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High Performance Liquid Chromatography Resolution of Ubiquitin Pathway Enzymes from Wheat Germ[1]

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ABSTRACT

The highly conserved protein ubiquitin is involved in several cellular processes in eukaryotes as a result of its covalent ligation to a variety of target proteins. Here, we describe the purification of several enzymatic activities involved in ubiquitin-protein conjugate formation and disassembly from wheat germ (*Triticum vulgare*) by a combination of ubiquitin affinity chromatography and anion-exchange high performance liquid chromatography. Using this procedure, ubiquitin activating enzyme (E1), several distinct ubiquitin carrier proteins (E2s) with molecular masses of 16, 20, 23, 23.5, and 25 kilodaltons, and a ubiquitin-protein hydrolase (isopeptidase) were isolated. Purified E1 formed a thiol ester linkage with ^{125}I-ubiquitin in an ATP-dependent manner and transferred bound ubiquitin to the various purified E2s. The ubiquitin protein hydrolase fraction was sensitive to hemin, and in an ATP-independent reaction, was capable of removing the ubiquitin moiety from both ubiquitin ^{125}I-lysozyme conjugates (ϵ-amino or isopeptide linkage) and the ubiquitin 52-amino acid extension protein fusion (α-amino or peptide linkage). Using this procedure, wheat germ represents an inexpensive source from which enzymes involved in the ubiquitin pathway may be isolated.

The covalent attachment of the highly conserved, 76-amino acid protein ubiquitin to various target proteins is involved in a number of cellular processes in eukaryotes (13, 24). Best characterized of these is its role in protein breakdown, where attachment of ubiquitin serves to commit proteins to degradation. Less well understood functions of ubiquitin conjugation include DNA repair (17) and cell cycle progression (6), both possibly mediated by altering chromatin structure through the conjugation of ubiquitin to histones. The ubiquitination of histones has also been implicated in the modulation of gene expression (7). Several cell surface receptors have been shown to be conjugated with ubiquitin (26), although the functional significance of this modification remains to be elucidated.

Ligation of ubiquitin to proteins is an ATP-dependent process catalyzed by a pathway consisting of at least three families of enzymes. The pathway was initially characterized from rabbit reticulocyte lysates (14) and similar pathways have subsequently been described in yeast (*Saccharomyces cerevisiea*) (17) and wheat (*Triticum vulgare*) (12). In the initial step of ligation, ubiquitin activating enzyme (E1[3]) adenylates the carboxy terminus of ubiquitin using ATP. The activated ubiquitin is then attached via a thiol ester linkage to a cysteine on E1 with the concomitant release of AMP. The ubiquitin moiety is next transferred to a cysteine on a ubiquitin carrier (E2) protein by transesterification. The E2s comprise a family of low mol wt proteins (with one exception [18]) that differ in their recognition of specific target proteins and in their requirement for a third enzyme, ubiquitin protein ligase (E3), for ubiquitin conjugate formation (6, 9, 11, 17, 22, 23, 27). Finally, ubiquitin is covalently attached to target proteins with or without the participation of E3. The bond thus formed is an isopeptide linkage between the carboxy terminus of ubiquitin and lysyl ϵ-amino groups within the target protein.

Once a protein is conjugated with one or more ubiquitin moieties, it has three possible fates. It may be degraded by an ATP-dependent protease specific for ubiquitin conjugates, releasing amino acids and intact ubiquitin. The protease responsible has been identified recently as being related to the multicatalytic protease or proteosome (16, 19). In this way, ubiquitin serves as a recognition signal for proteolysis.

Alternatively, the ubiquitin moiety may be removed from the target protein by ubiquitin-protein hydrolases (also known as ubiquitin isopeptidases) to yield free intact target protein and intact ubiquitin (24). Removal of the ubiquitin moiety from a conjugated protein may serve to correct errors in the conjugation system and/or regulate the activities of ubiquitinated proteins (histones or cell surface receptors, for example). Additionally, ubiquitin protein hydrolases are likely required to release free ubiquitin from proteolytic breakdown products (lysyl ϵ-linked) and to process ubiquitin gene products that are synthesized as protein fusions (α-amino linked) into ubiquitin monomers (2, 5). Ubiquitin protein hydrolase activities have been detected in a number of tissue extracts derived from mammalian (20), fungal, and plant sources (12, 28). Recently, a family of hydrolases has been isolated from calf thymus, with various members differing in their specificity toward α- and ϵ-ubiquitin linkages (20). DNA clones for a 30-kD form have been obtained from yeast (21), bovine, and human sources (30) and have been found to share substantial amino acid sequence similarity. Because of their specificity

