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# Purification and Characterization of a New Ubiquitin C-Terminal Hydrolase (UCH-1) with Isopeptidase Activity from Chick Skeletal Muscle[1]

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**We have previously shown that chick muscle extracts contain at least 10 different ubiquitin C-terminal hydrolases (UCHs). In the present studies, one of the enzymes, called UCH-1 was partially purified by conventional chromatographic procedures using $^{125}$I-labeled ubiquitin-$\alpha$NH-MHISPPEPESEEEEEHYC as a substrate. The purified enzyme behaved as a 35-kDa protein under both denaturing and nondenaturing conditions, suggesting that it consisted of a single polypeptide chain. It was maximally active at pHs between 8 and 9, but showed little or no activity at pH below 6 and above 11. Like other UCHs, its activity was strongly inhibited by sulfhydryl blocking reagents, such as iodoacetamide, and by ubiquitin-aldehyde. In addition to Ub-PESTc, UCH-1 hydrolyzed ubiquitin-$\alpha$NH-protein extensions, including ubiquitin-$\alpha$NH-carboxyl extension protein of 80 amino acids, ubiquitin-$\alpha$NH-dihydrofolate reductase, and poly-His-tagged di-ubiquitin. This enzyme was also capable of generating free ubiquitin from mono-ubiquitin-$\varepsilon$NH-protein conjugates and from branched poly-ubiquitin chains that are ligated to proteins through $\varepsilon$NH-isopeptide bonds. These results suggest that UCH-1 may play an important role in the generation of free ubiquitin from ubiquitin-ribosomal protein fusions and linear poly-ubiquitin, as well as in recycling of Ub molecules after degradation of poly-ubiquitinated protein conjugates by the 26S proteasome.**

**Key words: isopeptidase, ubiquitin C-terminal hydrolase, ubiquitin specific protease.**

Ubiquitin (Ub) is a highly conserved 76-amino acid polypeptide. This small protein is involved in a variety of cellular functions, including regulation of intracellular protein breakdown, cell cycle regulation, and stress response (*1-6*). Ub is covalently ligated to target proteins through an isopeptide linkage between the C-terminal Gly residue of Ub and the $\varepsilon$-amino group of Lys residue(s) of the proteins. Ubs by themselves or that have already been conjugated to proteins may also be ligated to additional Ub molecules to form branched poly-Ub by linkage between the $\varepsilon$-amino group of Lys-48 of one Ub and the C-terminus of the other. Proteins ligated to multiple units of Ub are degraded by the 26S proteasome (*1-3, 7-9*).

Ubs are encoded by two distinct classes of gene, none of which encodes the monomeric form of Ub (*10, 11*). One is a poly-Ub gene which encodes a linear polymer of Ubs that are linked through peptide bonds between the C-terminal Gly and N-terminal Met of contiguous Ub molecules. The other encodes a fusion protein in which a single Ub is linked to a ribosomal protein consisting of 52 or 76-80 amino acids (*12*). Thus, generation of free Ub from the linear poly-Ub and Ub-fusion proteins and recycling of Ub from the branched poly-Ub ligated to proteins should be essential for Ub-requiring processes, such as intracellular ATP-dependent proteolysis.

A number of Ub C-terminal hydrolases (UCHs) that release Ub molecules that are conjugated to proteins by $\alpha$NH-peptide bonds and/or $\varepsilon$NH-isopeptide linkages have been identified from different sources. *Saccharomyces cerevisiae* contains at least 5 different UCHs, including YUH1 and UBP-1 to -4 (*13-16*). A family of UCHs, named L1, L2, L3, and H2, has been identified from bovine calf thymus (*17-19*). Isopeptidase T in human red blood cells has been purified, and its cDNA has been isolated (*20, 21*). In addition, the *fat facets* gene in *Drosophila*, which is required in eye development, and the *DUB-1* gene in murine pro-B cell line, which is specifically induced by IL-3 and involved in growth suppression, have recently been shown to encode a UCH (*22, 23*). We also have identified at least 10 different UCHs in chick skeletal muscle using Ub-$\alpha$NH-MHISPPEPESEEEEEHYC (referred to as Ub-PESTc) (*24-26*). Since the Tyr residue next to the C-terminal Cys can be exclusively radio-iodinated, we could

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Abbreviations: Ub, ubiquitin; UCH, ubiquitin C-terminal hydrolase; YUH1, yeast ubiquitin hydrolase 1; UBP, ubiquitin specific protease; Ub-PESTc, ubiquitin-$\alpha$NH-MHISPPEPESEEEEEHYC; CEP80, carboxyl extension protein 80; DHFR, dihydrofolate reductase; His-di-Ub, poly-His-tagged di-ubiquitin; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine.

assay the UCH activity by simple measurement of the radioactivity of the peptide portion that is released into acid-soluble products.

