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Purification and partial characterization of ubiquitin-activating enzyme from *Saccharomyces cerevisiae*

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Ubiquitin-activating enzyme was purified from the yeast *Saccharomyces cerevisiae* by covalent affinity chromatography on ubiquitin-Sepharose followed by HPLC anion-exchange chromatography. Enzyme activity was monitored by the ubiquitin-dependent ATP: $^{32}PP_i$ exchange assay. The purified enzyme has a specific activity of 1.5 μmol $^{32}PP_i$ incorporated into ATP·min^{-1}·mg^{-1} at 37°C and pH 7.0 under standard conditions for substrate concentrations as described by Ciechanover et al. (1982) J. Biol. Chem. 257, 2537–2542. The catalytic activity showed a maximum at pH 7.0. Its molecular weight both in non-denaturing and in SDS-gel electrophoresis was estimated to be 115 kDa, suggesting a monomeric form. The isoelectric point determined by gel electrofocusing was approximately 4.7. Two protein bands differing slightly in electrophoretic mobility could be distinguished when SDS gels were loaded with very small amounts of purified E1 and immunoblotted, the one with higher molecular weight being clearly predominant. The same two bands were also found in anti-E1 immunoblots of crude yeast lysates prepared under broad protease inhibition.

Yeast; Ubiquitin-activating enzyme

1. INTRODUCTION

Almost all functions of ubiquitin are mediated by its covalent attachment to target proteins [2,3]. In the course of the conjugation to acceptors the initial reaction is the activation of ubiquitin by ubiquitin-activating enzyme, E1. During this reaction ubiquitin is first adenylated at its C terminus in the presence of ATP with the release of pyrophosphate. In a second step of the activation mechanism ubiquitin is bound by a thiol site on E1 to form a high-energy thiol ester with the liberation of AMP [1,4]. After this central event, the ubiquitin moeity can be transferred to thiol sites on several different E2 proteins (ubiquitin carrier proteins or ubiquitin conjugating enzymes) by a transesterification reaction [5]. Some of these E2 proteins are capable of directly conjugating ubiquitin to acceptor proteins, others require the action of E3 (ubiquitin–protein ligase) [6]. The resulting ubiquitin-protein conjugate formed between the C-terminus of ubiquitin and ε-amino groups of lysine residues of the substrate protein is of isopeptide nature.

The importance of E1 is underscored by its absolute requirement for cell viability [6,7] and its key-position in the ubiquitin-conjugating pathway.

We report a purification table based on activity measurements by the ubiquitin-dependent ATP:PP_i exchange assay [1,8] and some characteristics of E1 from yeast.

Abbreviations: E1, ubiquitin activating enzyme; E2, ubiquitin carrier protein; PEI-TLC, polyethyleneimine thinlayer chromatography; Ub, ubiquitin

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2. MATERIALS AND METHODS

2.1. *Materials*

Auxiliary enzymes and biochemicals were purchased from Boehringer Mannheim (Mannheim, Germany) and Sigma (Deisenhofen, Germany). Ubiquitin was obtained from Sigma (Deisenhofen, Germany). Ubiquitin-Sepharose was synthesized using activated CH-Sepharose 4B (Pharmacia, Freiburg, Germany) according to the manufacturer's instructions. The final concentration of immobilized ubiquitin was 10 mg/ml gel. Polyethyleneimine thin-layer chromatography (PEI-TLC) plates 'F1440/PEI 20×20 cm' were from Schleicher and Schuell (Dassel, Germany). [^{32}P]Sodium pyrophosphate was from New England Nuclear (Dreieich, Germany).

2.2. *Assay for enzyme activity*

For assaying E1 the ATP:$^{32}PP_i$ exchange reaction described by Ciechanover et al. [8] was used. All substrate concentrations were as described [1] except for the assay buffer which was 0.1 M HEPES-NaOH pH 7.0. Incubation times were chosen to measure only within the linear range of the progress curve, up to approximately 15% relative radioactivity in ATP. A unit of enzyme activity is defined as the amount catalyzing the incorporation of 1 μmol of PP_i into ATP per minute at 37°C under standard conditions as described above. The reactions were started by the addition of enzyme preparation and stopped by spotting 3 μl aliquots (~ 10^4 cpm) onto PEI-TLC plates. After chromatography in 1.2 M potassium phosphate pH 3.14 the plates were dried and the ATP spots were identified under UV light. The ATP spots were excised and liquid-scintillation counted. Assays

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for fraction II were run in the presence and absence of ubiquitin, and enzyme activity was calculated by the difference. After the ubiquitin affinity chromatography step the reaction was completely dependent on the presence of ubiquitin.