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[3] Abbreviations: E1, ubiquitin activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin protein ligase; kD, kilodalton.

for ubiquitin linkages, ubiquitin-protein hydrolases are proving to be useful biotechnological tools for the production of large amounts of proteins and peptides with defined amino termini (21).

Stable ubiquitin-protein conjugates may also exist in the cell, being a substrate for neither proteases nor hydrolases. Arthrin, a ubiquitin-actin conjugate found in insect flight muscle, is an example of such a conjugate, where neither the ubiquitin moiety nor the target protein appear to turn over (1). The functions of such stable conjugates are unknown.

To understand better the ubiquitin pathway in plants, we have begun to purify and characterize enzymes involved in ubiquitin conjugate formation and dissassembly from wheat germ. Previously, a method for the purification of ubiquitin activating enzyme, E1, was described (12). Here we report the purification and resolution of E1, a family of E2s, and a ubiquitin protein hydrolase activity from wheat germ by a combination of ubiquitin affinity chromatography and anion exchange HPLC. Using this method, several E2s were purified to near homogeneity, which will allow both the biochemical and molecular characterization of individuals in this enzyme family.

MATERIALS AND METHODS

Biological Materials and Reagents

Untoasted wheat germ (*Triticum vulgare*) was a gift of General Mills (Minneapolis, MN) and was stored at 4°C. Bovine ubiquitin and inorganic pyrophosphatase were purchased from Sigma.

Ubiquitin Affinity Chromatography

Several enzymes involved in the ubiquitin pathway were copurified by the covalent affinity procedure of Ciechanover *et al.* (4) as modified by Hatfield and Vierstra (12). The affinity procedure was carried out as described with the following exceptions: (a) for the initial $(NH_4)_2SO_4$ precipitation, proteins precipitating between 0.15 and 0.30 g/mL of $(NH_4)_2SO_4$ were collected; (b) the affinity column used contained 50 mg bovine ubiquitin coupled to 25 mL of Affi-Gel 10 (Bio-Rad Laboratories); (c) after elution from the ubiquitin affinity column, the eluate buffer was exchanged for HPLC equilibration buffer (see below) and concentrated approximately 200-fold by ultrafiltration with an Amicon YM10 membrane.

Anion Exchange HPLC Resolution of Ubiquitin Pathway Enzymes

E1, a family of E2s, and ubiquitin-protein hydrolase activity present in the ubiquitin affinity column eluate were resolved from each other by anion-exchange HPLC at room temperature using a 75 × 7.5 mm Bio-Gel TSK DEAE-5 PW column (Bio-Rad Laboratories). The column was preequilibrated with buffer containing 35 mM NaCl, 20 mM KH_2PO_4 (pH adjusted to 6.9 with NaOH), and 0.5 mM DTE. Affinity column samples (up to 2 mg) were loaded at a flow rate of 1 mL/min, and the column was washed with equilibration buffer until the absorbance at 210 nm returned to baseline. Proteins were eluted from the column with a linear 35 to 360 mM NaCl gradient with a slope of 5.15 mM/min. One mL fractions were collected across the gradient or individual peaks were collected manually. The buffer of fractions collected manually was exchanged for 20 mm KH_2PO_4, 0.5 mM DTE (pH adjusted to 8.0 with NaOH), and the fractions were concentrated 20- to 50-fold using Centricon-10 microconcentrators (Amicon). Protein concentrations were determined using Bio-Rad protein assay reagent with bovine γ-globulin as the standard.

Size Exclusion HPLC of E1 Containing Fractions

An E1 containing fraction from the DEAE column was further purified using a Beckman 0.75 × 30 cm Spherogel TSK 3000SW column equilibrated with 300 mM NaCl, 20 mM KH_2PO_4 (pH adjusted to 8.0 with NaOH), and 0.5 mM DTE. The column flow rate was 0.5 mL/min and A_{214} nm was monitored.

