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Improved method for preparation of ubiquitin-ligated lysozyme as substrate of ATP-dependent proteolysis

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A simple method was developed for preparation of proteins conjugated with ubiquitin. Heat-denatured ^{125}I-labeled lysozyme was highly ubiquitinated by incubation at pH 9.0 with a ubiquitin–protein ligase system consisting of E1, E2 and E3 that had been partially purified from rabbit reticulocytes by affinity chromatography with ubiquitin as a ligand. The resulting conjugates were separated from free lysozyme and other proteins by successive chromatographies on anion and cation ion-exchange resins. The ubiquitinated ^{125}I-lysozymes recovered in the fraction not adsorbed to either resin served as an efficient substrate for ATP-dependent proteolysis in a reticulocyte lysate or with a purified 26 S protease complex. By the present method, ^{125}I-lysozyme–Ub conjugates can be prepared in 3 h with a high yield of 15–20%.

ATP; Ubiquitin; Lysozyme; Ubiquitin–protein ligase; ATP-dependent 26 S protease

1. INTRODUCTION

Ubiquitin (Ub) is a cofactor in an ATP-dependent pathway for intracellular protein breakdown [1]. It is attached ATP-dependently to target proteins to form a signal for their selective degradation by a Ub–protein ligation system consisting of Ub-activating enzyme (E1), Ub-carrier protein (E2) and Ub–protein ligase (E3) [2]. Recently a novel cytosolic protease, named the 26 S proteolytic complex, was shown to cause ATP-dependent degradation of proteins conjugated with Ub [3–5]. Moreover, a 20 S proteasome, a multicatalytic proteinase, was found to associate ATP-dependently with other protein factors to form the 26 S complex [6]. However, little is known about the molecular mechanism of degradation of proteins ligated to Ub. Ubiquitinated substrates are necessary for in vitro studies on the ATP-dependent proteolytic pathway. Lysozyme was shown to be a good substrate for ATP/Ub-dependent proteolysis and two methods for preparation of ubiquitinated proteins have been reported [7,8]. However, these procedures involve complex steps and give low yields. To develop a more efficient method, we re-examined these reported methods to determine the optimal conditions. In this paper, we report a simple method for preparation in high yield of ubiquitinated lysozyme that was degraded rapidly by a mammalian ATP-dependent proteolytic complex.

Abbreviations: Ub, ubiquitin; E1, Ub-activating enzyme; E2, Ub-carrier protein; E3, Ub–protein ligase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; BSA, bovine serum albumin.

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2. MATERIALS AND METHODS

Rabbit reticulocyte extracts were prepared as described [9]. Lysozyme (Seikagaku Kogyo Co.) was iodinated with Na^{125}I (Amersham) by the Chloramine-T method [10]. Ub (Sigma Chem. Co.) was coupled to activated CH-Sepharose (Pharmacia LKB Biotechnology Inc.) by the procedure recommended by the manufacturer. Protein concentration was measured by the method of Bradford [11] with BSA as a standard.

For analysis of Ub–protein conjugates, aliquots of ^{125}I-labeled proteins conjugated with Ub (for preparation, see text) were precipitated by the addition of 5 vols. of cold acetone, collected by centrifugation and subjected to SDS-PAGE (10–20% gradient gel). The gels were dried and exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

For assay of degradation of conjugates, samples of about 5000–10 000 cpm of ^{125}I-lysozyme–Ub conjugates were incubated at 37°C for 15–90 min in a total volume of 100 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP and an ATP-regenerating system (10 μg/ml of creatine phosphokinase and 10 mM phosphocreatine), 1 mM DTT and a suitable amount of the 26 S protease complex. Then, the proteins were subjected to SDS-PAGE, and autoradiographed as mentioned above. For measuring the degradation of ^{125}I-lysozyme–Ub conjugates with acid-soluble fragments by reticulocyte lysates and purified 20 S and 26 S proteases, the reaction was terminated by addition of 575 μl of 10% trichloroacetic acid with 125 μl of 4% BSA as a carrier, and the radioactivity recovered in the acid-soluble fraction was determined in a γ-counter. The method for purification of 20 S proteasomes from rat liver was as reported [12]. The purifications and characterizations of the ATP-dependent 26 S proteolytic complex from rat liver will be described elsewhere (to be published).