Although the number of UCHs identified from various organisms is rapidly growing, only a few of them have been purified and characterized *in vitro*. In the present studies, we have purified a new UCH in chick skeletal muscle, named UCH-1, and shown that the enzyme hydrolyzes not only Ub-$\alpha$NH-protein extensions and linear poly-Ub precursors, but also branched poly-Ub that is ligated to proteins though $\varepsilon$NH-isopeptide bonds.

## MATERIALS AND METHODS

*Materials*—Ub-PESTc, Ub-$\alpha$NH-carboxyl extension protein of 80 amino acids (Ub-CEP80), Ub-$\alpha$NH-dihydrofolate reductase (Ub-DHFR), and poly-His-tagged di-Ub (His-di-Ub) were purified as described previously (*25*). The purified Ub-PESTc was radiolabeled with $Na^{125}I$ using Iodo-Beads (Pierce) (*27*). Ub-aldehyde was prepared by borohydride reduction of Ub in the presence of YUH1 as described (*28*). Reticulocytes were obtained by subcutaneous injections of phenylhydrazine into albino rabbits (*29*). From the cells, fraction II containing the Ub-conjugating system was prepared as described (*30*).

*Preparation of Poly-Ub-Protein Conjugates*—To prepare $^{125}$I-labeled poly-Ub-$\varepsilon$NH-protein conjugates, 1 $\mu$g of $^{125}$I-labeled Ub ($1\text{-}2\times10^6$ cpm) was incubated with 720 $\mu$g of fraction II, and an ATP-regenerating system consisting of 10 mM Tris-HCl (pH 7.8), 15 units/ml creatine phosphokinase, 6.5 mM phosphocreatine, 1.5 mM ATP, 1 mM DTT, 0.5 mM $MgCl_2$, and 1 mM KCl in a final volume of 0.3 ml (*30*). The incubation was performed for 2 h at 37°C in the absence and presence of 1 mM hemin to prevent degradation of poly-Ub-protein conjugates by the 26S proteasome. After incubation, half of each sample was heated for 10 min at 55°C for inactivation of endogenous UCHs and/or the 26S proteasome in fraction II. The other half was incubated at 4°C for the same period. The resulting samples were directly used as a substrate or freed of excess $^{125}$I-labeled Ub by treatment of 0.2 N NaOH and 100 mM DTT followed by gel filtration on a Sephadex G-75 column ($1\times40$ cm) equilibrated with Tris-HCl (pH 7.8), 1 mM DTT, and 1 mM EDTA.

*Assay of UCH Activity*—Reaction mixtures (0.1 ml) contained a suitable amount of the purified UCH-1 or chromatographic fractions and 1 $\mu$g of $^{125}$I-labeled Ub-PESTc ($10^4$ cpm/$\mu$g) in 100 mM Tris-HCl (pH 7.8), 1 mM DTT, 1 mM EDTA, and 5% (v/v) glycerol. The mixture was incubated for various periods at 37°C, then the reaction was terminated by addition of bovine serum albumin and trichloroacetic acid to final concentrations of 0.3% (w/v) and 10% (w/v), respectively. The samples were centrifuged, and the resulting supernatants were counted for radioactivity using a gamma counter. The enzyme activity was expressed as a percentage of $^{125}$I-Ub-PESTc hydrolyzed to acid-soluble products.

When assaying for hydrolysis of Ub-$\alpha$NH-carboxyl extension proteins, His-di-Ub or $^{125}$I-labeled poly-Ub-$\varepsilon$NH-protein conjugates, incubations were performed as above, but in the presence of 1 $\mu$g of the substrates. After incubation for appropriate periods, the samples were subjected to discontinuous gel electrophoresis (see below). Proteins in the gels were then visualized by staining with Coomassie blue R-250 or by exposing the gels to X-ray films (Fuji) at −70°C.