Assay for Thiol Ester Adduct Formation

Formation of E1 and E2 thiol ester adducts with ubiquitin was determined as described by Haas *et al.* (10). Reaction mixtures contained various HPLC column fractions, 0.45 μg purified E1 (where indicated), 0.56 μg ^{125}I-ubiquitin (approximately 4×10^5 cpm), and 1 unit inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) in 20 μl of 50 mM Tris-HCl (pH 7.6 at 25°C), 10 mM $MgCl_2$, 1 mM ATP, and 0.1 mM DTE. Following a 2.5 min incubation at 30°C, reactions were terminated either by boiling the samples for 10 min in 25 mM Tris-HCl, 5% (v/v) glycerol, 4% (w/v) lithium dodecyl sulfate, and 4% (v/v) 2-mercaptoethanol (pH 6.8) or by incubating the samples for 15 min at 30°C in the above buffer containing 4 M urea instead of 2-mercaptoethanol. Samples were subjected to SDS-PAGE at 4°C. Gels were stained with Coomassie blue, dried between cellophane, and used for autoradiography.

Assay for Ubiquitin-Protein Hydrolase Activity

Ubiquitin-protein hydrolase activity was assayed using two different substrates. Ubiquitin-^{125}I-lysozyme conjugates were synthesized in wheat germ extracts using a previously described modification (28) of the method of Hough and Rechsteiner (15). Hydrolase reaction mixtures contained 10 μL of various HPLC column fractions, 1 μL of ubiquitin-^{125}I-lysozyme conjugates (approximately 2000 cpm), 11.5 μL H_2O, and 2.5 μL of 500 mM Tris-HCl, 5 mM $MgCl_2$ (pH 8.2) (final pH of the reaction was 8.0). The reactions were performed at 30°C for 15 min and terminated by boiling for 5 min with an equal volume of SDS-PAGE sample buffer (29). The samples were subjected to SDS-PAGE and the gels were stained with Coomassie blue and dried between cellophane. Free and conjugated lysozyme were localized by autoradiography, excised, and quantified by liquid scintillation counting.

Alternatively, a ubiquitin extension protein fusion was used as a substrate for the hydrolase activity assay. The fusion protein, encoded by the *Arabidopsis thaliana UBQ1* gene, consists of a single ubiquitin moiety fused to a 52-amino acid protein (2). A plasmid expressing the extension protein fusion

under the control of the T7 promoter was made by ligating a *UBQ1* cDNA restriction fragment into pET3c (25). The extension protein fusion expressed in *Escherichia coli* differs from the *A. thaliana UBQ1* initial translation product by the addition of 11 amino acids to the N terminus of the ubiquitin moiety and replacement of the ubiquitin N-terminal Met with Arg. Following induction (25), cells were harvested by centrifugation, and resuspended to 1/50 the original culture volume in lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 14 mM 2-mercaptoethanol). The bacteria were disrupted by sonication on ice and the resulting extract was clarified by centrifugation.

For the hydrolase assay with the ubiquitin extension protein fusion, reaction mixtures consisted of 15 μL of HPLC fractions, 3 μL of *E. coli* extract (diluted 1:30 with lysis buffer), and 2 μL of 500 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$. After a 30 min incubation at 30°C, the reactions were terminated by boiling for 5 min with an equal volume of SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE and immunoblot analysis according to the procedure of Vierstra *et al.* (29) using either anti-52-amino acid extension protein antisera (2) or anti-oat ubiquitin antisera and alkaline phosphatase conjugated goat anti-rabbit immunoglobulins (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in conjunction with the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Activity was quantified by scanning of blots using a reflective densitometer (Hoefer, San Francisco, CA).

