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Purification and partial characterization of an ATP-hydrolyzing serine protease from lettuce leaves

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Abstract

A novel ATP-hydrolyzing protease has been purified from lettuce leaves by combination of $(NH_4)_2SO_4$ fractionation, gel filtration and anionic exchange chromatography. The purified enzyme is made up of a single subunit with an apparent molecular weight of 40 000, even though a possible higher molecular organization might occur in vivo induced by the presence of ATP or protease's substrate. The lettuce protease showed caseinolytic, trypsin-like and, to a smaller extent, peptidyl glutamyl hydrolase activities. It is a serine protease as both peptidase activities are highly sensitive to tosyl-L-lysinechloromethylketone (TLCK) and leupeptin. The trypsin-like activity of the enzyme was not affected by MgATP complex or ATP alone. Peptidyl glutamyl hydrolase activity, instead, and ATP hydrolysis were strictly correlated as incubation of the enzyme with MgATP, but not with ATP alone, stimulated the peptidase activity of the enzyme, while peptide substrate as well as TLCK enhanced ATPase activity. Moreover, the ATPase inhibitor vanadate, which also blocked the peptidyl glutamyl hydrolase activity, caused a strong activation of the trypsin-like activity of the enzyme. Therefore, these studies could indicate the existence of multiple functional states of the enzyme achieved in vivo by ATP hydrolysis. The cytosolic localization of the enzyme is finally discussed.

Keywords: *Lactuca sativa*; Asteraceae; Lettuce; Leaves; Purification; ATP hydrolyzing protease; Proteolysis; Serine protease

1. Introduction

In eukaryotes and prokaryotes degradation of proteins is a key regulatory mechanism involved in many aspects of cells growth and development (Callis, 1995). Proteolysis is, in fact, essential for the removal of mutated or damaged proteins, for supplying aminoacids needed to synthesize new proteins, for zimogens activation and for controlling metabolism by decreasing the amount of rate-limiting enzymes or by modelling the levels of receptors (Vierstra, 1996). Moreover, in plant cells, proteolysis is implicated during seed storage proteins utilization (Wilson, 1986), leaf senescence (Lohman, Gan, John, & Amasino, 1994) and environmental stress response (Garbino, Rockhold, & Belknap, 1992). It has been shown that selective proteins removal is accomplished by large multimeric complexes which require ATP hydrolysis coupled with proteolytic degradation (Goldberg, 1992). Enzymatic complexes involved in such ATP-stimulated proteolytic cleavage have been purified from bacteria (Hwang, Woo, Goldberg, & Chung, 1985; Katayama-Fujimura, Gottesman, & Maurizi, 1987), yeast (Rubin et al., 1996), mammals (Tanaka, Ii, Ichiara, Waxman, & Goldberg, 1986; Hough, Pratt, & Rechsteiner, 1987; Waxman, Fagan, & Goldberg, 1987; Kanayama et al., 1992; Sawada, Muto, Fujimuro, Akaishi, & Sawada, 1993), and higher plants (Fujinami et al., 1994). In *Escherichia coli* several, both soluble and membrane-associated, endoproteases have been described, whose function is linked to ATP hydrolysis: La protease, Ti (Clp) protease, Hsl VU and FtsH proteases. La protease is a tetrameric complex of a single 87 k polypep-

