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Protease activities in the chloroplast capable of cleaving an LHCII N-terminal peptide

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Two protease activities of pea chloroplasts, one located in the stroma and the other associated to the thylakoid membrane, are described. Both proteases catalyse the endo-proteolytic cleavage of a peptide corresponding to the N-terminal loop and the first turn in helix-B of light-harvesting complex II (Lhcb1 from pea). The stromal protease cleaves preferentially on the carboxy-side of glutamic acid residues. Inhibitor studies indicate that this protease is a serine-type protease. The protease was partially purified and could be correlated to a 95-kDa polypeptide band on SDS-polyacrylamide gels. The 95 kDa protein was partially sequenced and showed similarity to an 'unknown protein' from *A. thaliana* (in the NCBI

public database) as well as to a glutamyl endopeptidase purified from crude extract of cucumber leaves. It is concluded that the stromal protease is a chloroplast glutamyl endopeptidase (cGEP). The protease localized in the thylakoid membrane, cleaved the peptide at only one site, close to its N terminus. The activity of the thylakoid-associated protease was found to be drastically increased in the presence of the reducing agent 1,4-dithiothreitol. Inhibitor studies suggest that this protease is a cysteine- or serine-type protease. The possible roles of these proteases in the regulation of photosynthetic electron transport and in the chloroplast homeostasis are discussed.

Introduction

Proteolysis is involved in a multitude of cellular events. With a single proteolytic cleavage, proteases can activate or inactivate regulatory enzymes. A more extensive proteolytic digestion can completely remove certain proteins. The complete digestion of key regulatory enzymes is an important strategy to control enzymatic pathways. Proteolysis is also employed for the removal of miss-targeted, miss-folded, or damaged proteins as well as for maintaining correct stoichiometry of multi-subunit complexes by digesting proteins which are in excess.

Several reports on protein degradation in the chloroplasts of plants have been presented, including degradation of unassembled proteins (Ostersetzer and Adam

1997) and apoproteins lacking their chlorophyll molecules (Bennett 1981, Mullet et al. 1990). Of special importance for chloroplast homeostasis is the activities of proteases in the regulation and maintenance of photosynthetic electron transport and its components. Excess light can lead to inhibition of photosynthetic electron transport due to damage of its components. For example, in order to avoid photoinhibition, the co-ordination of synthesis and proteolysis is important in the turnover of photodamaged D1 protein of the photosystem II reaction centre, reviewed in (Aro et al. 1993, Andersson and Aro 1997, Adir et al. 2003). Other proteins, such as PsbW, have also been shown to be degraded in the chloroplasts. PsbW was shown to be

Abbreviations – cGEP, chloroplast glutamyl endopeptidase; DTT, 1,4-dithiothreitol; LHCII, light harvesting complex II; MALDI, matrix assisted laser desorption/ionization; MS, mass spectrometry; MS-MS, tandem mass spectrometry; TFA, trifluoroacetic acid; TOF, time-of-flight.

degraded in a light-dependent manner with similar rates and to almost the same extent as the D1 protein (Hagman et al. 1995, Hagman et al. 1997). Another example of the importance of proteolysis in chloroplasts, is the acclimative degradation of LHCII in response to increased light intensities (Lindahl et al. 1995).

Chloroplasts contain their own set of proteolytic enzymes and the presence of several protease activities and proteases localized in this organelle have been reported, reviewed in (Adam 2001, Adam and Clarke 2002). Many of the proteases in the chloroplast have bacterial homologues, such as the serine-type proteases Clp (Shanklin et al. 1995, Sokolenko et al. 1998), DegP (Itzhaki et al. 1998, Haußühl et al. 2001) and SppA (Lensch et al. 2001), and the metallo-protease FtsH (Lindahl et al. 1996, Chen et al. 2000). DegP2 has from in vitro experiments been shown to carry out the initial cleavage of the D1 protein (Haußühl et al. 2001) and FtsH1 has previously been shown to in vitro be able to carry out the degradation of the 23-kDa cleavage product, arising from the initial cleavage of photo-damaged D1 protein (Lindahl et al. 2000). FtsH2 (Var2) has however, recently been demonstrated to be required for cleavage and turnover of photodamaged D1 protein in vivo (Bailey et al. 2002). In *Escherichia coli*, Clp proteases have been shown to be involved in chaperone activity as well as the degradation of large polypeptides in an ATP-dependent manner (Katayama-Fujimura et al. 1987, Hwang et al. 1988, Wickner et al. 1994). The Clp proteases in the chloroplasts are also ATP dependent, probably degrading miss-targeted, unassembled, and miss-folded proteins (Halperin and Adam 1996, Majeran et al. 2000, Halperin et al. 2001).