RESULTS

Ubiquitin-Affinity Purification of Ubiquitin Pathway Enzymes

Using the covalent affinity procedure of Ciechanover *et al.* (4) as modified by Hatfield and Vierstra (12), several wheat germ proteins involved in ubiquitin conjugation were copurified (Fig. 1, bottom panel, lane U). These included E1 (117, 123, and 126 kD), and several E2s with apparent molecular masses of 16, 20, 23, and 25 kD. Also present in the affinity column eluate was a ubiquitin protein hydrolase activity, although we were unable to assign this activity to a distinct protein species. Relative yields of E1 and E2s from the ubiquitin column were largely dependent on the ratio of column bound ubiquitin to the amount of partially purified protein applied (data not shown). As more protein was applied to the ubiquitin affinity column and/or less ubiquitin was linked to the column support, E2s bound preferentially, appearing to displace E1 from the column. Under these conditions, an eluate enriched in E2s was obtained. E1 relatively free of E2s could be subsequently recovered from the affinity column flow through material by a second application to the affinity column (P Hatfield, personal communication). Fold purification and percent yield for E1 and E2s could not be determined because quantitative activity assays for the individual enzymes were not possible using crude extracts.

The E1 and E2s eluted from the affinity column were resolved from each other by anion exchange HPLC using a DEAE column and a linear gradient of NaCl (Fig. 1, Table I). Several E2s were purified to near homogeneity, including

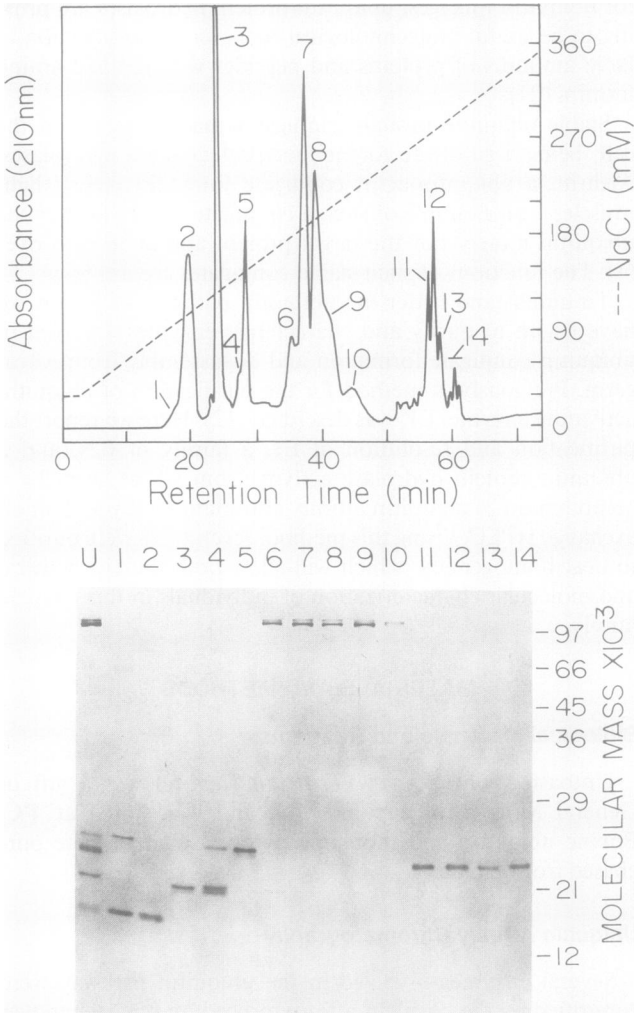

Figure 1. HPLC resolution of ubiquitin conjugating enzymes purified by ubiquitin covalent affinity chromatography. Ubiquitin affinity column eluate (1.3 mg) was fractionated by DEAE-HPLC with a NaCl gradient as described in "Materials and Methods." (*Top panel*) Elution was monitored by absorbance at 210 nm and numbered fractions were collected manually as indicated. (*Bottom panel*) Analysis of the HPLC fractions described in the *top panel* by SDS-PAGE on a 13.5% acrylamide gel and silver staining. Lane U contains approximately 2 μg of unfractionated ubiquitin affinity eluate.

species with molecular masses of 16 kD (fraction 2), 20 kD (fraction 3), 25 kD (fraction 5), 23 kD (fractions 11, 12, 13), and 23.5 kD (fraction 14). Additionally, several E2s were partially resolved, including a 25-kD E2 (fraction 1), and two additional 20-kD E2s, one eluting in fraction 4 and resolving as a closely spaced doublet by SDS-PAGE ($E2_{20kD}$b), and the other coeluting with E1 in fractions 6 to 10 ($E2_{20kD}$c). It was possible to separate the latter $E2_{20kD}$ from E1 by size exclusion HPLC (data not shown). The designation and elution position of each E2 is described in Table I.