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tide chain involved in the degradation of most abnormal proteins (Goldberg, 1992). ATPase as well as proteolytic activities are associated with this single subunit. Instead, Ti (Clp) protease is made up of two different subunits Clp A (81 k) and Clp P (21 k) which correspond respectively to the ATPase and proteolytic moiety of the complex. Two hexamer of ClpA assemble on either side of two stacked seven-membered rings of Clp P to constitute the Clp AP complex. It has been shown that ClpAP catalyses the specific hydrolysis of protein with abnormal aminotermini (Goldberg, 1992). Protease Hsl VU is a two component enzymatic complex more recently isolated in *Escherichia coli* as product of the heat-inducible operon hsl VU (Rohrwild et al., 1996; Yoo et al., 1996). It is constituted by a 50 k protein, Hsl U with ATPase activity, and a 19 k protein, Hsl V, which represents the proteolytic subunit (Wickner et al., 1994). The Hsl VU structure resembles the ClpAP protease since it forms cylindrical four ring particle in which two central HslV hexameric rings are flanked, at either ends, by one HslU hexameric ring. Hsl VU seems to play a role in the degradation of incomplete proteins occurred by puromycin treatment as well as of mutated polypeptides (Huang & Goldberg, 1997). FtsH is a monomeric (71 k), membrane-anchored, ATP-dependent protease which supplies both ATPase and proteolytic activities (Suzuki et al., 1997). It is involved in the rapid and selective degradation of unassembled SecY, a component of a membrane translocase (Akiyama, Kihara, Tokuda, & Ito, 1996). Recent studies have pointed out that also chloroplasts contain ATP-dependent proteolytic pathways. Immunological analysis indicated, in fact, that chloroplasts of different plant sources, such as tomato, barley and spinach, might contain proteases homologous both to the *E. coli* Clp AP and FtsH (Shanklin, DeWitt, & Flanagan, 1995; Ostersetzer, Tabak, Yarden, Shapira, & Adam, 1996; Benešová, Ďurčová, Kužela, Kutejová, & Pšenák, 1996; Lindahl et al., 1996). The cytosol and nucleus of eukaryotes contains an ATP-dependent proteolytic complex called proteasome 26S, consisting of 15–20 different polypeptides, for reviews see (Hershko & Ciechanover, 1992; Vierstra, 1993; Vierstra, 1996; Coux, Tanaka, & Goldberg, 1996). Proteasome has been purified from yeast (Rubin et al., 1996) and several mammalian cells (Tanaka et al., 1986; Hough et al., 1987; Waxman et al., 1987; Kanayama et al., 1992; Sawada et al., 1993). The existence of the 26S proteasome has been also suggested in several plant sources (Vierstra & Sullivan, 1988; Hatfield & Vierstra, 1989), and its purification to homogeneity was obtained from spinach leaves Fujinami et al., 1994). Since it require ubiquitin to target the substrate, proteasome 26S was considered part of the ubiquitin-dependent pathway which catalyses the degradation of rate-limiting enzymes, transcriptional factors, regulatory proteins as well as mutated or damaged proteins (Hershko & Ciechanover, 1992; Vierstra, 1993; Vierstra, 1996; Coux et al., 1996). Proteasome 26S is a symmetric structure that contains a barrel-shaped central core, the 20S proteasome, composed of four stacked seven membered rings, which is implicated in proteolytic activity. On both ends of the 20S proteasome there is a large complex, PA 700 activator, which is responsible for ATP hydrolysis (Coux et al., 1996). Unlike the bacterial FtsH, all ATP-dependent proteases described previously, in both prokaryotes and eukaryotes, as well as in eukaryotes organelles, are multisubunits complexes assembled in a well-organized structure of high molecular mass. In this wide repertoire of ATP-dependent protease, we report the purification and partial characterization of a new type, monomeric, small-sized, serine, ATP-hydrolyzing and ATP-stimulated endoproteinase from *Lactuca sativa* leaves, whose features, for certain aspects, resemble that of other ATPase-dependent proteinases. Experimental data are also shown to support the hypothesis that the lettuce leaves enzyme is localized in the cytosol.

2. Results

2.1. Proteolytic activity purification

An ATPase-associated protease was isolated from lettuce leaves by a procedure described in Section 4. The results are summarized in Table 1. After ammonium sulfate fractionation proteolytic activity on casein was recovered in the 55% saturation fraction. The ammonium sulfate precipitate was subjected to gel filtration onto Sephacryl S-300. Fractions eluted from gel filtration column were assayed for both proteolytic and ATPase activities as reported in Section 4. The elution pattern showed two well-separated proteolytic activities. In contrast, only one peak of ATPase activity was obtained and it was associated with the second proteolytic peak. The first proteolytic activity emerged in the void volume, showed multicatalytic nature, and, it was inhibited by *N*-carbobenzyloxy–leucine–leucine–leucinale (*N*-cbz–leu–leu–leucinale) and chlorophyllin (data not shown). These findings could account for a proteasome like activity in lettuce leaves which presence is currently under active investigation in our laboratory. The further purification of the ATPase associated proteolytic activity was performed by anionic exchange chromatography on DEAE Sepharose CL 6B column. The proteolytic activity as well as the ATPase activity were eluted as single peak at 1.1 M of NaCl concentration. Proteolytic active fractions were subsequently purified by a second gel filtration step on Sephacryl S-300. The

Table 1
Summary of the purification of the lettuce leaves protease

Purification step	Protein (mg)	Activity[a]		Recovery (%)	Purification fold
		total μkat	specific nkat mg^{-1}		
Crude extract	133	27.6	208	100	1
$(NH_4)_2SO_4$	56	18.3	328	66	1.6
First gel-filtration	8.4	3.0	366	11	1.8
Anionic exchange	4.0	1.7	433	6	2
Second gel-filtration	0.16	0.16	1033	0.6	5

[a] Proteolytic activity was followed during enzyme purification using 2% casein as substrate.

purified enzyme showed ATPase activity along with proteolytic activity both emerged in the same elution volume. As shown in Table 1 the purified enzyme had a specific activity against casein of 1033 nkat mg^{-1} with 0.6% recovery of the starting material protein and a purification fold of 5. The inherent lability of the proteolytic activity during the purification procedure account for such a low purification fold obtained. SDS-gel electrophoresis of the purified enzyme (Fig. 1, lane 5) showed the presence of a single protein band with an apparent molecular mass of 40 k. Determination of the molecular mass of the purified protease was also performed by gel filtration on Sephacryl S-300 previously calibrated as described in Section 4. Both proteolytic and ATPase activity emerged in the same elution volume corresponding to an apparent molecular mass of 40 k thus confirming the value obtained with SDS-PAGE analysis. The