*Preparation of Muscle Extracts*—Chick pectoralis muscle tissues (3 kg) were minced and homogenized using a Waring blender in buffer A [25 mM Tris-HCl buffer (pH 7.8) containing 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol] in the presence of 100 mM NaCl. The homogenates were centrifuged at $10{,}000\times g$ for 1 h to remove cell debris, and their supernatants were centrifuged again at $100{,}000\times g$ for 2 h. The resulting supernatants were titrated with 1 M Tris base to pH 7.8 and referred to as the muscle extracts.

*Electrophoresis*—Polyacrylamide gel electrophoresis in the presence and absence of SDS and 2-mercaptoethanol was performed as described by Laemmli (*31*) or using Tris-Tricine buffer as described by Schägger and Von Jagow (*32*). The discontinuous slab gels contained 4, 10, and 16% (w/v) polyacrylamide to improve resolution of small proteins. The sample buffer contained 150 mM Tris-HCl (pH 6.8), 1.5% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue, and 7% glycerol.

## RESULTS

*Purification of UCH-1*—To separate UCH-1 from the other UCHs, extracts (100.9 g protein) were prepared from 3 kg of chick skeletal muscle (*25*). The activity of UCH-1 in chromatographic fractions obtained during purification was monitored by determining its ability to hydrolyze $^{125}$I-labeled Ub-PESTc. The muscle extracts were applied to a DEAE-Sepharose column ($5\times30$ cm) equilibrated with buffer A. Proteins that did not bind to the column were precipitated by adding solid ammonium sulfate to the eluate to give 50% saturation. The precipitated proteins were resuspended and dialyzed in buffer A, and loaded onto a S-Sepharose column ($2\times20$ cm) equilibrated with the same buffer. The bound proteins were eluted with a linear gradient of 0-150 mM NaCl, and the active fractions were pooled and dialyzed against buffer B (pH 7.4) consisting of 25 mM $NaH_2PO_4/Na_2HPO_4$ buffer, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol. The dialyzed sample was applied to a hydroxylapatite column ($1\times12$ cm) equilibrated with the same buffer, and the proteins bound to the column were eluted with a linear gradient of 20-150 mM phosphate. The active fractions were pooled, dialyzed against buffer B, and loaded onto a phospho-cellulose column ($1\times6$ cm). The bound proteins were eluted as above for the hydroxylapatite chromatography. The fractions with high activity were pooled, dialyzed against buffer A, diluted 2-fold with buffer A containing 2 M ammonium sulfate, and loaded onto a phenyl-Superose column ($0.5\times5$ cm). The bound proteins were separated by a reverse gradient of 1.0-0.5 M ammonium sulfate. The active fractions were pooled, dialyzed against buffer A containing 0.1 M NaCl, and concentrated to 2 ml using a Centricon (Amicon). The sample was then subjected to gel filtration chromatography on a Superdex-75 column ($1.6\times60$ cm).

A single peak of the activity against $^{125}$I-labeled Ub-PESTc was eluted in the fractions corresponding to about 35 kDa (Fig. 1A). Upon analysis by polyacrylamide gel electrophoresis under denaturing conditions, the fractions under the activity peak contained three proteins of 44, 35,

and 24 kDa (Fig. 1B). However, the elution pattern of the enzyme activity closely overlapped that of the 35-kDa protein band, but not those of the other polypeptides, suggesting that the 35-kDa protein is responsible for the activity of UCH-1. In order to clarify further whether the UCH activity is due to the 35-kDa protein, the peak fractions (*i.e.*, number 58-62) were pooled and concentrated to 0.5 ml as above. An aliquot of the concentrated sample was subjected to gel electrophoresis under nondenaturing conditions (*i.e.*, in the absence of SDS and 2-mercaptoethanol). After electrophoresis, the gel was cut into 3 mm slices, then each slice was crushed, and incubated overnight at 4°C in buffer A. Aliquots of the samples were assayed for the hydrolysis of $^{125}$I-labeled Ub-PESTc. As shown in Fig. 2A, slice number 4 showed the highest activity. Therefore, the rest of the same gel fraction was again subjected to electrophoresis, but under denaturing conditions, followed by silver-staining. Figure 2B shows that the gel fraction contains only a single protein of 35 kDa. Thus, it is likely that the 35-kDa protein is responsible for the UCH-1 activity.