Enzymatic Activities of the Purified E1 and E2s

The purified proteins were confirmed to be E1 and E2s by their ability to form a thiol ester adducts with ^{125}I-ubiquitin

Table I. *DEAE Resolution of Ubiquitin Conjugating Enzymes*

HPLC Fraction[a]	[NaCl]	Species Present	Yield
	mM		*μg*[b]
1	105	$E2_{16kD}$, $E2_{25kD}$a	55
2	120	$E2_{16kD}$	330
3	140	$E2_{20kD}$a	450
4	155	$E2_{20kD}$b, $E2_{25kD}$b	60
5	165	$E2_{25kD}$b	280
6–10	190–230	E1, $E2_{20kD}$c	775
11–13	280–290	$E2_{23kD}$a-c	345
14	305	$E2_{23.5kD}$	35

[a] Fraction numbers are as in Figure 1. [b] Yield per 100 g of wheat germ.

in an ATP-dependent reaction (Fig. 2). All these adducts were sensitive to 2-mercaptoethanol and hydroxylamine (data not shown), indicating the linkage was a thiol ester (3). When incubated with ^{125}I-ubiquitin and ATP, E1 (fractions 6 through 10) formed ubiquitin adducts with a molecular mass of approximately 130 kD. The formation of a lower molecular mass adduct (28 kD) by these fractions resulted from the presence of contaminating $E2_{20kD}$c which could be separated from E1 by size exclusion HPLC (Fig. 3).

The remaining fractions from the DEAE-HPLC purification required the addition of purified E1 and ATP to form ubiquitin adducts (Fig. 2, fractions 1–5, 11–14, and data not shown). This E1 requirement was used to define these species as E2s. We detected multiple thiol ester species for the 16-, 23-, 25b-, and 20c-kD E2s and noted that some of them migrated anomalously (especially $E2_{25kD}$b-Ub) (Figs. 2 and 3). These observations are similar to those described for several E2s purified from rabbit reticulocytes (9). This unexpected migration pattern may be the result of multiple ubiquitins attached to individual E2s or the anomalous migrations of E2-ubiquitin thiol ester adducts in the nonreducing gel system used.

Ubiquitin-Protein Hydrolase Activity in the Affinity Eluate

In addition to E1 and E2, the ubiquitin affinity column eluate also contained ubiquitin protein hydrolase activity. This enzyme catalyzed the disassembly of both types of ubiquitin protein linkages—the isopeptide (ϵ-amino) linkage of ubiquitin-lysozyme conjugates and the peptide (α-amino) linkage of the *A. thaliana UBQ1* gene product (Fig. 4). The ubiquitin-^{125}I-lysozyme conjugates were disassembled to yield free ^{125}I-lysozyme and unlabeled ubiquitin, whereas the ubiquitin 52-amino acid extension protein fusion was cleaved to yield the intact 52-amino acid extension protein and a free ubiquitin moiety with an N-terminal extension of 11 amino acids (see "Materials and Methods"). The hydrolase activity did not require ATP and was substantially inhibited by 100 μM hemin. Hemin has been shown to inhibit ubiquitin protein hydrolase activity in mammalian, yeast, and plant extracts without substantially affecting conjugation (8, 28).

To characterize further the hydrolase activity present, ubiquitin affinity eluate was subjected to DEAE-HPLC. Fractions collected across the salt gradient were assayed for ubiquitin-protein hydrolase activity using both ubiquitin-^{125}I-lysozyme and ubiquitin extension protein fusion as substrates. (The concentration of NaCl present in the column elution buffer had little or no effect on the hydrolase activity assays [data not shown].) Two closely spaced peaks of hydrolase activity were detected, the first eluting just prior to $E2_{23kD}$ and the second coeluting with $E2_{23kD}$ (Fig. 5). The two peaks of activity were capable of disassembling both types of ubiquitin-protein linkages, although the latter peak appeared to be more effective against ubiquitin-lysozyme conjugates. Using SDS-PAGE and silver staining, we were unable to definitively