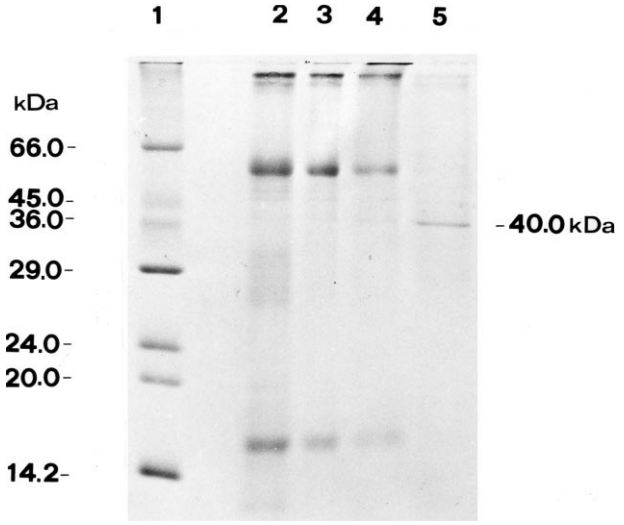

Fig. 1. SDS-PAGE (12.5%) slab gel analysis of the purification procedures of the lettuce leaves protease. Lane 1: molecular mass markers; lane 2: ammonium sulphate precipitated fraction, 10 μg; lane 3: 48–57 pooled fractions from the first gel filtration step on Sephacryl S-300, 10 μg; lane 4: 30–38 pooled fractions of the DEAE–Sepharose CL-6B column, 10 μg; lane 5: fraction 49 from the second gel filtration step, 10 μg.

results obtained from both SDS-PAGE and gel filtration chromatography account for the presence in the lettuce leaves of a small sized (40 k), monomeric, ATP-hydrolyzing protease. However, due to our analytical procedure we cannot rule out the possibility that the enzyme is made up of two different subunits with similar molecular weight which associate in vivo forming multimeric complexes.

2.2. Substrate specificity

The purified enzyme was tested for substrate specificity using three synthetic peptides *N*-carbobenzyloxy–glycine–glycine–arginine–β-naphthylamide (*N*-cbz–GGR–βNA), *N*-carbobenzyloxy–glycine–glycine leucine–*p*-nitroanilide (*N*-cbz–GGL–*p*NA) and *N*-carbobenzyloxy–leucine–leucine–glutamic acid–β-naphthylamide (*N*-cbz–LLE–βNA), which are model substrates for trypsin-like, chymotrypsin-like and peptidyl glutamyl hydrolase activities (SV8-like), respectively. Table 2 showed that the lettuce protease hydrolyzed *N*-cbz–GGR–βNA more efficiently than the other substrates examined (specific activity 83 pkat mg^{-1}). It did not exhibit chymotrypsin-like activity, whereas was slightly active against *N*-cbz–LLE–βNA (specific activity 11 pkat mg^{-1}).

2.3. Effect of inhibitors on the protease activity

Specific inhibitors were used to characterize the proteolytic and peptidase activities of the purified protease. As shown in Table 3, both trypsin-like and SV8-like activities were strongly inhibited by tosyl-L-lysine-

Table 2
Substrate specificity of the purified lettuce leaves protease

Substrate	Specific activity (pkat mg^{-1})
N-cbz–GGR–βNA 0.4 mM	83
N-cbz–GGL–*p*NA 0.4 mM	0
N-cbz–LLE–βNA 0.4 mM	11

Table 3
Effect of different inhibitors on both peptidase and caseinolytic activities of the lettuce leaves enzyme

	Activity % of control		
	casein	*N*-cbz–GGR–βNA	*N*-cbz–LLE–βNA
Control[a]	100	100	100
TLCK 0.1 mM	65	17	20
Leupeptin 0.1 mM	78	33	35
PMSF 2 mM	80	100	100
NEM 2 mM	85	80	33
SDS 0.01%	100	100	100

[a] The specific activity of the untreated enzyme (control) was 1033 nKat mg^{-1} against 2% casein, 83 pkat mg^{-1} against 0.4 mM *N*-cbz–GGR–βNA, and 11 pkat mg^{-1} using 0.4 mM *N*-cbz–LLE–βNA as substrate.

chloromethylketone (TLCK) and leupeptin whereas these two inhibitors had only slight effects upon the caseinolytic activity of the enzyme. Phenylmethylsulphonylfluoride (PMSF) was only a weak inhibitor of the caseinolytic activity of the enzyme. The thiol reagent such as NEM had little effect on either trypsin-like and caseinolytic activities whereas it strongly inhibited the peptidyl glutamyl hydrolase activity. Proteolytic as well as peptidase activities were unaffected by addition of 0.01% (w/v) SDS.