2.3. *Preparation of fraction II*

S. cerevisiae strain M1 was grown for 24 h at 30°C in YEPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose) to stationary phase. Performance of the ATP depletion and preparation of the clarified lysate was as described by Jentsch et al. [9], except that the lysate was made from a 33% (w/v) cell suspension in 50 mM Tris-HCl pH 7.5. Following an overnight dialysis against 20 vol 1 mM DTT in 50 mM Tris-HCl pH 7.5 the preparation of fraction II was performed as described in [10,11] except that the basis-buffer used in all steps was 50 mM Tris-HCl pH 7.5. The protein content of Frx II was typically 11–11.5 mg/ml.

2.4. *Ubiquitin affinity chromatography and FPLC*

Covalent affinity purification on ubiquitin-Sepharose was mainly as described [11,12]. Binding of E1 and E2 proceeded for 15 min at room temperature and was performed as a batch adsorption. The ratio of fraction II to (immobilized) ubiquitin was 13:1. For the elution proce dure the ubiquitin-Sepharose was poured into a column with a resulting volume of 25 ml. AMP/PP_i-elution and a consecutive DTT-elution was performed essentially as described by Haas and Bright [12], also the concentration of the eluates by ultrafiltration and subsequent FPLC anion-exchange chromatography on a MonoQ HR 5/5 column, from which E1 is eluted by a linear salt gradient of 0–500 mM KCl.

2.5. *Electrophoresis, isoelectric focusing*

Polyacrylamide electrophoresis in the presence of SDS was carried out as described by King and Laemmli [13]. Non-denaturing gel-electrophoresis using polyacrylamide gradient (8–25%) gels and isoelectric focusing using Phast-IEF-gels (4–6.5) were done with a Pharmacia PhastSystem according to the company's recommendations. Gels were stained for protein with Coomassie brilliant blue R-250 and destained by diffusion. Molecular mass standards for SDS-gel electrophoresis were prestained (with apparent molecular mass in kDa), myosin H-chain (211), phosphorylase B (107), BSA (69), ovalbumin (46), carbonic anhydrase (29), β-lactoglobulin (18), lysozyme (14).

2.6. *Antiserum, immunoblotting*

A rabbit was immunized against SDS-denatured E1 [14] and boosted after 3, 5 and 7 weeks. Antiserum was collected 2 weeks thereafter. Immunoblotting was done after electrophoresis of the respective samples as described [15].

2.7. *Protein determination*

Protein concentrations were determined by the method of Bradford [16] using BSA as standard.

3. RESULTS AND DISCUSSION

The procedure for isolating factors of the ubiquitin-conjugating system by the preparation of fraction II from ATP-depleted cells to remove endogenous ubiquitin, followed by covalent affinity chromatography on ubiquitin-Sepharose was developed by Hershko and colleagues [1,11]. This method has proved to be applicable to a great variety of eukaryotic organisms like rabbit reticulocytes [11] and liver [17], human erythrocytes [1], chicken muscle [18], wheat germ [19] and yeast [9]. The ubiquitin-activating enzyme E1 can be eluted in a relatively selective manner from the ubiquitin-Sepharose matrix by reversing its enzymatic reaction with the addition of AMP and pyrophosphate. This AMP/PP_i eluate still contains small contaminations of ubiquitin-conjugating enzymes E2 [20,12] that were probably released from the column by reversal of the ubiquitin-transesterification reaction from E1 to E2 during the elution of E1 [20]. To remove those residual E2 contaminants an HPLC anion-exchange chromatography described by Haas and Bright for the resolution of E2 enzymes [12] on a MonoQ column proved to be very effective. In contrast to the situation found in preparations of reticulocytes where E1 co-elutes with one of the E2s from the MonoQ column [12], we did not observe this elution pattern in yeast. Hence, additional E1 (see below) could easily be separated from the DDT eluate of the ubiquitin affinity chromatography by this step. Polyacrylamide electrophoresis of the purified E1 is shown in Fig. 1. Table I shows the purification procedure where the ubiquitin-Sepharose column was first eluted with AMP + PP_i (2 mM of each) and successively with DTT (10 mM). As can be seen, the AMP/PP_i elution yielded only approximately 2/3 of the column-bound E1. The remainder 1/3 of the total bound E1 could be released by a consecutive DTT-eluate (with the disadvantage of including all E2-proteins whose thiolesters are also cleaved). However, this impure preparation can be purified further by FPLC anion-exchange chromatography to a specific activity almost as high as that obtained for the further purified AMP/PP_i eluate (Table I).