The concentrated enzyme sample containing the 35-kDa was kept frozen at −70°C until use. Approximately 70% of total protein in the concentrated enzyme preparation was the 35-kDa UCH-1, as determined by electrophoresis of the sample as above followed by scanning of the gel with a densitometer. The purification of UCH-1 is summarized in Table I. Since the size of UCH-1 estimated by gel electrophoresis under denaturing conditions and by gel filtration analysis under nondenaturing conditions is identical, the enzyme appears to consist of a single polypeptide.

*Biochemical Properties*—Since all of the UCHs so far identified are sensitive to inhibition by sulfhydryl blocking agents, such as iodoacetamide (*13-17*), we also examined the effect of this reagent on the activity of the purified UCH-1 against $^{125}$I-labeled Ub-PESTc. As shown in Fig. 3, the enzyme activity was strongly inhibited by iodoacetamide. Thus, it appears that UCH-1 also contains a conserved Cys residue for catalysis, like other known UCHs (*13-17*). The Ub-PESTc-cleaving activity of UCH-1 was

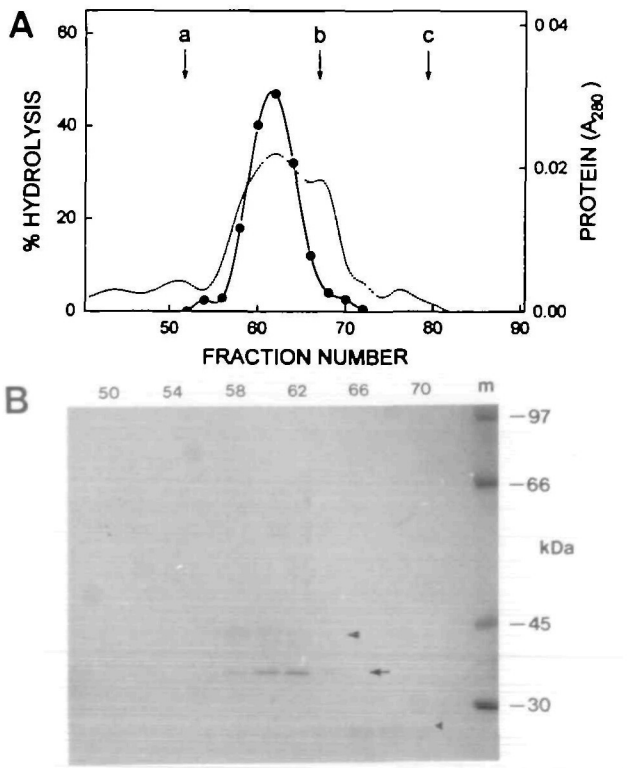


Fig. 1. **Separation of UCH-1 on a Superdex-75 column.** (A) The UCH-1 preparation from the phenyl-Superose chromatography was loaded onto a Superdex-75 column as described in the text. Fractions of 1 ml were collected, and aliquots (10 $\mu$l) of them were assayed for the ability to hydrolyze $^{125}$I-labeled Ub-PESTc (●) as described under "MATERIALS AND METHODS." The size markers used are: a, bovine serum albumin (66 kDa); b, carbonic anhydrase (29 kDa); c, cytochrome *c* (12.4 kDa). The dotted line indicates the protein profile. (B) Aliquots (80 $\mu$l) of the same fractions were electrophoresed on a 12% polyacrylamide gel containing SDS and 2-mercaptoethanol. Proteins in the gel were then visualized by staining with Coomassie Blue R-250.