2.4. ATPase activity associated with the lettuce leaves protease

Purified protease from lettuce leaves showed considerable ATPase activity. As shown in Table 4 in our experimental conditions the specific activity was around 20 nkat mg^{-1}. ATPase activity associated with our preparation was insensitive to different typical ATPases inhibitors such as NaN_3, $NaNO_3$, whereas Na_3VO_4 a specific inhibitor of the P-type ATPases showed 60% inhibition of the hydrolytic activity.

2.5. Interdependence between proteolytic and ATPase activities of the purified proteinase

To investigate whether the proteolytic and peptidase activities were correlated to the associated ATPase activity, we tested the effect of ATPase's substrate (MgATP) and the inhibitor sodium vanadate upon the casein and peptides degradation activities of the enzyme; similarly, ATPase activity was measured in the presence of substrates (2% casein, 0.4 mM peptides) and inhibitors (0.1 mM leupeptin, 0.1 mM TLCK, 2 mM NEM) of both caseinolytic and peptidase activities. As shown in Table 5 the proteolytic activity of the enzyme against casein was not affected by both ATP or MgATP complexes. Conversely, peptidase activity against *N*-cbz–LLE–βNA was markedly stimulated by the presence of MgATP. It is worthwhile to point out that activation was strictly dependent on the presence of MgATP complexes since ATP alone did not cause any stimulatory effect thus suggesting that ATP hydrolysis is necessary to stimulate the SV8-like activity of the enzyme. Moreover, a strong inhibition was observed when *N*-cbz–LLE–βNA hydrolysis was measured in the presence of the ATPase inhibitor sodium vanadate and the addition of MgATP did not removed the vanadate effect. In contrast, trypsin-like activity was not markedly stimulated by the presence of MgATP and the observed stimulation was independent by the presence of Mg^{2+} (Table 5). Interestingly, sodium vanadate, a strong inhibitor of the ATPase activity as well as of the SV8-like peptidase activity of the enzyme, widely enhanced the trypsin-like activity measured with *N*-cbz–GGR–βNA as substrate, thus suggesting a possible correlation between the two putative peptidase active sites and the ATPase moiety of the enzyme. As can be seen in Table 6, ATPase activity of the lettuce leaves protease was unaffected by the presence of 2% casein, 0.4 mM *N*-cbz–GGR–βNA as well as by 1 mM NEM and 0.1 mM leupeptin; ATP hydrolysis was, instead, stimulated by the presence of the substrate of the SV8-like activity (*N*-cbz–LLE–βNA). Strong stimulation of ATPase activity was also obtained by addition of 0.1 mM TLCK which, as already shown, completely inhibited either trypsin-like and peptidyl glutamyl hydrolase activities.

Table 4
Effect of inhibitors upon the ATPase activity associated with the lettuce leaves proteinase

Compound	ATPase activity % of control
Control[a]	100
$NaNO_3$ 50 mM	92
NaN_3 2 mM	98
Na_3VO_4 1 mM	40

[a] The specific activity of the enzyme measured without any addition (control) was 20 nkat mg^{-1}. As reported in Section 4 ATPase activity was assayed using 3 mM ATP as substrate.

Table 5
Effect of different compound on the peptidase and proteolytic activities of the purified enzyme

	Activity % of control		
	casein	*N*-cbz–GGR–βNa	*N*-cbz–LLE–βNA
Control[a]	100	100	100
ATP 1 mM	90	118	107
MgATP 1 mM	90	125	142
Vanadate 1 mM	90	168	14
MgATP 1 mM + Vanadate 1 mM	90	156	14

[a] The specific activity of the untreated enzyme (control) measured according the procedures described in Section 4 were 1033 nkat mg^{-1} against casein, 83 pkat mg^{-1} using *N*-cbz–GGR–βNA as substrate, and 11 pkat mg^{-1} against *N*-cbz–LLE–βNA.