In order to identify proteases in the chloroplast, in particular those potentially active on LHCII, we have used an overexpressed and purified peptide corresponding to the N-terminal loop and the first turn in helix-B of LHCII (Lhcb1 from pea) as a substrate for proteolysis. We here describe two chloroplastic proteases involved in the proteolysis of this LHCII peptide and the specific breakdown products they create.

Materials and methods

Plant material

Peas (*Pisum sativum* L. cv Oregon sugar pod) were grown for 12 to 14 days on vermiculite in a growth chamber at 20°C with a light intensity of 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 12 h dark/12 h light cycle. Chloroplast and thylakoid isolation was carried out as described below [essentially according to (Walker 1971) with some modifications]. Pea leaves were homogenized in ice-cold grinding medium (0.33 M sorbitol, 50 mM HEPES pH 7.6, 5 mM MgCl_2 , 10 mM NaCl, 2 mM EDTA and 10 mM ascorbate). The homogenate was filtered through four layers of nylon filter (20 μm pore size) and intact chloroplasts were isolated by centrifugation of the filtrate for 20 s at 3700 $\times g$ at 4°C. The intact chloroplasts were washed

once and re-suspended in the same medium. Broken chloroplasts were obtained by rigorous vortexing of the intact chloroplasts in the presence of glass beads (425–600 μm), just prior to protease assays or purification. Thylakoid membranes were prepared by lysing intact chloroplasts in lysis medium (10 mM HEPES pH 7.6, 5 mM MgCl_2) at room temperature. The membranes were pelleted by centrifugation for 2 min at 8000 $\times g$ at 4°C, washed once with lysis medium and finally re-suspended in grinding medium. Chlorophyll concentrations were estimated by the method of (Arnon 1949).

Partial purification of the stroma-localized protease

After breaking isolated intact chloroplasts by vortexing in the presence of glass beads, the suspension was centrifuged at 10 000 $\times g$ for 8 min. The supernatant was run through centrifugal molecular weight cut-off filter (Microsep 300K; Pall Gelman Sciences, East Hills, NY). The flow-through was filtered through a 0.2- μm filter and fractionated using a HiLoad Superdex[®] 200 (16/60) column (Amersham Biosciences, Uppsala, Sweden). Fractions containing protease activity towards the N terminus of LHCII were pooled and further purified on a MonoQ HR 5/5 anion exchange column (Pharmacia Biotech). Elution was performed with a linear gradient of 5–285 mM NaCl over 27 ml elution volume. Collected fractions were assayed for protease activity as described below and analysed by SDS-PAGE.

Overexpression and purification of the LHCII N terminus

Plasmid construction

To express amino acids 2–71 of the N terminus of pea-LHC II (Lhcb1/AB80) its corresponding cDNA was amplified by polymerase chain reaction. The PCR was carried out in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl_2 , 0.1% Triton X-100, 0.25 μg of each primer, cDNA corresponding to 0.4 μg total RNA (cDNA was synthesized by reverse transcription from total RNA), 200 μM of each dATP, dCTP, dTTP and dGTP, and 2.5 units of Taq polymerase (Promega, Madison, WI) in a total volume of 100 μl (25 cycles, 94°C for 1 min, 52°C for 2 min and 72°C for 1.5 min per cycle). The forward and reverse primers used were: 5'-AGC ATC CGG AAG TCT GCT ACC ACC and 5'-ATG GAA TTC TCA TCT GGA GTG GAT CAC TTC AAG C, respectively. The primers were designed to introduce a *AceIII* site at the 5'-end and an *EcoRI* site at the 3'-end, of the coding sequence to allow subcloning into the glutathione S-transferase gene fusion expression vector – pGEX 3X (Pharmacia Biotech). Calcium-mediated transformation (Cohen et al. 1972) was used to transform the ligation product into *E. coli* HB101. Transformed cells containing the insert were detected by screening of the transformants using a [³²P]-labelled specific oligonucleotide complementary to the fusion junction and confirmation of the sequence was achieved by sequencing of the insert in both directions. For optimal expression of the LHCII N

terminus, the recombinant plasmid was purified on a Wizard column (Promega) according to the manufacturer's instructions and transformed into *E. coli* BL21 by electroporation (Bio-Rad Gene Pulser; 25 μ F; 2500 V; 200 Ω ; 0.2 cm cuvette; Bio-Rad, Hercules, CA).