3. RESULTS

3.1. *Purification of Ub–protein ligation system*

The enzymes E1, E2 and E3 catalyzing the ligation of

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Ub to proteins were partially purified from a rabbit reticulocyte extract by Ub–Sepharose affinity chromatography. The procedure used was as described by Hershko et al. [2]. A sample of 50–100 ml of reticulocyte extract was used as starting material, and fraction II, the fraction bound to DEAE-cellulose, which contains the enzymes required for conjugation of Ub to proteins and various other proteins, was separated as reported previously [9]. Fraction II was treated with 90% saturation of ammonium sulphate and precipitated material was dialyzed against buffer A (50 mM Tris-HCl, pH 7.5, containing 1 mM ATP, 5 mM $MgCl_2$ and 0.2 mM DTT) supplemented with 5% glycerol. The dialyzate was concentrated to approximately 20–30 mg protein/ml by ultrafiltration with a PM-10 membrane (Amicon). A volume of 10 ml of the concentrated fraction II, supplemented with 5 mM ATP and 10 mM $MgCl_2$, was mixed with about 7 ml of Ub–Sepharose resin (approximately 20 mg of Ub/ml of swollen gel) equilibrated with buffer A containing 5 mM ATP and 10 mM $MgCl_2$ in a 50 ml Falcon tube and rotated slowly for 30 min at room temperature. Then the resin was packed in a small column and washed with 5 bed volumes of buffer A. The column was first developed with 3 bed volumes of 50 mM Tris-HCl (pH 7.5) containing 20 mM DTT. The resulting eluate contained E1 and E2 and a small amount of E3 (this fraction is named 'E1 + E2' hereafter). Then the column was developed with 6 bed volumes of 50 mM Tris-HCl (pH 9.0) containing 2 mM DTT and 1 M KCl. The resulting eluate, containing mainly E3 with a small amount of E2, was quickly neutralized by addition of 10 ml of 1 M Tris-HCl (pH 7.0) (this preparation is named 'E3'). These two enzyme solutions were concentrated by ultrafiltration to protein concentrations of about 1.0 mg/ml and 3.0 mg/ml, respectively, dialyzed against 50 mM Tris-HCl (pH 7.5) containing 1 mM ATP, 1 mM DTT and 20% glycerol, and stored at −70°C until use.

3.2. *Preparation of ^{125}I-lysozyme–Ub conjugates*

^{125}I-Lysozyme (1.7×10^7 cpm, 12 μg) was incubated for 30–90 min at 37°C in 600 μl of reaction mixture containing 100 mM Tris-HCl (pH 9.0), 500 μg of Ub, 1.2 U of inorganic pyrophosphatase, 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, an ATP-regenerating system, 200 μg of 'E1 + E2' and 600 μg of 'E3'. After incubation, the pH in the solution was adjusted to 7.5 with 2 N HCl. The conjugated products were then separated by SDS-PAGE and analyzed by autoradiography. As shown in Fig. 1, the amounts of various sized ^{125}I-lysozyme conjugates with one or more Ub molecule increased with an increase in the amounts of the conjugating enzymes, 'E1 + E2' and 'E3', added. The heterogeneity in size of the conjugates indicated the ligation of various numbers of Ub moieties to a single ^{125}I-lysozyme molecule. The conjugation of Ub to ^{125}I-lysozyme was much greater at pH 9.0 than in the neutral pH range (data not shown).