2.6. Intracellular localization of the ATP-stimulated protease

The NADP-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13), a chloroplast's stroma marker enzyme (Quail, 1979), was used to establish whether any stromal contamination was present in our lettuce leaves crude extract. Chloroplast's stromal fraction and crude extract were prepared separately as described in Section 4 and both assayed for NADP-glyceraldehyde-3-phosphate dehydrogenase, ATPase and proteolytic activities. Table 7 showed that in the crude extract no detectable NADP-glyceraldehyde-3-phosphate dehydrogenase activity was associated to ATPase associated proteolytic activity. As expected, NADP-glyceraldehyde-3-phosphate dehydrogenase activity was instead detected in the stromal fraction and it was associated to reasonable levels of chloroplastic endogenous proteolytic activity; ATPase activity was instead not observed in this fraction. The presence of ATPase activity in the soluble stromal fraction, due possibly to either chloroplast's contamination or to released soluble CF_1, was actually not expected. In our experimental conditions, in fact, the high speed centrifugation used to obtain the stromal fraction could assure a perfect membrane sedimentation, moreover, ATPase activity was carried out in absence of trypsin, calcium and dithiothreitol which are known to be necessary for CF_1 activation (Lien & Racker, 1971). Therefore, these results indicated that the crude extract used for the isolation of the ATPase associated protease was not strongly contaminated by chloroplast's stromal fraction since NADP-glyceraldehyde-3-phosphate dehydrogenase activity and ATPase-associated proteolytic activity were observed in different cellular fraction.

Table 6
Effect of different compound on the ATPase activity of the purified protease

Compound	ATPase activity % of control
Control[a]	100
Casein 2%	100
N-cbz–GGR–βNA 0.4 mM	100
N-cbz–LLE–βNA 0.4 mM	145
Leupeptin 0.1 mM	100
NEM 1 mM	100
TLCK 1 mM	266

[a] The specific activity of the enzyme measured against 3 mM ATP without any further addition (control) was 20 nkat mg^{-1}.

3. Discussion

In eukaryotes and prokaryotes removal of most proteins requires ATP hydrolysis. ATP-hydrolyzing endoproteases have been isolated from bacteria (Hwang et al., 1985; Katayama-Fujimura et al., 1987), yeast (Rubin et al., 1996), and a variety of mammals sources (Tanaka et al., 1986; Hough et al., 1987; Waxman et al., 1987; Kanayama et al., 1992; Sawada et al., 1993). More recently it has been purified to homogeneity from spinach leaves (Fujinami et al., 1994), and immunologically detected in several plant's chloroplasts (Shanklin et al., 1995; Ostersetzer et al., 1996; Benešová et al., 1996; Lindahl et al., 1996). Requirement of ATP hydrolysis has in such system distinct functions; in La protease ATP binding allowed the formation of peptidase site and successive ATP hydrolysis inactivated the enzyme (Goldberg, 1992). ATPase activity could promote the assembly of the bacterial Clp AP protease (Goldberg, Moerchell, Chung, & Maurizi, 1994; Maurizi, Thompson, Singh, & Kim, 1994) as well as of the 26S proteasome in eukaryotic cells (Coux et al., 1996). ATP hydrolysis has been proposed to assist as an 'unfoldase' coupling protein unfolding to its degradation (Vierstra, 1996). Therefore, Clp A ATPase, FtsH and 26S proteasome can exhibit a chaperon-like activity presenting to the proteolytic moiety of the enzyme a completely unfolded, more degradable, substrate (Vierstra, 1993, 1996; Wickner et al., 1994; Suzuki et al., 1997). Obviously, chaperon-like activity cannot justify

Table 7
Localization of enzymatic activities[a] in the crude extract and chloroplast's stroma from lettuce leaves

	ATPase activity (nkat) mg^{-1}	Proteolytic activity (nkat mg^{-1})	NADP-glyceraldehyde-3- phosphate dehydrogenase activity (nkat mg^{-1})
Crude extract	0.25	190	–
Chloroplast's stroma	–	50	63.3

[a] As reported in Section 4 ATPase activity was assayed using 3 mM ATP as substrate; the proteolytic activity was measured against 2% casein and NADP-glyceraldehyde-3-phosphate dehydrogenase activity was determined using 1,3-diphosphoglyceric acid as substrate according the coupled assay described in Heber, Pon, & Heber.

