A yeast enzyme that cleaves ubiquitin from a genetically engineered ubiquitin–fusion polypeptide has recently

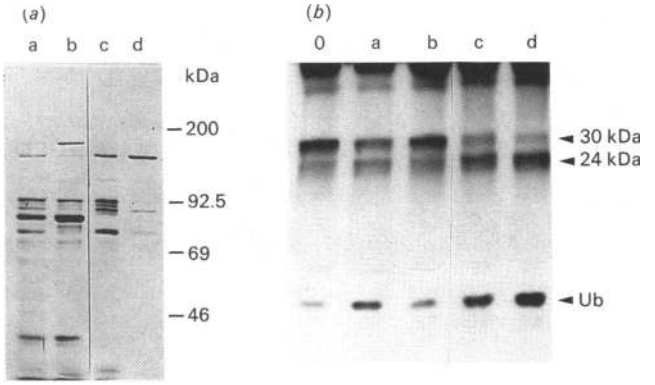

Fig. 3. Separation of hydrolases by Thiol–Sepharose 4B affinity chromatography

Pooled and concentrated fractions with hydrolase activity from sucrose gradients (total 2.5 mg) were adjusted to a final concentration of 0.5 M-NaCl and applied to a 6 ml thiol affinity column (3.5 cm × 1.5 cm). The unbound protein was collected and the column was washed successively with 30 ml of buffer (0.1 M-Tris/HCl, pH 7.2, 2 mM-EDTA and 0.5 M-NaCl), with 30 ml of the buffer plus 3 mM-DTT and with 30 ml of the buffer plus 10 mM-DTT. The three fractions were concentrated to 1 ml each by using Centriprep-30 concentrators (Amicon) and dialysed against 50 mM-Tris/HCl (pH 7.2)/2 mM-EDTA/10% glycerol. Protein concentrations were estimated by using the A_{280} for the unbound material and for the 3 mM-DTT eluate. For the 10 mM-DTT eluate, proteins were separated by SDS/PAGE in a 7.5%-acrylamide gel [26]. Protein concentration was determined by comparing band intensity after silver staining with known amounts of β-galactosidase electrophoresed on the same gel. (*a*) SDS/PAGE analysis: lane (a), 2 μg of the sucrose gradient fraction applied to the column; lane (b), 2 μg of the unbound protein; lane (c), 2 μg of the 3 mM-DTT eluate; lane (d), 0.3 μg of the 10 mM-DTT eluate. The gel was silver-stained. (*b*) Hydrolase activity of thiol-column eluates measured with 5 μl of translation mixture containing the Ub1.6 substrate and incubated for 10 min at 37 °C. Lane (a), 0.1 μg of the sample applied; lane (b), 0.1 μg of the unbound protein; lane (c), 0.1 μg of the 3 mM-DTT eluate; lane (d), 0.015 μg of the 10 mM-DTT eluate. The lane (0) had no additional protein added. The total reaction volume was 10 μl. Samples were analysed by electrophoresis in a phosphate/urea 15%-acrylamide/SDS gel system, followed by fluorography.

been purified and its sequence determined from a cDNA clone [20]. This highly purified enzyme did not process the Ub1.6 30 kDa conjugate, and the most purified enzyme fraction described here had weak activity against the engineered fusion protein (results not shown).

There are two major types of ubiquitin–protein conjugates; one has ubiquitin in isopeptide bond to the ϵ-amino group of a lysine residue, and the other has ubiquitin in a normal peptide bond to different amino acids in proteins. In yeast, there are three of the latter: a ubiquitin-(ubiquitin)$_4$ in which a Gly–Met bond would be at the site cleaved to produce ubiquitin, a ubiquitin fusion protein with a Gly–Ile bond at the cleavage site, and a ubiquitin fusion protein with a Gly–Gly bond at the cleavage site [30]. Enzyme specificity could be based on the amino acids at the cleavage site, but it might also depend on the nature of the polypeptide to which ubiquitin is attached. The two natural fusion proteins in yeast are highly basic polypeptides with a 'zinc finger' motif that appear to interact with rRNA. These are clearly distinct from the polyubiquitin molecule that would arise from translation of the yeast polyubiquitin mRNA. Isoubiquitin bonds are found in the ubiquitinated histones and in the polyisoubiquitin formed during targeting of a protein for proteolytic degradation. Although the isopeptide bonds for both these type of conjugates are the same, a Gly–Lys (ϵ-amino) linkage, the proteins are vastly different: one is a positively charged histone, whereas the other is a compact globular ubiquitin. In addition, the two are in different cell compartments: the ubiquitinated histone is in the nucleus and the polyisoubiquitinated protein is in the cytoplasm. Thus it is likely that two distinct isopeptide hydrolases exist, one that removes ubiquitin from histone–ubiquitin conjugates and one that recycles ubiquitin from the polyisoubiquitin–protein conjugates.

In the purification system described here, the enzymic activity that removes ubiquitin from histones was lost during the sucrose-gradient fractionation. The hydrolase(s) that remove ubiquitin from the ribosomal fusion proteins and cleave a ubiquitin dimer fractionated differently on the DE 52 and thiol-affinity columns. From these results, we postulate that the hydrolase purified in this study is distinct from those previously described. The ubiquitin released from the multi-ubiquitinated 30 kDa protein to form the 24 kDa product is bound to the substrate by an isopeptide bond [23]; thus, we postulate that the natural substrate for this hydrolase is the polyisoubiquitin structure on proteins undergoing degradation in the 26 S proteasome complex [31]. Possibly the hydrolase purified here is part of the 26 S complex, and functions both as a receptor for the polyubiquitin moiety and as an enzyme for removing ubiquitins so they can be further utilized. This model could be tested by sequencing peptides from the 160 kDa protein in order to isolate a yeast cDNA that can be used for cloning and genetic manipulations of this ubiquitin hydrolase gene.

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