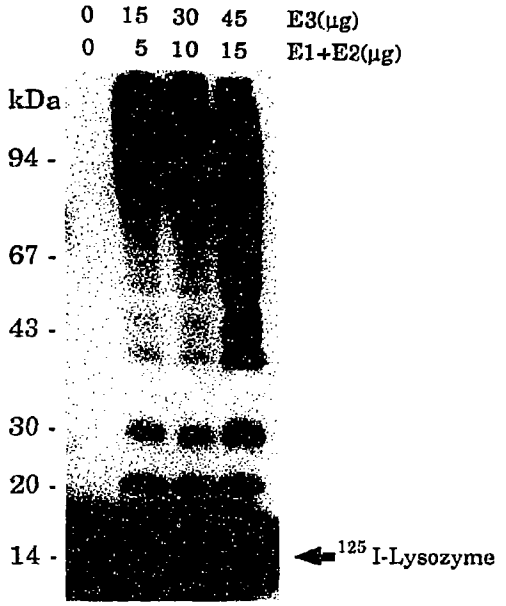

Fig. 1. Electrophoretic analysis of ^{125}I-lysozyme–Ub conjugates synthesized by the Ub-protein ligase system consisting of 'E1 + E2' and 'E3'. The two enzyme fractions 'E1 + E2' and 'E3' were prepared as described in Materials and Methods. The conjugation reaction was carried out for 90 min with the indicated amounts of the enzymes, and conjugates were detected by autoradiography after separation by SDS-PAGE. Details of experimental conditions are described in the text. The arrow indicates the position of free ^{125}I-lysozyme.

Moreover, we found that heat-denaturation of ^{125}I-lysozyme caused a marked increase in its formation of conjugates with Ub. When ^{125}I-lysozyme was incubated for 20 min in boiling water and then quickly chilled in an ice-water bath before use for ligation, numerous conjugates with molecular weights of 70–200 kDa were formed during the first 15 min of the reaction (Fig. 2).

As the ^{125}I-lysozyme–Ub conjugates prepared by this method were not good substrates for the purified ATP-dependent 26 S protease (data not shown), we tried to improve the conditions for their purification. The ^{125}I-lysozyme–Ub conjugates synthesized were put into 2 ml Eppendorf tubes with 0.3 ml of swollen DE52 cellulose gel that had been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, and mixed in a rotary mixer for 5 min at room temperature. The mixtures were then centrifuged at 10 000 rpm for 0.5 min, the resin was washed once with 200 μl of the same buffer and the supernatants were combined. By this procedure, various proteins containing E1, E2 and E3 became associated with the resin and so could be removed from the conjugates. The supernatants were then mixed under the same conditions as described above with CM52 cellulose. Free ^{125}I-lysozyme, which was tightly associated with the resin, was removed at this step. The resulting solution including unbound materials was re-centrifuged at 14 000 rpm for 10 min to remove essentially all the resin. In this chromatographic operation, it was necessary to keep the ratio of the volumes of the resin and the solution containing ^{125}I-lysozyme–Ub conjugates at

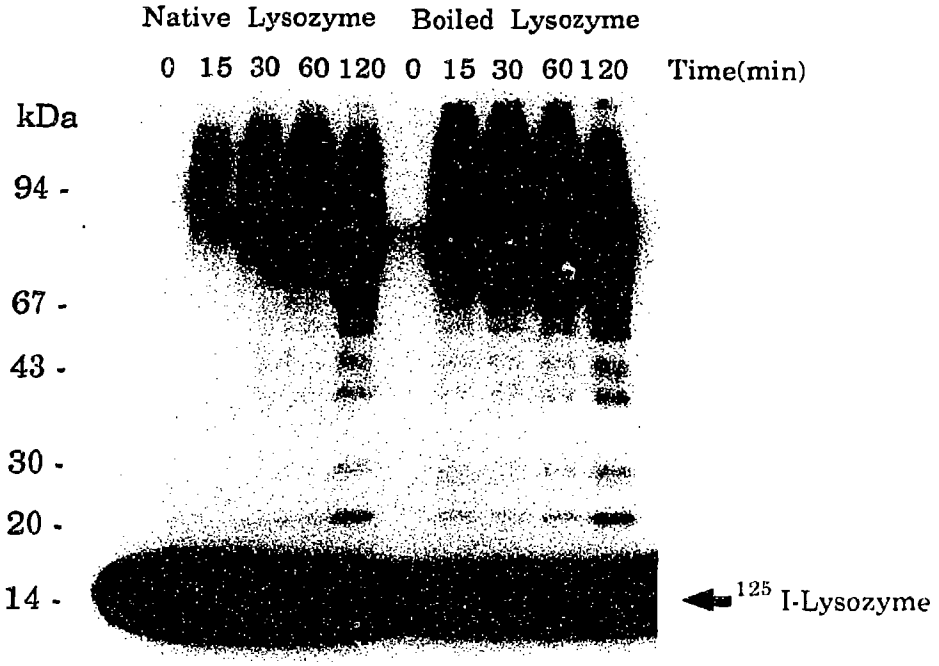

Fig. 2. Effect of heat-treatment of ^{125}I-lysozyme on its conjugation with Ub. The conjugation reaction was carried out for the indicated times with native or boiled (20 min) ^{125}I-lysozyme.