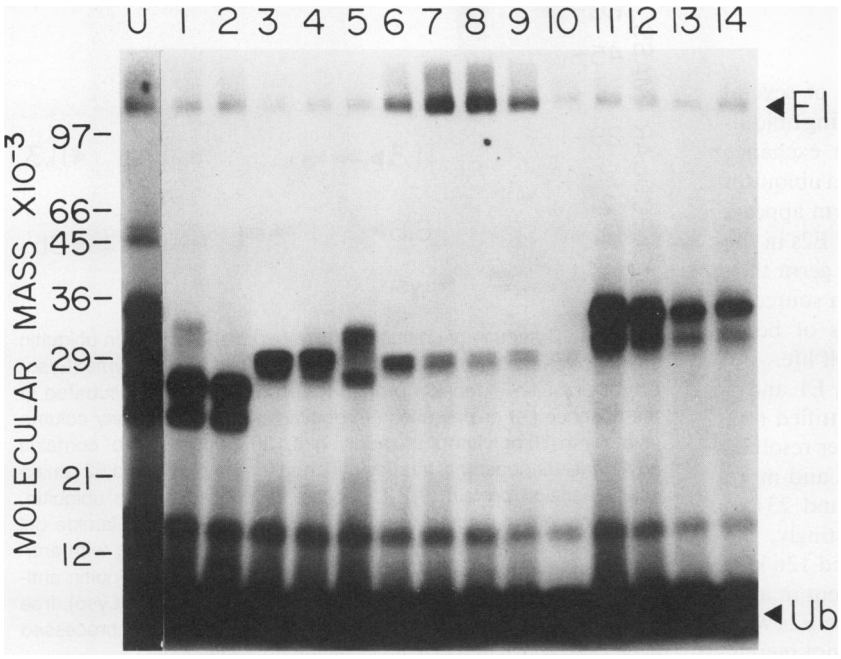

Figure 2. Thiol ester adduct formation between ubiquitin and E1 or E2s resolved by DEAE-HPLC. Thiol ester reactions contained ^{125}I-ubiquitin, ATP, and either approximately 1.0 μg of the unfractionated ubiquitin affinity column eluate (U) or 0.5 to 2.0 μg of the DEAE-HPLC fractions as indicated (lanes 1–14). Purified E1 (0.5 μg) was added to the reactions in lanes 1 to 5 and 11 to 14. Reaction mixtures were subjected to SDS-PAGE on a 13.5% acrylamide gel in the absence of 2-mercaptoethanol followed by autoradiography. The *arrowheads* indicate the migration positions of the E1 thiol ester adduct and free ubiquitin.

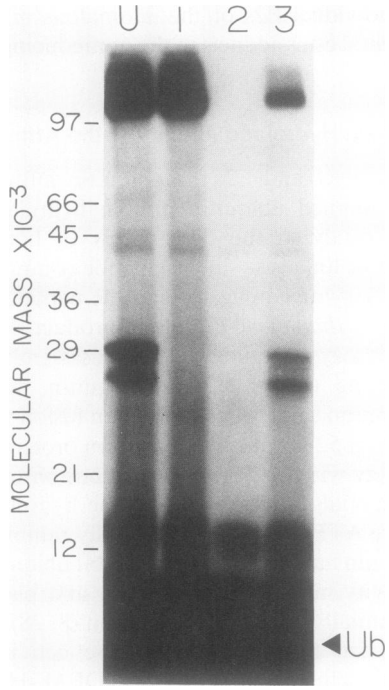

Figure 3. Thiol ester adduct formation between ubiquitin and purified E1 or $E2_{20kD}c$. E1 and $E2_{20kD}c$ present in DEAE-HPLC fraction number 7 (see Fig. 1) were purified by size exclusion HPLC as described in "Materials and Methods." Thiol ester reactions contained ^{125}I-ubiquitin, ATP, and either 3.0 μg of DEAE-HPLC fraction number 7 (lane U), 3.0 μg of purified E1 (lane 1), 0.5 μg of purified $E2_{20kD}c$ (lane 2), or 0.5 μg of purified E1 plus 0.5 μg purified $E2_{20kD}c$ (lane 4). Reaction mixtures were subjected to SDS-PAGE on a 13.5% acrylamide gel in the absence of 2-mercaptoethanol, followed by autoradiography. The *arrowhead* indicates the migration position of free ubiquitin.

assign the hydrolase activities in these HPLC fractions to a specific protein.

