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SPINACH CHLOROPLAST ATP-DEPENDENT ENDOPEPTIDASE: Ti-LIKE PROTEASE

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Abstract—The soluble fraction of spinach chloroplast was used for purification and characterization of an ATP-dependent protease. Purification included Q Sepharose Fast Flow, hydroxylapatite and FPLC Superose 6 column chromatography. The isolated enzyme requires ATP and Mg^{2+} for stimulation and represents a ubiquitin independent serine protease, containing essential sulphydryl group(s). By using fluorogenic peptides a similarity of chloroplast protease to *Escherichia coli* Ti protease was observed. The chloroplast protease is immunochemically cross – reactive with the bacterial protease Ti.

INTRODUCTION

Protein degradation appears to be an essential component of cellular functions [1–3]. Originally the vacuolar proteases were thought to be responsible for protein degradation in plant tissues [4, 5]. The recently described occurrence of proteases in plant cell organelles indicates the complexity of proteolytic events during the life cycle of plant cells [6–9].

Chloroplasts represent the major protein-containing component of photosynthetic tissues [10]. Different aminopeptidases [11, 12] and endopeptidases [13] have been identified in stroma and thylakoids. It has been suggested that the ubiquitin-dependent pathway occurs in plant chloroplasts [14], although some have disputed this. [15, 16]. An ATP-dependent proteolysis of phosphorylase was reported in spinach leaves [17]. Following the fate of labelled *de novo* formed proteins in isolated pea chloroplasts it was found that the breakdown of these proteins is stimulated by ATP [18, 19]. Moreover the DNA-sequence analysis indicates that chloroplasts contain a protease similar to ATP-dependent protease Ti from *Escherichia coli* [20]. ATP-dependent proteolytic systems have been characterized in detail in *E. coli* and are represented by proteases La and Ti [1]. They control the availability of certain short-lived regulatory proteins, ensure the proper stoichiometry for multi-protein complexes and protect the accumulation of abnormal proteins [9, 21, 22]. Both La and Ti proteases are serine type endopeptidases containing essential sulphydryl group(s) sensitive to NEM [1]. La protease is composed of four identical M_r 87 k subunits, each with an ATP binding site [24, 25]. Enzymes with similar basic characteristics have been purified from rat liver [26] and yeast mitochondria [27]. The gene sequences for coding this type of protease have been recently published [28–30]. Unlike protease La, protease Ti [31] consists of two different subunits, both of which are essential for proteolytic reaction [32]. Subunit A (Clp A) is a homodimer (subunit M_r 89 k) possessing the ATP-ase activity [33]. Component P (Clp P: a heat shock protein) is a multimer of 21 k subunits. Subunits P contain the serine active site for proteolysis. DNA sequence analysis [20] has shown that the amino acid sequence of Clp P in *E. coli* closely resembles a protein encoded by chloroplast DNA, which is well conserved between chloroplasts of different plant species. The chloroplast Clp P homologs should be functional proteases, since regions around the two active site residues are among the most highly conserved. Given the previous observations [34] that the chromosomally encoded Clp A homologs in tomatoes carry chloroplast transit sequences, it seems likely that a Clp-like ATP-dependent protease is active within chloroplasts. Protein degradation is, in fact, known to occur in chloroplast; for example, the small subunit of ribulose-1,5-bisphosphate carboxylase is rapidly degraded when present in excess over the larger subunit [18]. Moreover, an ATP-dependent proteolytic activity has been found in chloroplasts [18, 19, 35] but has not yet been characterized biochemically. Whether either of these proteolytic activities, are dependent on a Clp-like protease remains unknown. In this paper the procedure for the isolation of ATP-dependent protease from spinach chloroplasts is described and

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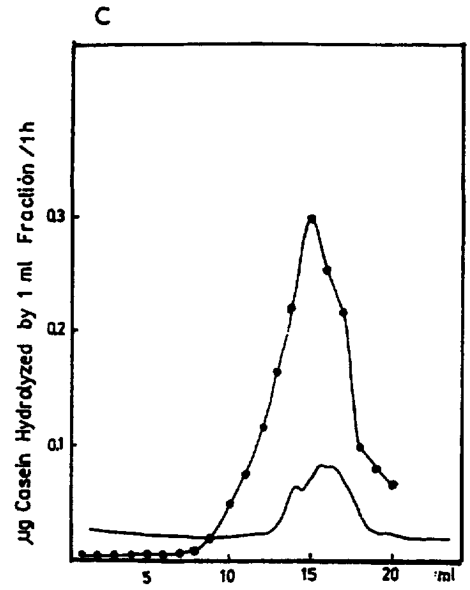
some of the basic characteristics of this enzyme are compared with those of the bacterial protease Ti.