Expression and purification

LB-medium (500 ml) containing ampicillin (100 μ g ml⁻¹) was inoculated with a 5-ml overnight culture of recombinant cells. After the cells were grown for 2–3 h at 37°C (125 r.p.m) isopropyl-b-D-thiogalactopyranosid (IPTG) to a final concentration of 0.08 mM was added. The cells were grown for another 3–5 h before being harvested by centrifugation (10 000 \times *g* for 7 min). The harvested cells were re-suspended in PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.3) containing 1% (v/v) Triton X-100 and were sonicated on ice by four pulses of 10 s (high voltage; Amp 2), with 1 min intervals, using a 150-W Ultrasonic Disintegrator (MSE, London, UK). The sonicated material was then centrifuged again as above. The GST-fusion protein was isolated from the resulting supernatant using Glutathione Sepharose[®] 4B (Pharmacia Biotech) according to the manufacturer's instructions. The GST-fusion protein was further purified by gel filtration on a Superose[®] 12 column (Pharmacia Biotech). The fusion protein was then cleaved with Bovine Factor Xa (Haematologic Technologies Inc., Essex Jct, VT) in a buffer containing 20 mM Tris-HCl (pH 8.0) and 100 mM NaCl. The resulting cleavage products (the LHCII N terminus and the GST-tag) were separated by gel filtration using a Sephacryl[®] S-200 HR column (Pharmacia Biotech). All gel filtrations were operated with an FPLC[®] system (Pharmacia Biotech).

The sequence of the overexpressed and purified LHCII N-terminal peptide was KSATTKKVAS SGSPWYGPD R VKYLGPFSGE SPSYLTGEFP GDYGWDTAGL SADPETFSKN RELEVIHSR.

Protease assays

Isolated thylakoid membranes, freshly broken chloroplasts, or intact chloroplasts were incubated, with or without the over-expressed LHCII N terminus, for 20 min in the dark (low intensity green safe light) at 37°C, in protease assay medium containing 0.2 M Sorbitol, 50 mM HEPES (pH 7.6) and MgCl₂, NaCl, and NH₄Cl, all at 5 mM. The samples were then centrifuged at 10 000 \times *g* for 5 min and protease activity was stopped by addition of 1.5 volumes of 0.5% TFA to the supernatants and cooled to 4°C. Proteolysis was detected by reduction of the original LHCII N-terminal peptide and the concomitant occurrence of its breakdown products using reversed-phase HPLC. Essentially the same protocol was followed when assaying proteolytic activity of the partially purified stromal protease.

Protease inhibitor studies

In order to investigate the effects of various protease inhibitors on the chloroplast proteases, isolated thylakoids or the partially purified protease was pre-incubated in the presence of protease inhibitors for 30 min at 4°C in protease assay medium (described above). The protease substrate, overexpressed LHCII N terminus, was then added. Protease assays and further sample processing was carried out as described above. The inhibitors used were antipain (50 μ g ml⁻¹), bestatin (40 μ g ml⁻¹), E-64 (10 μ g ml⁻¹), leupeptin (5 μ g ml⁻¹), pepstatin (0.7 μ g ml⁻¹), phosphoramidon (330 μ g ml⁻¹), Pefabloc SC (1 mg ml⁻¹), EDTA-Na₂ (0.5 mg ml⁻¹), aprotinin (2 μ g ml⁻¹), and Complete (1/125 tablet). As control, sample incubation was carried out with no inhibitor present. All inhibitors were from Roche Diagnostics (Mannheim, Germany) and used according to their recommendations.

Light, redox (DTT), ATP and GTP dependency studies

Incubations of recombinant LHCII N terminus with thylakoid membranes or partially purified protease was as above but in the presence of either 10 mM DTT, 200 μ M ATP, or GTP. For the light-dependency studies, recombinant LHCII N terminus was incubated with thylakoids and treated as above in white light (80 μ mol m⁻² s⁻¹). All the other thylakoid samples were incubated in the dark.

Reversed phase HPLC

Reversed phase HPLC was performed using a system from Waters (Waters 600 controller; Waters 996 photodiode array detector; Waters 717 autosampler; Waters, Milford, MA). Proteins were separated on a C18-column (218TP; Vydac, Hesperia, CA) at a flow of 1 ml min⁻¹ with a mobile phase consisting of acetonitrile in water containing 0.08–0.1% (v/v) TFA. For analysis of protease assays, peptides were separated with a linear gradient from 21 to 48% (v/v) acetonitrile in 11 ml elution volume. For the purification of tryptic fragments or LHCII N terminus breakdown products, a more shallow elution gradient of 4–70% (v/v) acetonitrile in 50 ml elution volume, was used.