DISCUSSION

Here we report a method for the purification of several ubiquitin pathway enzymes from wheat germ utilizing ubiquitin covalent affinity chromatography and anion exchange HPLC. Using this method, E1, a family of E2s, and a ubiquitin protein hydrolase activity were isolated. Wheat germ appears to be a rich source of E2s, with yields of individual E2s in the range of 35 to 450 μg per 100 g of tissue. Wheat germ thus offers an attractive alternative to reticulocytes as a source of conjugating enzymes with the added advantages of being inexpensive, easy to handle, and having a long shelf life.

By ubiquitin covalent affinity chromatography, E1 and 5 different molecular mass classes of E2s were identified (16, 20, 23, 23.5, and 25 kD). These species were further resolved by DEAE-HPLC into 5 differentially retained E1s and more than 14 differentially retained E2s with the 20- and 23-kD species each eluting in 3 separate peaks. Interestingly, the different molecular mass classes of E1 (117, 123, and 126 kD) (12) were not resolved from each other, being present in all 5 E1-containing fractions. It is clear that several of the E2s with different molecular masses are distinct species and not merely proteolytic degradation products of larger E2s. This conclusion is demonstrated by (a) the failure of antibodies raised against HPLC-purified $E2_{16kD}$ and $E2_{23kD}$ to significantly cross react with other purified E2s, and (b) differences in the deduced amino acid sequence of wheat cDNAs corresponding to the $E2_{16kD}$ and $E2_{23kD}$ species (ref. 27 and data not shown). It is uncertain how related the differentially eluting E2s of similar molecular masses are to each other. The existence of multiple E2s of similar size is supported by the recent identification of *A. thaliana* E2 cDNAs encoding proteins of similar size but slightly different amino acid composition (data not shown). Alternatively, it is possible that at least some of these differentially eluting species are the result of posttranslational and/or posthomogenization modifications of single E2s or anomalous elution caused by protein-protein interactions.

All wheat germ E2s isolated appear to be active as judged by their ability to form ubiquitin thiol ester adducts in the presence of E1 and ATP. Similar to that reported for several E2s from rabbit reticulocytes (9), multiple thiol ester adduct species are observed for $E2_{23kD}$, $E2_{16kD}$, and $E2_{20kD}c$. These multiple species may be the result of either multiple attachment of ubiquitin to the E2 or an anomalous migration pattern of the adducts in the gel system used. cDNA clones encoding $E2_{23kD}$ and $E2_{16kD}$ have been sequenced and both encode proteins with two cysteine residues (ref. 27 and data not shown). Whether both cysteines are capable of forming a thiol ester adduct with ubiquitin is currently being examined.