RESULTS AND DISCUSSION

Ti-like ATP dependent protease from spinach chloroplasts has been isolated by anion exchange chromatography, hydroxylapatite and gel filtration (Fig. 1, Table 1). Proteolytic activity of purified enzyme is stimulated by ATP and Mg^{2+} while ADP and nonhydrolysable ATP analogs AMP-PCP (β,τ-methylene adenosine 5-triphosphate) and AMP-CPP, (α,β-methylene adenosine 5-triphosphate) cannot replace ATP in protein degradation. Moreover the activity of the spinach chloroplast protease is inhibited by vanadate (Table 2), a potent inhibitor of ATPases [36]. This indicates that ATP hydrolysis is required for degradation by the enzyme. The pH optimum of chloroplast matrix protease is 7.9 and its K_m for ATP is about 60 μM.

To examine the requirement of ubiquitin for the stimulation of proteolysis by ATP, the enzyme solution was made ubiquitin-free by filtration on Sephadex G 50 [37]. No changes in ATP-dependent proteolytic activity in ubiquitin-free enzyme sample were observed. The subsequent addition of ubiquitin did not exhibit any effect on the ATP-dependent breakdown of caseine. Since the chloroplast enzyme is inhibited by TLCK, TPCK and PMSF (Table 2) it appears to be a serine ATP dependent protease such as La or Ti in *E. coli* [1]. Its sensitivity to



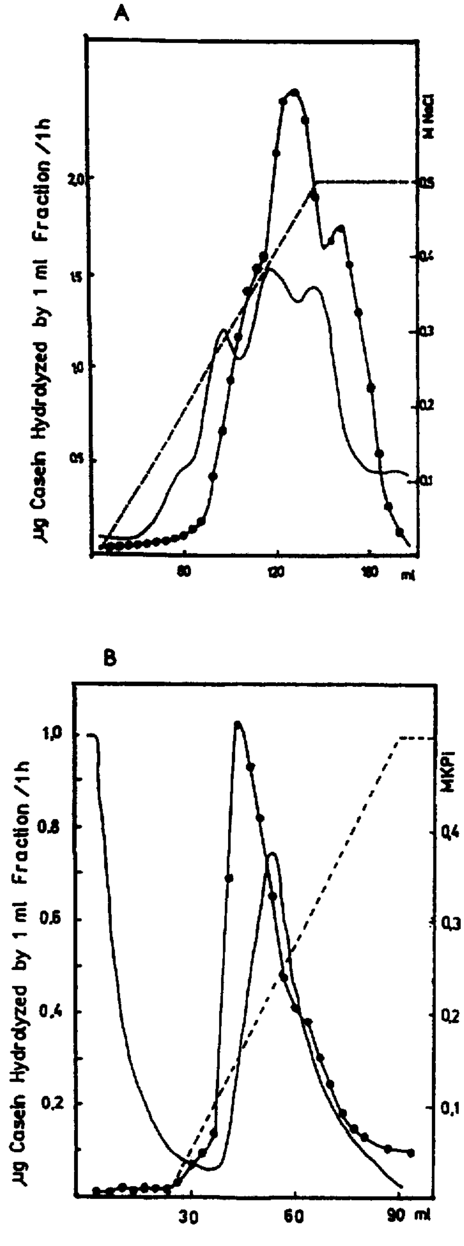


Fig. 1. Protein and protease activity elution profiles during purification of the chloroplast matrix ATP-dependent protease. Separations on Q Sepharose Fast Flow (A), hydroxylapatite (B) and FPLC Superose 6 (C) were done as described in Experimental. A_{280} in appropriate scales (——), salt concentration (- - -), protease activity in the presence of 2 mM ATP (-O-).

Table 1. Summary of the purification of the ATP-dependent protease from matrix of spinach chloroplast

Purification step	Protein (mg)	μg [^{14}C]casein hydrolysed by 1 mg protein per 1 hr	
		− ATP	+ ATP
Chloroplast fraction	7 800	0.025	0.096
Chloroplast matrix	1 100	0.058	0.122
Q Sepharose Fast Flow	162	0.350	0.529
Hydroxylapatite	16.8	0.746	1.09
FPLC Superose 6	0.088	2.24	5.69

Experimental details are given in the text. Results of a typical purification are presented.

Table 2. Effect of some inhibitors on the activity of ATP-dependent protease from matrix of spinach chloroplast

Effector	Concentration (mM)	Remaining protease activity (%)
None		100
TPCK	0.10	76.8
TLCK	0.10	73.5
PMSF	1.00	47.6
Vanadate	5.00	66.9
Leupeptin	0.20	46.6
NEM	1.00	47.0
Hemin	0.05	39.8
Ubiquitin	3.75	89.3

Experimental details are given in the text. Results of a typical experiment are presented. The protease activity was determined in the presence of 2.5 mM ATP.