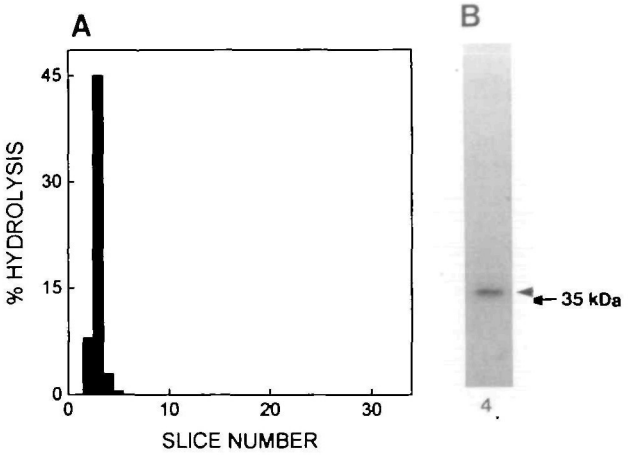


Fig. 2. **Elution of the 35-kDa UCH-1 from gels after electrophoresis under nondenaturing conditions.** An aliquot (5 $\mu$g) of the proteins obtained after the final purification step was electrophoresed at 4°C on an 8% polyacrylamide slab gel as described (*31*), but in the absence of SDS and 2-mercaptoethanol. After electrophoresis, the gel was cut into 3 mm slices, and each slice was crushed and incubated overnight at the same temperature in 0.2 ml of buffer A. (A) Aliquots (20 $\mu$l) of the gel fractions were then incubated with 1 $\mu$g of $^{125}$I- labeled Ub-PESTc ($1\text{-}2\times10^4$ cpm/$\mu$g) for 2 h at 37°C. After incubation, the radioactivity released into acid-soluble products was determined. (B) The remaining sample of the gel slice number 4 was again subjected to gel electrophoresis as above but on a 12% slab gel containing SDS and 2-mercaptoethanol. After electrophoresis, proteins were visualized by silver-staining.

TABLE I. **Summary of purification of UCH-1.**

| Steps | Protein (mg) | Total activity (units[a]) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|
| Crude extract | 100,880 | —[b] | — | — |
| 1st DEAE-Sepharose | 76,704 | — | — | — |
| $(NH_4)_2SO_4$ precipitation | 15,801 | — | — | — |
| 2nd DEAE-Sepharose | 10,271 | 14,094 | 1.4 | 100 |
| S-Sepharose | 137 | 9,396 | 69 | 67 |
| Hydroxylapatite | 13 | 4,324 | 333 | 31 |
| Phospho-cellulose | 2.3 | 1,078 | 468 | 8 |
| Phenyl-Superose | 0.14 | 108 | 771 | 0.8 |
| Superdex-75 | 0.03 | 41 | 1,358 | 0.3 |

[a]One unit was defined as 1 $\mu$g of Ub-PESTc hydrolyzed to acid-soluble products per h. [b]Not determined.

also inhibited by Ub-aldehyde, which is known to inhibit the activity of certain mammalian UCHs, such as isopeptidase T (*21*). However, inhibitors of serine proteases, such as phenylmethylsulfonyl fluoride (1 mM), or metal chelating agents, including EDTA (10 mM) and *o*-phenanthroline (1 mM), showed little or no effect (data not shown). We also examined the effects of varying pHs on the hydrolysis of $^{125}$I-labeled Ub-PESTc by UCH-1 using various buffers. UCH-1 was maximally active at pH near 8.5, but was inactive at pH below 6 and above 11 (Fig. 4).

*Substrate Specificity*—To determine the substrate specificity of UCH-1, the purified enzyme was incubated with various Ub-$\alpha$NH-protein extensions, such as Ub-DHFR, Ub-CEP80, and His-di-Ub. After incubation, the samples were subjected to discontinuous gel electrophoresis under denaturing conditions, followed by staining with Coomassie Blue R-250. Assays were also performed with Ub-PESTc as a control. As shown in Fig. 5, UCH-1 was capable of generating free Ub from all of the substrates tested. We have previously shown that the crude preparation of UCH-1 is capable of removing Ub from mono-Ub-$\varepsilon$NH-conjugate of Ub(RGA)-P-ARRKWQKTGHAVRAIGRLSS,

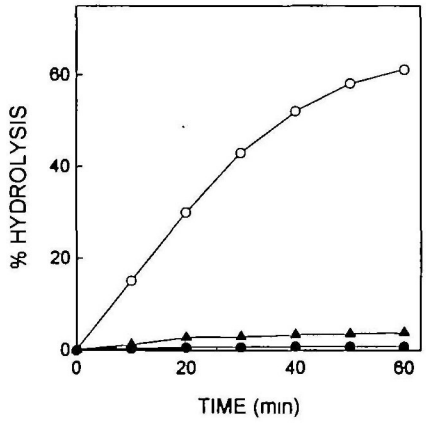


Fig. 3. **Effects of iodoacetamide and Ub-aldehyde on the hydrolysis of Ub-PESTc by UCH-1.** Reaction mixtures containing 0.1 $\mu$g of UCH-1 and 1 $\mu$g of $^{125}$I-labeled Ub-PESTc were incubated in the absence (○) and presence of 1 mM iodoacetamide (●) or 0.1 $\mu$M Ub-aldehyde (▲). After incubation for the indicated periods, the enzyme activity was determined as in Fig. 2.