ATPase activity if short peptides are used as substrates. Cleavage of such peptides by La and Clp proteases was, in fact, not affected by the presence of non-hydrolyzable analog of ATP (Katayama-Fujimura et al., 1987; Van Melderen et al., 1996). In our study we reported the purification and characterization of a novel ATP-hydrolyzing endoprotease from lettuce leaves which showed caseinolytic, trypsin-like and, to a smaller extent SV8-like activities. The specific activity of the purified enzyme was 1033 nkat mg^{-1} on casein, whereas it was 83 pkat mg^{-1} when *N*-cbz–GGR–βNA was used as substrate (trypsin-like activity), and 11 pkat mg^{-1} against *N*-cbz–LLE–βNA (SV8-like activity). The enzyme appears to be a serine protease as it is strongly inhibited by TLCK and leupeptin. Moreover NEM, a sulfhydryl specific reagent, inhibits specifically the peptidyl glutamyl peptidase activity of the enzyme suggesting a possible role for cysteines in the catalytic mechanism. The caseinolytic activity as well as trypsin-like activity were not affected by ATP hydrolysis. Surprisingly, as reported for Hsl VU protease (Huang & Goldberg, 1997), the SV8-like activity was markedly stimulated by ATP hydrolysis as well as the ATPase activity was significantly enhanced by peptide substrate or by TLCK. Since we cannot postulate either a role in subunits assembling or a chaperon-like activity of the enzyme in the presence of short peptides, it is likely that ATP hydrolysis induced some conformational change in the enzyme's structure so that the active site could better accommodate the peptide substrate (*N*-cbz–LLE–βNA). Moreover, the ATPase inhibitor sodium vanadate blocked the SV8-like activity but caused an high activation of the trypsin-like activity, thus suggesting the existence of a putative functional state of the enzyme fully active against *N*-cbz–GGR–βNA occurring when both ATPase and SV8-like activity were blocked. Although many similarity with other ATP-dependent proteases have been found, such as proteolytic and ATPase activities associated to unique polypeptide chain (Goldberg, 1992), ATPase-stimulated peptidase activity (Goldberg, 1992), peptide-enhanced ATPase activity (Goldberg, 1992), vanadate sensitive ATPase and peptidase activities (Goldberg, 1992), the lettuce enzyme markedly differed from the other ATP-dependent system in its molecular mass and structural organization. As far as we know this is the first evidence for the purification in higher plants of a 40 k, soluble, single subunit enzyme involved in ATP-stimulated proteolytic process. Only another case of monomeric ATP-dependent protease has been reported in bacteria and chloroplasts (Lindahl et al., 1996; Suzuki et al., 1997), that is the membrane anchored, 71 k protein FtsH. However, since ATP or protease's substrate were not present in the isolation buffers of the purification procedure here reported as well as during molecular weight determination, we cannot exclude that the proteolytic and ATPase activity may be associated with different subunits of the enzyme with similar molecular weight. Finally the experimental data showed in Table 7 excluded any chloroplastic contamination of our starting material (crude extract) suggesting a cytosolic localization of the enzyme.

4. Experimental

4.1. Enzyme extraction and purification

Fresh lettuce leaves (*Lactuca sativa* L. cv Romana) (100 g) were obtained from a local market, washed with bidistd H_2O and homogenized with 125 ml of standard buffer (220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM cysteine, 5 mM HEPES-KOH, pH 7.5) with a Polythron homogenizer at half maximum power for 2 min. The homogenate was filtered through two layers of cheesecloth and centrifuged at 27 200*g* for 30 min. The resulting surnatant was again centrifuged at 150 000*g* for 60 min and the surnatant obtained, representing the crude extract, was precipitated with solid $(NH_4)_2SO_4$ at 55% of satn. The ppt was resuspended in 2 ml of standard buffer and loaded onto a Sephacryl S-300 gel filtration column (1 × 50 cm) previously equilibrated with 150 ml of 25 mM HEPES-KOH, pH 7.5, and eluted with 80 ml of the same buffer. Frs of 1 ml were collected and assayed for proteolytic activity; the frs of the second peak with the highest proteolytic activity were pooled and

applied onto a DEAE Sepharose CL 6B column (1.5× 10 cm) equilibrated with 100 ml of 25 mM HEPES-KOH, pH 7.5. The column was developed with a 50 ml linear gradient of NaCl (0–2 M) in HEPES-KOH, pH 7.5, and 2 ml frs were collected. The active proteolytic fractions were combined and precipitated with solid $(NH_4)_2SO_4$ at 55% of satn. After centrifugation the pellet was redissolved in 1.5 ml of 25 mM HEPES-KOH, pH 7.5 and further purified on a Sephacryl S-300 gel filtration column as described above. The active proteolytic frs were pooled and used for enzyme's characterization. All purification procedures described above were performed at 4°. Frs eluted from gel filtration as well as those from anionic exchange chromatography were also assayed for ATPase activity as described below.

4.2. Assay of proteolytic activity

Proteolytic activity was routinely assayed using casein as substrate according to the method described in (Kaneda, Arima, Yonezawa, & Uchikoba, 1994), except that the surnatant was recovered after centrifugation at 10 000 rpm in a bench-top microfuge. For inhibition assay the enzyme was preincubated for 20 min with 0.1 mM TLCK, 0.1 mM leupeptin, 2 mM PMSF, 2 mM NEM, 0.01% SDS in the reaction medium without substrate. The reaction was started by addition of casein. To test the effect of 1 mM ATP, 1 mM MgATP as well as 1 mM Na_3VO_4 on the proteolytic activity, the enzyme was used to start the reaction in assay buffer. Enzyme activity was expressed in nanokatal and it was defined as the amount of enzyme that yielded 10^{-6} unit of absorbance change per s.