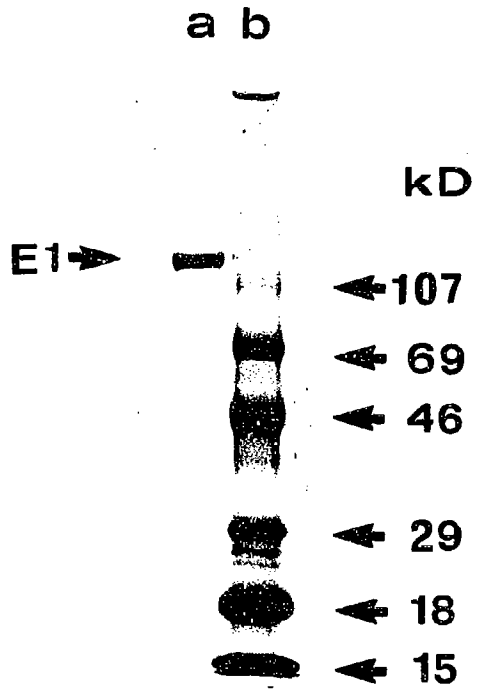

Fig. 1. SDS-polyacrylamide gel electrophoresis of E1. 6 μ_g E1 (purified from the AMP/PP_i-eluate) were loaded on a 10–20% gradient gel, lane a; molecular mass marker proteins, lane b.

Table I

Purification of ubiquitin-activating enzyme from *Saccharomyces cerevisiae* starting with 140 g yeast (wet weight). Values marked with an asterisk are related to the activity measured in fraction II (see text).

Step	Volume (ml)	Protein-conc. (mg/ml)	Total protein (mg)	Spec. activity (mU/mg)	Total activity (mU)	Purification* (-fold)	Yield* (%)
Crude extract	400	20	8000				
Fraction II	300	11	3300	0.67*	2211*	1	100
Ub-Aff. chrom.:							
AMP/PP_i eluate	10	0.045	0.45	1380	621	2060	28.1
DTT eluate	10.5	0.037	0.37	930	344	1388	15.5
FPLC:							
of AMP/PP_i eluate	1	0.23	0.23	1560	359	2328	16.2
of DDT eluate	1	0.115	0.115	1520	175	2269	7.9

From the MonoQ column E1 eluted as a single peak at 225 mM KCl.

The purification described here yielded 378 mU E1 (0.25 mg) from 100 g wet weight yeast with approximately 1.5 U/mg.

Calculations of 'purification' and 'yield' are related to fraction II because the ATP:PP_i exchange assay is not applicable in the crude extract due to endogenous ubiquitin. Moreover, the validity of the assay in fraction II is hampered by the permanent removal of ubiquitin from the isotope-exchange reaction by conjugation to endogenous acceptor proteins of fraction II. Nevertheless, such information has been given before [1] and is useful for comparison with other organisms or purification protocols. Values relating on the activity in fraction II are therefore included but marked with an asterisk to indicate that in Frx II the actually measured activity is always an inevitable underestimate of the true E1-activity.

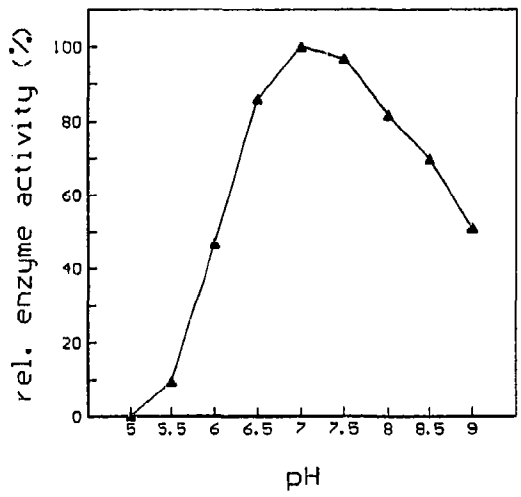

Fig. 2. Effect of pH on the ATP:PP_i exchange activity of purified E1. Each assay was performed with 115 ng E1. The maximal activity observed was set as 100% relative enzyme activity. Buffers were 0.1 M Na-acetate pH 5.0; 0.1 M MES-NaOH at 5.5–6.5; 0.1 M HEPES-NaOH pH 7.0–7.5; 0.1 M Tris-HCl pH 8.0–9.0.