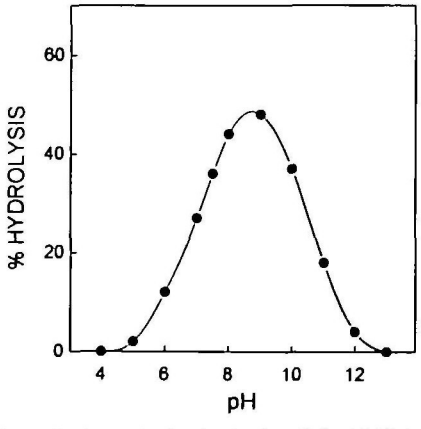


Fig. 4. **Effect of pH on the hydrolysis of Ub-PESTc by UCH-1.** The effect of pH on the Ub-PESTc-cleaving activity of UCH-1 was determined using various buffers. The buffers used were: Na-acetate (for pH 4-5), $NaH_2PO_4/Na_2HPO_4$ (for pH 6-7), Tris-HCl (pH 7.5-8.5), and Na-glycine (pH 9-11). The reaction mixtures were incubated at 37°C for 30 min.

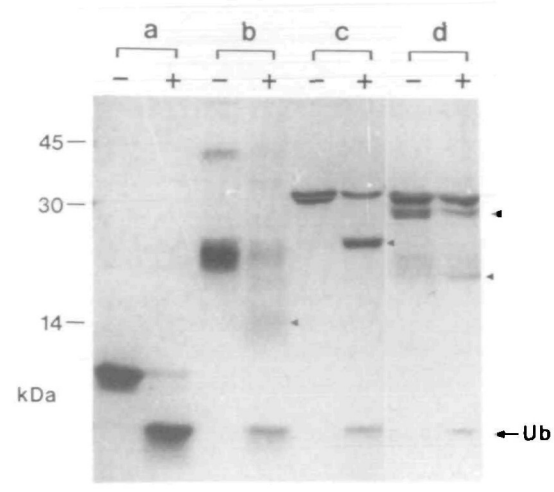


Fig. 5. **Hydrolysis of various Ub-$\alpha$NH-protein extensions by UCH-1.** Aliquots (5 $\mu$g each) of the purified Ub-PESTc (a), Ub-CEP80 (b), Ub-DHFR (c), and His-di-Ub (d) were incubated for 2 h at 37°C in the absence ("−" lanes) and presence of 0.4 $\mu$g of the purified UCH-1 ("+" lanes). After incubation, the samples were subjected to discontinuous gel electrophoresis as described under "MATERIALS AND METHODS." Proteins in the gels were then visualized by staining with Coomassie Blue R-250. The small arrowheads in b, c, and d indicate the positions to which CEP80, DHFR, and His-mono-Ub migrated, respectively. The large arrowhead in d indicates His-di-Ub. The protein band seen just above His-di-Ub (in d) is an unknown protein contaminant left during preparation of the substrate.

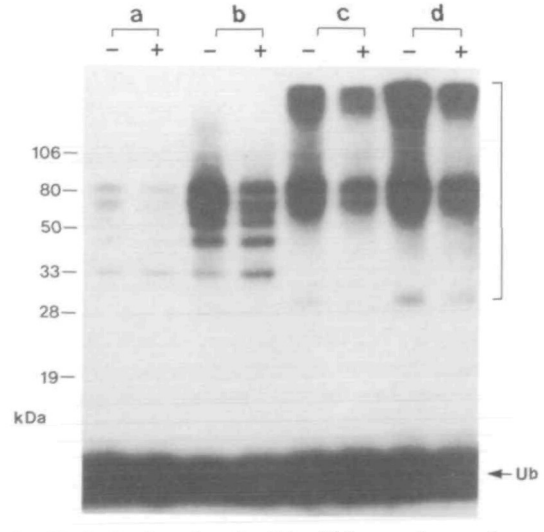


Fig. 6. **Hydrolysis of poly-Ub-$\varepsilon$NH-protein conjugates by UCH-1.** Poly-ubiquitinated protein conjugates were prepared by incubating $^{125}$I-labeled Ub and fraction II in the absence (a and b) and presence of 0.1 mM hemin (c and d) followed by incubation for 10 min at 4°C (a and c) or 55°C (b and d). After incubation, the samples were further incubated without ("−" lanes) and with 0.4 $\mu$g of the purified UCH-1 ("+" lanes) for 4 h at 37°C. They were then subjected to electrophoresis on a discontinuous gel containing SDS and 2-mercaptoethanol, followed by autoradiography. The bracket on the right side of the gel indicates poly-Ub-protein conjugates.