4.3. Peptidase assay

Peptidase activity was estimated by the cleavage of the synthetic peptides *N*-cbz–GGL–βNA, *N*-Cbz–GGR–βNA and *N*-Cbz–LLE–βNA. The assay mixture (0.25 ml) contained 100 mM Tris–HCl, pH 8.0 and 0.4 mM of the synthetic peptide as substrate. After incubation at 30° for suitable periods, the reaction was stopped by adding 0.25 ml of 20% (w/v) TCA. Samples were centrifuged at 10 000 rpm for 5 min, then the released *p*NA or βNA were measured following the diazotization procedure according to (Bratton & Marshall, 1939) as modified by (Goldbarg & Rutemburg, 1958). To test the effect of different proteases inhibitors as well as of 1 mM Na_3VO_4, 1 mM ATP and 1 mM MgATP on peptidase activity, assays were performed as described above except that the enzyme was added to start the reaction. Enzyme activity was expressed in nanokatal (nkat).

4.4. ATPase assay

The enzyme was incubated for 20 min in a reaction medium containing 3 mM $MgSO_4$, 3 mM ATP, 20 mM HEPES-KOH, pH 7.5 (final volume 0.5 ml) at 30°. ATP hydrolysis was stopped by adding 20 μl of 10% (w/v) SDS soln. After incubation on ice for 5 min, the released Pi was measured as reported in (Lanzetta, Alvarez, Reinach, & Candia, 1979). For inhibitors studies, the enzyme was preincubated for 20 min at room temp with 2 mM NaN_3, 2 mM $NaNO_3$, 2 mM Na_3VO_4 in the reaction medium lacking ATP. Similarly, the enzyme was preincubated for 20 min at room temp with 2% (w/v) casein, 0.1 mM leupeptin, 0.1 mM TLCK, 1 mM NEM, 0.4 mM *N*-cbz–GGR–βNA and 0.4 mM *N*-cbz–LLE–βNA, respectively, and then ATP was added to start the reaction.

4.5. Molecular mass determination

The M_r of the purified enzyme was estimated by gel filtration on a Sephacryl S-300 column (1 × 50 cm) equilibrated with 25 mM HEPES-KOH, pH 7.5. The column was eluted with the same buffer at flow rate of 0.75 ml min^{-1}. M_r markers used to calibrate the column were: blue dextran (2000 k), thyroglobulin (669 k), apoferritin (443 k), β-amylase (200 k), alcohol dehydrogenase (150 k), bovine albumin (66 k), carbonic anhydrase (29 k). M_r was also determined by SDS-PAGE under reducing conditions as described below.

4.6. Electrophoresis

SDS-PAGE (12.5% slab gel) was performed according to (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250 and destained with MeOH–HOAc–H_2O soln (2:1:10). M_r markers were bovine albumin (66 k), egg albumin (45 k), glyceraldehyde-3-phosphate dehydrogenase (36 k), carbonic anhydrase (29 k), trypsinogen (24 k), soybean trypsin inhibitor (20 k), and α-lactalbumin (14.2 k).

4.7. Chloroplasts preparation

Isolation of lettuce leaves chloroplasts was performed as described in McCarty & Racker (1967). Purified chloroplasts were washed ×3 with 400 mM sucrose, 10 mM NaCl, 15 mM cysteine, 20 mM Tris–HCl, pH 8.0; the stromal fr was obtained by resuspending chloroplasts in 50 ml of 10 mM HEPES-KOH, pH 7.5 under gentle stirring for 30 min at 4°, followed by centrifugation at 150 000*g* for 45 min. The resulting surnatant containing the stromal proteins was assayed for both proteolytic and ATPase activities as described above. NADP-glyceraldehyde-3-phosphate

dehydrogenase (EC. 1.2.1.13) was determined according to (Heber, Pon, & Heber).

4.8. Protein assay

Protein content was measured by the method of Bradford (1976) using bovine serum albumin as standard.