exactly 1:2 (v/v), because recovery of the conjugates was greatly decreased when larger amounts of resin were used. The resulting ^{125}I-lysozyme–Ub conjugates were stored in the presence of 20% glycerol at −70°C. Repeated freezing-thawing also reduced the amount of conjugates. Fig. 3 shows the electrophoretic pattern of the purified conjugates. Comparison with the pattern of unpurified materials (Fig. 1, arrow) shows that the amount of free ^{125}I-lysozyme was greatly reduced (Fig. 3, arrow). The purified ^{125}I-lysozyme–Ub conjugates appeared to be fairly stable during incubation for 80 min (Fig. 3, lane 2), although small amounts of the largest conjugates appeared to be dissociated during the incubation, suggesting that the preparation was slightly contaminated with isopeptidase. From the radioactivities of ^{125}I-lysozyme used and ^{125}I-lysozyme–Ub recoverd as conjugates, the recovery of conjugates was estimated as 7.0–10.0% with normal lysozyme and 15–20% with heat-denatured lysozyme.

3.3. *ATP-dependent degradation of ubiquitinated lysozyme*

Next, we examined whether the Ub–lysozyme conjugates served as a substrate in ATP-dependent proteolysis. Reticulocyte extracts are known to have high activity for ATP-dependent degradation of various proteins [9]. As shown in Table I, degradation of ^{125}I-lysozyme–Ub conjugates by a reticulocyte lysate from rabbits was much higher than that of ^{125}I-lysozyme, although ^{125}I-lysozyme itself was also degraded actively in an ATP-dependent fashion. The degradation of unmodified lysozyme may be mediated by the Ub pathway, because the lysates contain an active Ub–protein ligation system [7,8]. We have recently obtained nearly homogeneous preparations of 26 S proteolytic complexes from various mammalian tissues (to be published) that appear to be similar to the enzymes reported by others [3–5]. A preparation of the 26 S protease complex from rat liver rapidly degraded ^{125}I-lysozyme–Ub conjugates to the acid-soluble fragments and this degradation was markedly stimulated by ATP, but the 26 S protease complex

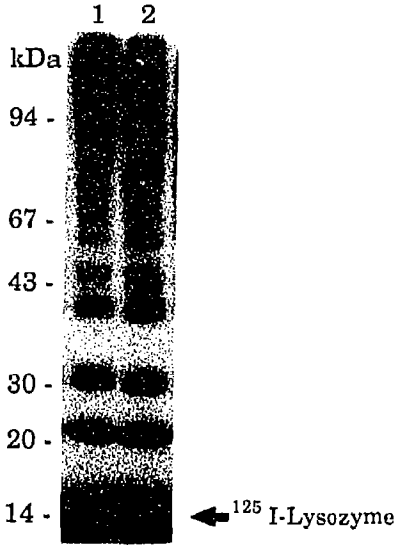

Fig. 3. Rapid purification of ^{125}I-lysozyme–Ub conjugates on ion-exchange resins. ^{125}I-Lysozyme–Ub conjugates were prepared as described for Fig. 1, and purified as described in the text. Lane 1, freshly purified ^{125}I-lysozyme–Ub conjugates. Lane 2, purified ^{125}I-lysozyme–Ub conjugates after incubation for 80 min.

Table I

Degradation of ^{125}I-lysozyme and ^{125}I-lysozyme–Ub conjugates by reticulocyte lysates, an ATP-dependent 26 S protease and 20 S proteasomes

Substrate	% Hydrolysis per h					
	Reticulocyte lysate		26 S Protease complex		20 S Proteasomes	
	−ATP	+ATP	−ATP	+ATP	−ATP	+ATP
^{125}I-Lysozyme	1.0	9.5	0.9	2.7	2.5	1.5
^{125}I-Lysozyme–Ub conjugates	14.6	43.8	5.0	20.4	4.2	4.8

Degradation of ^{125}I-lysozyme (5×10^4 cpm) and ^{125}I-lysozyme–Ub conjugates (8×10^3 cpm) was assayed with samples of 40 μl of reticulocyte lysates and 1.5 μg of purified 26 S protease complex or 20 S proteasomes in the presence or absence of 2 mM ATP. For assay of ATP-independent activity, an ATP-depleting system consisting of hexokinase (1 μg/ml) and glucose (10 mM) was added to the assay mixture, because the lysates and enzyme solutions were prepared in the presence of 2 mM ATP. Percent degradation denotes the ratio of trichloroacetic acid-soluble ^{125}I to total ^{125}I. Values are means of two independent determinations.