While the E1-yield of the AMP/PP_i-eluate from yeast (28.1%) is comparable to that from erythrocytes (36.6%, [1]), greater differences are seen in the specific activities. Yeast E1 isolated from the AMP/PP_i eluate exhibits a specific activity five times higher than the same preparation from human erythrocytes [1]. When comparing the difference in specific activity in the AMP/PP_i eluates one has to consider that we used 0.1 M HEPES-NaOH pH 7.0 instead of 0.05 M Tris-HCl pH 7.5. But this difference can only in part be attributed to the different buffer conditions used, because the specific activity of yeast E1 at pH 7.5 is only slightly lower (approx. 3%) than at pH 7.0, as can be seen in the pH-profile (Fig. 2). In addition yeast E1 activity at pH 7.5 was identical in both 0.05 M HEPES- or 0.1 M Tris-buffer in our hands. Dependence of ATP:PP_i exchange activity of purified E1 on the pH is shown in Fig. 2. Maximal

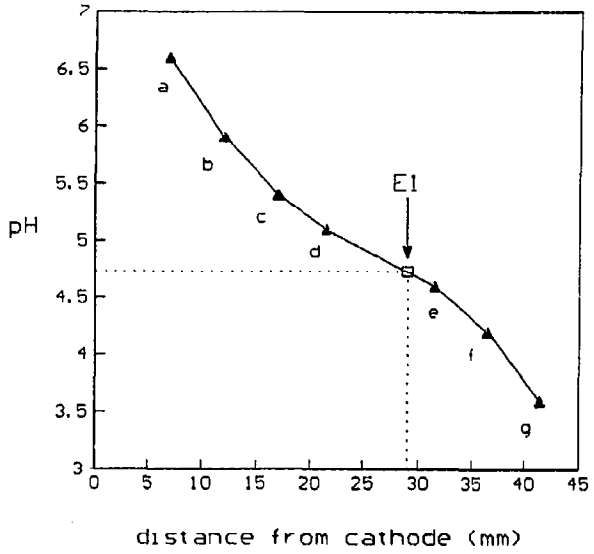

Fig. 3. Isoelectric focusing of E1. Estimated isoelectric point, 4.7. Isoelectric points of reference proteins are: a, carbonic anhydrase I (6.6); b, carbonic anhydrase II* (5.9); c, carbonic anhydrase II** (5.4); d, β-lactoglobulin A (5.1); e, soybean trypsin inhibitor (4.6); f, glucose oxidase (4.2); g, amyloglycosidase (3.6). *CA II (C6403), **CA II(C3666).

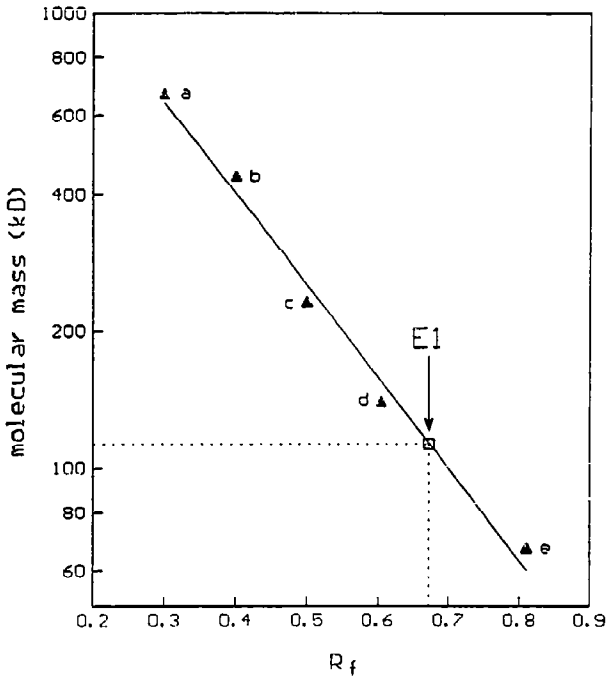

Fig. 4. Estimation of the molecular mass of purified E1 by non-denaturing gel electrophoresis. Estimated molecular mass, 15 kDa. The relative electrophoretic mobilities (R_f) of the protein-stained bands of E1 and reference proteins are compared. (molecular mass in kDa) a, thyroglobulin (669); b, ferritin (440); c, catalase (232); d, lactate dehydrogenase (140); BSA (67).

activity was observed at pH 7.0. To ensure that the pyrophosphate is not hydrolyzed during incubations at acid pH, a mock assay at pH 5 was done for 1 h without added E1. After PEI-TLC the TLC plate was autoradiographed and compared to identical runs with $^{32}PP_i$ and $^{32}P_i$ standards. There was no hydrolysis of pyrophosphate detectable. The pH optimum at pH 7 seems to be of physiological significance as the cytosolic pH in yeast is reported to be neutral [21]. The isoelectric point of E1 was measured by isoelectric focusing in a polyacrylamide gel. A pI of 4.7 was determined by comparison of the protein-stained band with marker proteins (Fig. 3).