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References

Akiyama, Y., Kihara, A., Tokuda, H., & Ito, K. (1996). *Journal of Biological Chemistry*, *271*, 31196.
Benešová, M., Ďurčová, G., Kužela, S., Kutejová, E., & Pšenák, M. (1996). *Phytochemistry*, *41*, 65.
Bradford, M. M. (1976). *Analytical Biochemistry*, *72*, 248.
Bratton, A. C., & Marshall, E. K. (1939). *Journal of Biological Chemistry*, *128*, 537.
Callis, J. (1995). *Plant Cell*, *7*, 845.
Coux, O., Tanaka, K., & Goldberg, A. L. (1996). *Annual Review of Biochemistry*, *65*, 801.
Fujinami, K., Tanahashi, N., Tanaka, K., Ichiara, A., Cejka, Z., Baumeister, W., Miyawaki, M., Sato, T., & Nakagawa, H. (1994). *Journal of Biological Chemistry*, *269*, 25905.
Garbino, J. E., Rockhold, D. R., & Belknap, W. R. (1992). *Plant Molecular Biology*, *20*, 235.
Goldbarg, J. A., & Rutemburg, A. M. (1958). *Cancer*, *11*, 283.
Goldberg, A. L. (1992). *European Journal of Biochemistry*, *203*, 9.
Goldberg, A. L., Moerchell, R. P., Chung, C. H., & Maurizi, M. R. (1994). *Methods in Enzymology*, *244*, 350.
Hatfield, P. M., & Vierstra, R. D. (1989). *Biochemistry*, *28*, 735.
Heber, U., Pon, N. G., Heber, M. *Plant Physiology*, 38, 355.
Hershko, A., & Ciechanover, A. (1992). *Annual Review of Biochemistry*, *61*, 761.
Hough, R., Pratt, G., & Rechsteiner, M. (1987). *Journal of Biological Chemistry*, *262*, 8303.
Huang, H.-C., & Goldberg, A. L. (1997). *Journal of Biological Chemistry*, *272*, 21364.
Hwang, B. J., Woo, K. M., Goldberg, A. L., & Chung, C. H. (1985). *Journal of Biological Chemistry*, *263*, 8727.
Kanayama, H. O., Tamura, T., Ugai, S., Kagawa, S., Tanahashi, N. et al. (1992). *European Journal of Biochemistry*, *206*, 567.
Kaneda, M., Arima, K., Yonezawa, H., & Uchikoba, T. (1994). *Phytochemistry*, *35*, 1395.
Katayama-Fujimura, Y., Gottesman, S., & Maurizi, M. R. (1987). *Journal of Biological Chemistry*, *262*, 4477.
Laemmli, U. K. (1970). *Nature*, *227*, 680.
Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979). *Analytical Biochemistry*, *100*, 95.
Lien, S., & Racker, E. (1971). *Methods in Enzymology*, *23*, 49.
Lindahl, M., Tabak, S., Cseke, L., Pichersky, E., Andersson, B., & Adam, Z. (1996). *Journal of Biological Chemistry*, *271*, 29329.
Lohman, K. N., Gan, S., John, M. C., & Amasino, R. M. (1994). *Physiologia Plantarum*, *92*, 322.
Maurizi, M. R., Thompson, M. W., Singh, S. K., & Kim, S. H. (1994). *Methods in Enzymology*, *244*, 314.
McCarty, R. E., & Racker, E. (1967). *Journal of Biological Chemistry*, *242*, 3435.
Ostersetzer, O., Tabak, S., Yarden, O., Shapira, R., & Adam, Z. (1996). *European Journal of Biochemistry*, *236*, 932.
Quail, P. H. (1979). *Annual Review of Plant Physiology*, *30*, 425.
Rohrwild, M., Coux, O., Huang, H.-C., Moerschell, R. P., Yoo, S. J., Seol, J. H., Chung, C. H., & Goldberg, A. L. (1996). *Proceedings of the National Academy of Sciences USA*, *93*, 5808.
Rubin, D. M., Coux, O., Wefes, I., Hengartnner, C., Young, R. A., Goldberg, A. L., & Finley, D. (1996). *Nature*, *379*, 655.
Sawada, H., Muto, K., Fujimuro, M., Akaishi, T., & Sawada, M. T. (1993). *FEBS Letters*, *335*, 207.
Shanklin, J., DeWitt, N. D., & Flanagan, J. M. (1995). *Plant Cell*, *7*, 1713.
Suzuki, C. K., Rep, M., van Dijl, J. M., Suda, K., Grivell, L. A., & Schatz, G. (1997). *Trends in Biochemical Sciences*, *22*, 118.
Tanaka, K., Ii, K., Ichiara, A., Waxman, L., & Goldberg, A. L. (1986). *Journal of Biological Chemistry*, *261*, 15197.
Van Melderen, L., Thi, M. H. D., Lecchi, P., Gottesman, S., Couturier, M., & Maurizi, M. R. (1996). *Journal of Biological Chemistry*, *271*, 27730.
Vierstra, R. D. (1993). *Annual Review of Plant Physiology and Plant Molecular Biology*, *44*, 385.
Vierstra, R. D. (1996). *Plant Molecular Biology*, *32*, 275.
Vierstra, R. D., & Sullivan, M. L. (1988). *Biochemistry*, *27*, 3290.
Waxman, L., Fagan, J. M., & Goldberg, A. L. (1987). *Journal of Biological Chemistry*, *262*, 2451.
Wickner, S., Gottesman, S., Skowyra, D., Hoskins, J., McKenney, K., & Maurizi, M. R. (1994). *Proceedings of the National Academy of Sciences USA*, *91*, 12218.
Wilson, K. A. (1986). In M. J. Dalling, *Plant proteolytic enzymes* (p. 19). Boca Raton, FC: CRC Press.
Yoo, S. J., Seol, J. H., Shin, D. H., Rohrwild, M., Kang, M. S., Tanaka, K., Goldberg, A. L., & Chung, C. H. (1996). *Journal of Biological Chemistry*, *271*, 14035.