Table 3. Hydrolysis of fluorogeic peptides by ATP-dependent protease from spinach chloroplast

Peptide	ATP	Rates of hydrolysis (nmol/hr)
Glu-Ala-Ala-Phe-MNA	+	1.54
	−	1.62
Suc-Leu-Tyr-AMC	+	23.9
	−	3.7

Experimental details are given in the text. Results of a typical experiment are presented.

NEM (Table 2) points to an essential role of sulphydryl group(s) in the enzyme activity and emphasizes the possible La- or Ti-like character of the enzyme.

To be able to distinguish between the above mentioned possibilities, the enzyme obtained after FPLC Superose 6 separation was tested for substrate specificity using two fluorogenic peptides Glu-Ala-Ala-Phe-MNA (GAAP-MNA) and Suc-Leu-Tyr-AMC (SLT-AMC) which are the specific substrates for La and Ti proteases respectively. SLT-AMC was found to be a good substrate for chloroplast protease; its proteolytic degradation was significantly stimulated in the presence of ATP. GAAP-MNA practically was not degraded by this enzyme (Table 3). These results compared with those published for Ti protease (Table 4) strongly support the idea that the ATP-dependent protease found in chloroplast is a Ti-like protease.

To extend these observations, the immunochemical relationship of the chloroplast protease with the bacterial proteases La and Ti has been tested. Chloroplast protease was recognized in the blot by both anti-protease Ti antibodies (Fig. 2), but not by anti-protease La antibody. These results show that the chloroplast ATP-dependent protease and protease Ti from *E. coli* are related not only functionally but also immunochemically.

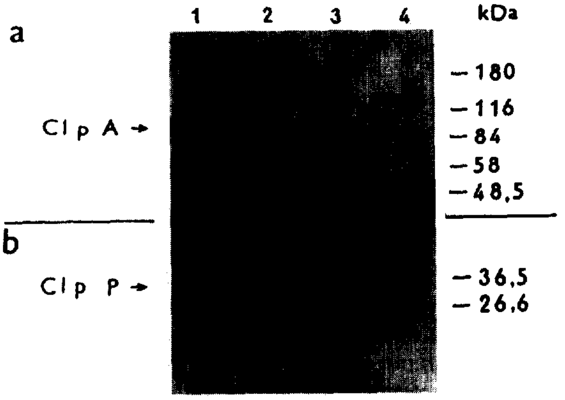


Fig. 2. Western blots of the partially purified ATP-dependent chloroplast protease on nitrocellulose. The blot was processed with two different antibodies against the ATP-dependent protease Ti (a – antibody against A subunit, b – antibody against P subunit). The *E. coli* protease Ti (lane 1), the chloroplast matrix (lane 2), prestained M_r standards (Sigma) (lane 3) and spinach chloroplast ATP-dependent protease (lane 4).

Table 4. Properties shared by ATP-dependent protease Ti from *E. coli* and the spinach chloroplast ATP-dependent protease

Property	Spinach chloroplast protease	*E. coli* protease
Action	endopeptidase	endopeptidase
pH optimum	7.9	7.5–9.5
ATP, Mg^{2+} dependency	yes	yes
ADP, nonhydrolysable analogs of ATP	no stimulation effect	no stimulation effect
Inhibitors	PMSF, NEM	DFP, NEM
Glu-Ala-Ala-Phe-MNA	not hydrolysed	not hydrolysed
Suc-Leu-Tyr-AMC	hydrolysed	hydrolysed
Stabilized by	ATP, glycerol	ATP, glycerol
K_m for ATP (protease)	60 μM	180 μM

Conclusions on protease Ti are based on other work [1, 31, 32, 42].

EXPERIMENTAL

Chloroplast stroma preparation. All procedures were carried out at 4°. 750 g of fresh spinach leaves (*Spinacia oleracea* L., 6-week-old, grown in the Garden of Medicinal Plants, Faculty of Pharmacy, Bratislava), were homogenized in 450 ml of preparation medium (300 mM sucrose, 50 mM Na-Pi, 5 mM $MgCl_2$, pH 7.4) by an ETA-type mixer (Elektro-Praga Hlinsko, 20 sec and 2 × 10 sec at 10000 rpm). After filtration (4-layers of cheese cloth) and centrifugation (1000 *g*, 3 min), the sediment was washed twice with preparation medium and resuspended in buffer A (20 mM Tris, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, pH 7.9). The crude chloroplast suspension was further purified by a sucrose density gradient (30–60%) centrifugation (Beckman L7, SW-14, 40000 rpm, 90 min) and disintegrated in precooled (0–4°) French pressure cell (SLM AMINCO, USA, 16000 psi). Supernatant after subsequent centrifugation (Beckman L7, Ti-50, 40000 rpm, 60 min) was frozen in liquid N_2 stored at − 70° and used as a chloroplast matrix.