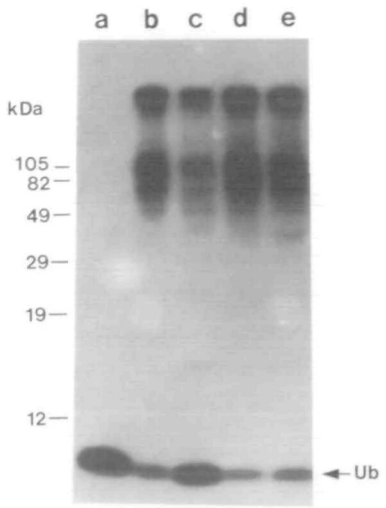


Fig. 7. **Hydrolysis of the branched poly-Ub chains by UCH-1.** $^{125}$I-Labeled poly-Ub protein conjugates that had been prepared in the presence of 0.1 mM hemin and with heat treatment at 55°C were chromatographed on a Sephadex G-75 column as described under "MATERIALS AND METHODS." The proteins eluted in the void volume were pooled, and aliquots of them were incubated alone (lane b) or with 0.4 $\mu$g of UCH-1 in the absence (lane c) and presence of 1 mM iodoacetamide (lane d) or 0.1 $\mu$M Ub-aldehyde (lane e) for 4 h at 37°C. After incubation, the samples were subjected to discontinuous gel electrophoresis under denaturing conditions, followed by autoradiography. Ub alone is also shown in lane a.

in which the C-terminal Gly residue of Ub is replaced by Ala, and a Pro residue is inserted at the N-terminus of the 20-amino acid peptide to prevent the enzyme preparation from cleaving the $\alpha$NH-peptide bond between Ub and the extension peptide (*25*). Similar results were obtained with the purified UCH-1 (data not shown).

To examine whether the purified UCH-1 can also act on branched poly-Ub chains that are ligated to proteins by isopeptide linkages, $^{125}$I-labeled poly-Ub-$\varepsilon$NH-protein conjugates were prepared by incubation of $^{125}$I-labeled Ub and fraction II of rabbit reticulocytes in the absence and presence of hemin followed by incubation for 10 min at 4 or 55°C. After incubation of the substrate with UCH-1, the samples were subjected to discontinuous gel electrophoresis followed by autoradiography. The size and amount of the radiolabeled poly-Ub protein conjugates prepared in the presence of hemin (Fig. 6, lanes c and d) were much higher than those of conjugates prepared in its absence (lanes a and b), indicating that the protein conjugates are rapidly degraded by the 26S proteasome. In addition, a significantly larger amount of the protein conjugates could be obtained when the incubation mixtures were heated at 55°C for 10 min prior to the treatment of UCH-1 ("−" lanes in b and d) as compared with those incubated at 4°C ("−" lanes in a and c). These results strongly suggest that endogenous isopeptidases in fraction II, such as isopeptidase T, act on poly-Ub-protein conjugates. Under all these conditions, further incubation with the purified UCH-1 at 37°C evidently decreased the amount of poly-Ub-protein conjugates ("+" lanes compared with "−" lanes). Thus, it seems clear that UCH-1 has an isopeptidase activity against poly-Ub-$\varepsilon$NH-protein conjugates.