The molecular mass of the native protein, determined by non-denaturing gel electrophoresis was estimated to be 115 kDa (Fig. 4). This result in conjunction with the molecular mass observed in SDS-gels (cf. Fig. 1) suggest E1 exists as a monomer.

Antiserum against SDS-denatured enzyme was raised in a rabbit. This anti-E1 antiserum recognized both SDS-denatured and native enzyme in immunoblot experiments (not shown). In contrast to antiserum against wheat germ E1 [19] the anti-yeast E1 antiserum did not crossreact with E1 from mammalian sources (human and rabbit tested, see Fig. 5b, lane b and c).

As shown in Fig. 5a, in immunoblotting experiments with the anti-E1 antiserum a faint second band with slightly lower molecular weight could be distinguished from the E1 band seen in Coomassie-stained gels with purified E1 (due to the better resolution when small amounts of protein are loaded in conjunction with the sensitive detection method). The observation of two or more closely related forms of E1 that differ only slightly in molecular weight has also been reported for E1 from humans [22], mouse [23], rabbit [19] and oat [19]. As this double band was also detected in crude extracts prepared under broad protease inhibition (antipain 50 μg/ml, aprotinin 20 μg/ml, chymostatin 100 μg/ml, EDTA

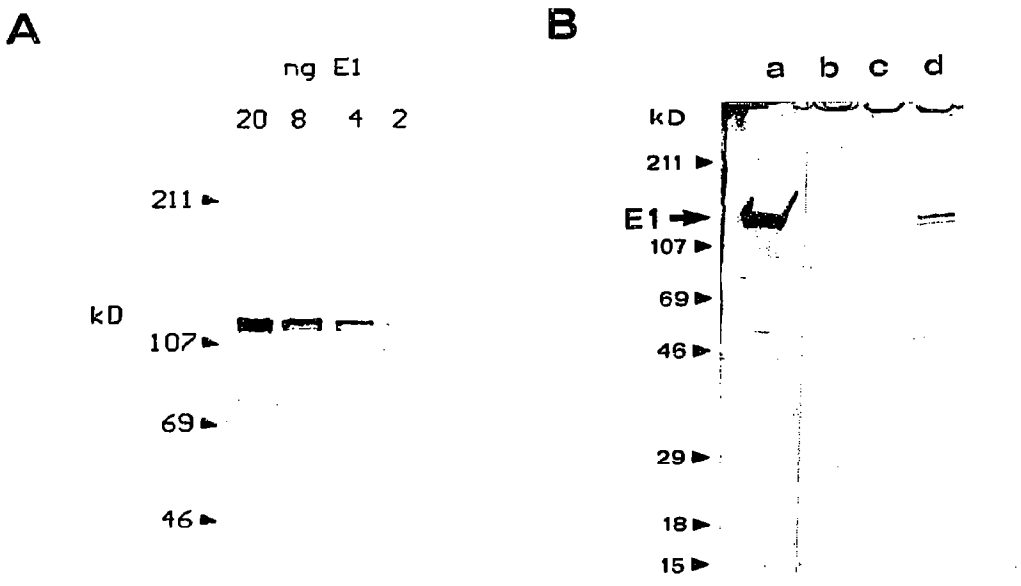

Fig. 5. Anti E1-immunoblotting. Panel A: resolution of two separate bands from purified E1. Small amounts of protein (ng E1 as indicated) were run in an 8% polyacrylamide gel in the presence of SDS and an immunoblot with anti-E1 antiserum was performed as described in section 2. Panel B: comparison of different E1-sources. Electrophoresis and immunoblotting was as described in Panel A. Lane a, 0.5 μg purified E1; lane b, 1 μg AMP/PP_i eluate from rabbit reticulocytes; lane c, 1 μg AMP/PP_i eluate from human erythrocytes; lane d, 30 μg crude yeast preparation (see text).

1 mM, EGTA 1 mM, leupeptin 20 μg/ml, pepstatin A 20 μg/ml), (Fig. 5b, lane d), it is probably not an artefact due to proteolysis during the purification procedure.

As there seems to be only one E1-encoding gene in yeast and its deletion results in cell death [3,7], the two E1 forms observed probably represent a covalent modification of the same enzyme. While this work was under way McGrath et al. reported some evidence for the existence of a ubiquitin-E1 adduct of isopeptide nature [7]. This could be a possible explanation for the observation of the two closely related bands in the immunoblotting experiments.

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