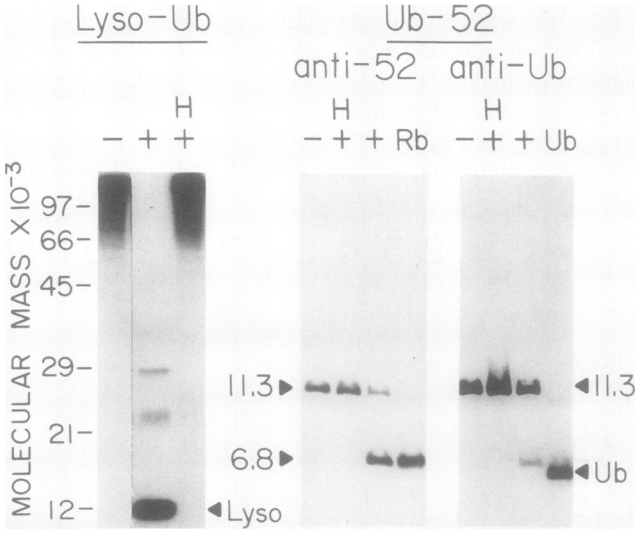

Figure 4. Detection of ubiquitin protein hydrolase activity in ubiquitin affinity column eluate. The substrates, ubiquitin-^{125}I-lysozyme (Lyso-Ub) or ubiquitin extension protein fusion (Ub-52) were incubated in the absence (−) or presence (+) of 1 μg of ubiquitin affinity column eluate with (H) or without the addition of 100 μM hemin. "Rb" contains an *A. thaliana* ribosomal fraction enriched for the processed 52-amino acid extension protein (2). "Ub" contains purified bovine ubiquitin. Samples were subjected to SDS-PAGE on a 13.5% acrylamide gel followed by autoradiography (Lyso-Ub) or immunoblotting with antibodies to the 52-amino acid extension protein or anti-ubiquitin antibodies (Ub-52). The migration positions of free lysozyme (Lyso), free ubiquitin (Ub), uncleaved fusion protein (11.3 kD), and processed extension protein (6.8 kD) are indicated by *arrowheads*.

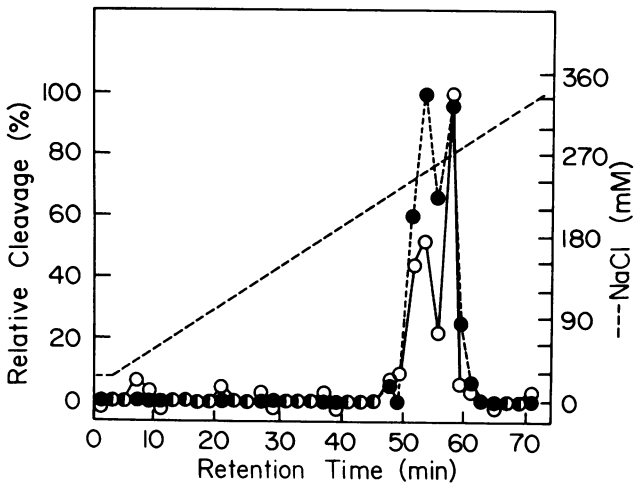

Figure 5. HPLC resolution of ubiquitin protein hydrolase activity bound to ubiquitin affinity column. Ubiquitin affinity column eluate (1.3 mg) was resolved by DEAE-HPLC as described in "Materials and Methods," and 1 mL fractions were collected across the gradient. Fractions were incubated with either ubiquitin-^{125}I-lysozyme conjugates (○) or the ubiquitin 52-amino acid extension protein fusion synthesized in *E. coli* (●) and then resolved by SDS-PAGE. Cleavage of the ubiquitin moiety from ubiquitin-lysozyme conjugates was detected and quantified by the generation of free ^{125}I-lysozyme using autoradiography and liquid scintillation counting. Cleavage of the ubiquitin extension protein fusion was detected by the production of free 52-amino acid extension protein using immunoblotting with anti-52-amino acid extension protein antibodies. Quantification was accomplished by scanning reflective densitometry of the immunoblots. Extent of cleavage is expressed as the percent of that observed for the most active fraction.

When expressed in *E. coli,* the 23- and 16-kD E2s also form multiple thiol ester adducts, eliminating the possibility that the two thiol ester adducts observed are the result of more than one E2 being present in the purified fractions.

Formation of ubiquitin protein conjugates involves the transfer of E2 bound ubiquitin to the target protein with or without the participation of E3. Recent studies indicate that the specificity of the ubiquitin system partially involves recognition of different target proteins by specific E2s (6, 11, 17, 18, 22, 23, 27). At present, we do not know the specific target proteins preferred *in vivo* by the isolated wheat E2s. Only $E2_{23kD}$ has been shown to have significant conjugating activity *in vitro,* specifically transferring ubiquitin to histones in an E3 independent reaction (27). It is likely that some of the isolated E2s have a requirement for E3, but until purification of wheat E3s is achieved, the E3 requirement will remain unclear.

In addition to E1 and E2s, a ubiquitin protein hydrolase activity bound to the ubiquitin affinity column. When fractionated by DEAE-HPLC, the affinity column eluate yielded two peaks of activity, each capable of cleaving both α- (peptide) and ϵ-amino (isopeptide) ubiquitin linkages. Yields of the ubiquitin protein hydrolase activity were relatively low, with most of the activity present in the ubiquitin affinity column flow through (data not shown). This is not surprising, however, as the purification procedure presented here was optimized for the recovery of E1 and E2s and not for the hydrolase. With an optimized purification procedure, wheat germ may prove to be a rich source of these enzymes as well.

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