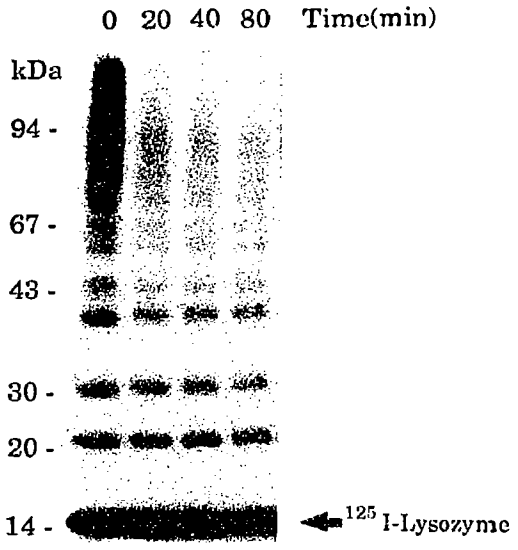

Fig. 4. Degradation of ^{125}I-lysozyme–Ub conjugates by an ATP-dependent 26 S proteolytic complex. The reaction was carried out for the indicated times with 4 μg of the purified 26 S proteolytic complex and ^{125}I-lysozyme–Ub conjugates (10 000 cpm) in the presence of ATP and Mg^{2+}.

did not degrade ^{125}I-lysozyme appreciably (Table I). In contrast, 20 S proteasomes did not show ATP-dependent activity for degradation of either ubiquitinated or unmodified lysozyme. We examined the mode of degradation of ^{125}I-lysozyme–Ub conjugates by the ATP-dependent 26 S protease electrophoretically. As shown in Fig. 4, ^{125}I-lysozyme–Ub conjugates were degraded time-dependently by 26 S protease in the presence of ATP and Mg^{2+}. The disappearance of the bands of the conjugates was probably due to proteolytic destruction of the ^{125}I-lysozyme moieties of the conjugates, not to reversible removal of Ub moieties from the conjugates by isopeptidase, because no appreciable accumulation of free ^{125}I-lysozyme was detected (Fig. 4, arrow). Thus ^{125}I-lysozyme–Ub conjugates prepared by the present method are a good substrate for ATP-dependent proteolysis.

4. DISCUSSION

Two groups have reported methods for preparation of ubiquitinated proteins for use as substrates in the ATP-dependent proteolytic system. Hough and Rechsteiner reported a procedure for chromatographic purification of Ub–lysozyme conjugates synthesized in reticulocyte extracts in the presence of hemin to suppress degradation of the conjugates [7]. However, we obtained the conjugates in only low yield by this method. Hershko et al. reported a procedure for preparing Ub–lysozyme conjugates using purified E1, E2 and E3 [2,8]. This method is essentially similar to that described in this paper, except that they purified the conjugates by gel filtration. We found that the conjugates seemed to be lost during the step of molecular-sieving chromatography owing to their non-specific adsorption to the resin. Therefore, in this work we developed a procedure for their purification by batch ion-exchange chromatography. The recovery of conjugates by the present method was 15–20%. Our improvements in the procedure of Hershko et al. for preparing Ub–lysozyme conjugates were as follows: (i) we used denatured lysozyme obtained by boiling it for 20 min. This improvement is consistent with our finding that heat-treatment of ^{125}I-lysozyme greatly increases its ATP-dependent degradation in reticulocyte extracts (unpublished data); (ii) the conjugating reaction was performed at pH 9.0. This modification was particularly important for preparing conjugates in high yield; (iii) we purified the conjugates by rapid batch ion-exchange chromatography in an Eppendorf tube. This improvement greatly increased the yield of ubiquitinated conjugates. By this improved method conjugates could be isolated in high yield (15–20%) in 3 h. No special technique or skill is required, so the present, simple method should be useful for preparing Ub–lysozyme conjugates in high yield for use in studies on the ATP-dependent proteolytic system in vitro.

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