In order to clarify further whether UCH-1 is capable of generating free Ub from branched poly-Ub chains that are ligated to proteins by $\varepsilon$NH-isopeptide bonds, $^{125}$I-labeled poly-Ub-$\varepsilon$NH-protein conjugates that had been prepared in the presence of hemin and with heat treatment were subjected to gel filtration on a Sephadex G-75 column to remove excess $^{125}$I-labeled Ub molecules in the substrate preparation. The proteins eluted in the void volume were then incubated with UCH-1, electrophoresed on a discontinuous gel, and autoradiographed as above. Although the gel filtration chromatography did not completely eliminate unincorporated $^{125}$I-Ub in the poly-Ub-protein conjugate preparation (Fig. 7, lane b), the amount of free Ub was significantly increased upon incubation with UCH-1 (lane c). Furthermore, treatment with iodoacetamide or Ub-aldehyde prevented the generation of free Ub (lanes d and e, respectively). In addition, we found that free Ub can also be generated from radiolabeled multi-Ub-lysozyme conjugates that were prepared as above, but using $^{125}$I-Ub and unlabeled lysozyme or $^{125}$I-lysozyme and unlabeled Ub (data not shown). These results indicate that UCH-1 has an isopeptidase activity against branched poly-Ub chains. Thus, it appears that UCH-1 can release Ubs that are conjugated to proteins or adjacent Ub molecules by $\varepsilon$NH-isopeptide linkages, as well as by $\alpha$NH-peptide bonds.

## DISCUSSION

In the present studies, UCH-1 was partially purified from the extracts of chick skeletal muscle using $^{125}$I-labeled Ub-PESTc as a substrate. It is interesting that UCH-1 has a slightly alkaline pH optimum for the hydrolysis of Ub-PESTc (*i.e.*, at pHs between 8 and 9), unlike the other muscle UCHs, including UCH-6, which are maximally active at or near neutral pH (*25*). In addition, UCH-1 is the only enzyme whose activity against Ub-PESTc is not stimulated by poly-L-lysine. We have previously shown that the Ub-PESTc-cleaving activities of the nine other UCHs identified from chick skeletal muscle are stimulated by the cationic reagent by 5- to 20-fold (*25*). Thus, UCH-1 appears to be distinct from other UCHs so far been reported (*13-17, 25*) in a number of respects, including chromatographic behavior, size, pH optimum, and sensitivity to poly-L-lysine.

In addition to Ub-PESTc, the purified UCH-1 is capable of generating free Ub from various Ub-$\alpha$NH-protein extensions, such as Ub-CEP80, Ub-DHFR, and His-di-Ub. Thus, UCH-1 may play an important role in processing of Ub-ribosomal protein fusions and linear poly-Ub precursors, both of which are Ub gene products, and hence in the generation of free Ub and certain ribosomal proteins in skeletal muscle cells. UCH-1 is capable of releasing free Ub from the mono-Ub-$\varepsilon$NH-conjugate of Ub(RGA)-PARRK-WQKTGHAVRAIGRLSS (*25*) and from branched poly-Ub-chains that are ligated to proteins through $\varepsilon$NH-isopeptide bonds. Hershko and coworkers (*33*) have shown that the mammalian isopeptidase T removes Ubs from high-molecular-weight poly-Ub-protein conjugates, but not from low-molecular-weight forms. They have also demonstrated that the 26S proteasome has inherent deubiquitinating activity against adducts in which a single Ub is linked to an $\varepsilon$NH-Lys group of a protein, as well as against conjugates containing multiple Ubs (*33*). Therefore, UCH-1 together with the 26S proteasome and isopeptidase T may be

involved in complete recycling of Ub molecules after degradation of poly-Ub-protein conjugates by the 26S proteasome in skeletal muscle cells.

However, the purified UCH-1 preparation used in the present studies contained at least two contaminating proteins of 44 and 24 kDa. Since the amount of the 35-kDa UCH-1 protein present in skeletal muscle is very small, no further attempt was made to achieve complete purification of the enzyme. Therefore, we could not completely rule out the possibility that the activity against Ub-$\alpha$NH protein extensions and/or branched poly-Ubs may be due to some other, unknown contaminating enzyme in the UCH-1 preparation. However, this seems very unlikely, because the elution pattern of the 35-kDa protein band in the final purification step (*i.e.*, from the Superdex-75 column chromatography) closely coincided not only with the activity against $^{125}$I-labeled Ub-PESTc, but also with that against Ub-CEP80 (see Fig. 1 and data not shown), and because the UCH activity eluted from the sliced gel after electrophoresis under nondenaturing conditions coincided with the 35-kDa protein band (see Fig. 2). Complete purification of UCH-1 and isolation of its cDNA will be required for detailed characterization and functional analysis of the enzyme.

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