Purification of protease. All chromatography steps were carried out at 4°.

Step 1. Q Sepharose Chromatograpy. Chloroplast matrix thawed on ice (about 1.1 g protein) was loaded on a Q Sepharose Fast Flow (Pharmacia) column (15 × 1.5 cm) equilibrated with buffer A. The column was washed with 50 ml of buffer A and eluted with 140 ml linear NaCl gradient (0–0.5 M) in buffer A. Fractions with ATP-dependent proteolytic activity were combined and dialysed against buffer A containing 10 mM K-Pi.

Step 2. Hydroxylapatite chromatography. The dialysed sample from step 1 was applied to a hydroxylapatite (Bio-Rad) column (10 × 1.1 cm) equilibrated with buffer A containing 10 mM K-Pi. The proteins were eluted with 60 ml linear K-Pi gradient (0.01–0.5 M) in buffer A. The active fractions were concentrated by Centricon (Amicon).

Step 3. Superose 6 chromatography. Conc. fractions from step 2 were further purified on a FPLC Superose 6 HR 10/30 (Pharmacia) column equilibrated with buffer A containing 0.15 M NaCl. Proteins were eluted with the same buffer. The fractions with ATP-dependent protease activity were used for characterization.

Assay of protease. Proteolytic activity was measured with [^{14}C]caseine as a substrate [29]. Reaction mixture contained 150 μl of mix (50 mM Tris-HCl, pH 7.9, 10 mM $MgSO_4$, 2.7 μg of [^{14}C]casein, 25800 cpm/μg of [^{14}C]casein), ± 5 μl 0.1 M ATP (neutralized to pH 7.5) and 50–100 μl of enzyme. After incubation at 37°, 30–60 min the reaction was terminated by 40 μl of 10% BSA and 220 μl 10% TCA. After centrifugation 300 μl of supernatant was transferred to 2.5 ml scintillation cocktail [38] for counting on LKB-scintillator. The enzyme activity is expressed in μg of casein hydrolysed by 1 mg protein per 1 hr.

Inhibition assays. We used 0.1 mM TLCK (*N*-α-*p*-tosyl-L-lysine chloromethyl ketone), 0.1 mM TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone), 1 mM PMSF (phenylmethylsulphonyl fluoride), 1 mM NEM (*N*-ethylmaleimide) and 5 mM vanadate for testing their inhibition effect on protease activity. For these experiments the enzyme obtained after gel filtration on Superose 6 was used. The enzyme was preincubated with various inhibitors (Table 2) in assay buffer for 10 min at 4°. Control containing no inhibitor was treated the same way. The reaction was started by the addition of ATP and [^{14}C]casein. Subsequently the samples were treated as described for the standard assay.

pH optimum was estimated in the same reaction condition as the protease assay using different buffers – MES (pH 5.4–6.8), Tris (pH 7.0–8.4), borate (pH 8.6–9.8).

Peptidase assay. Peptidase activity was estimated by cleavage of the synthetic fluorogenic peptides Glu-Ala-Ala-Phe-MNA (GAAP-MNA) and Suc-Leu-Tyr-AMC (SLT-AMC) (Bachem, Switzerland). The reaction mixture contained 175 μl of mix (50 mM Tris-HCl, pH = 7.9, 10 mM $MgSO_4$, 2,5 mM GAAP-MNA/or SLT-AMC), ± 5 μl 0.1 M ATP and 100 μl of protease. After 30–60 min incubation at 37° the reaction was stopped by 1 ml of 1% SDS in 0.1 M Na borate, pH 9.1 and the fluorescence was measured with a Perkin-Elmer 2B fluorometer. The hydrolysis was monitored for GAAP-MNA at 335 nm (extinction), 410 nm (emission) and for SLT-AMC at 380 nm (extinction), 440 nm (emission). Peptidolytic activity is expressed in nmol MNA or AMC (highly fluorescent moiety; AMC, 7-amido-4-methylcoumarin MNA, methoxynaphthylamine) released per hr.

Miscellanous. Protein estimation [39], PAGE in 10% acrylamide gels [40] and Western blotting [41] were done according to published procedures. The immune complex in Western blots were detected by the alkaline phosphatase reaction (ProtoBlot Promega).

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