SDS-PAGE

SDS-PAGE was carried out using the Bio-Rad Criterion PAGE-system. The gels used were either 4–20% gradient gels or 7.5% gels. Electrophoresis was carried out according to the manufacturers' instructions, essentially in agreement with (Laemmli 1970). Visualization of the proteins was made by staining with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO) or by silver staining (Bio-Rad).

MALDI-TOF MS, MS-MS and N-terminal sequencing

After protease assays the samples were applied and run on reversed phase HPLC as described above. Degradation

products, eluted from the HPLC, were collected for analysis by MALDI-TOF MS. The analysis was carried out with a Reflex III or with a Reflex IV mass spectrometer from Bruker (Shevchenko et al. 1996, Pandey et al. 2000). Peptides with masses lower than 3 kDa were analysed using alpha-cyano-4-hydroxy-cinnamic acid as a matrix, and for peptides with higher masses sinapinic acid was used as a matrix (Kusmann et al. 1997).

N-terminal micro sequencing was carried out with a Procise sequencer from Applied Biosystems. Proteins were sequenced from a polyvinylidene difluoride membrane (Prosorb, Applied Biosystems) essentially as described by (Matsudaira 1987).

After partial purification and SDS-PAGE, the stroma-localized protease was excised from the Coomassie-stained gel and subjected to in gel trypsination essentially as described by (Shevchenko et al. 1996). The trypsin used was sequencing grade modified trypsin (Promega; V5111). Tryptic peptides were sequenced by MS-MS using a Q-TOF mass spectrometer (Micromass, Manchester, UK).

Results

In an attempt to detect proteolytic activities against LHCII, a peptide substrate (recombinant peptide corresponding to the LHCII N terminus) was incubated with broken chloroplasts. The peptide was degraded and proteolytic fragments could be detected by reversed phase HPLC (data not shown). In order to localize and further investigate this degradation activity, intact chloroplasts were isolated and thylakoid membranes separated from the stroma. As shown in Fig. 1 and Table 1 distinctly different degradation pattern was seen when the peptide was incubated with stroma (Fig. 1b) and when the peptide was incubated with thylakoids (Fig. 1c). Only low activity was found when chloroplasts were kept intact (Table 1). Thus these activities appear to originate from the chloroplast and not from cytosolic contamination. The low activity seen with intact chloroplasts was probably due to breakage of chloroplasts during the final re-suspension and during the protease assays.

The breakdown products of the LHCII N-terminal peptide resolved by reversed phase HPLC were analysed with MALDI-TOF MS and N-terminal sequencing. The main fragments obtained by treatment with the stroma-localized protease showed that the protease is an endoprotease, cleaving its substrate on the carboxy-side of glutamic acid residues (Table 2).

The stroma-localized protease was partially purified from intact chloroplasts using a combination of a molecular weight cut off filter, gel filtration chromatography, and ion exchange chromatography. A substantial amount of Rubisco, the dominating protein in the stroma, was removed by the use of a 300 K molecular weight cut off filter. In the gel filtration the protease eluted at a position corresponding to 130 kDa. The protease eluted from the anion exchange column at a NaCl

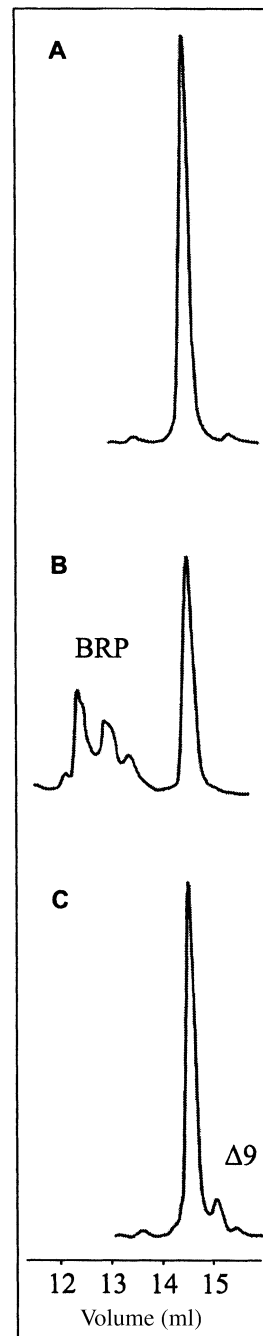


Fig. 1. HPLC separation of LHCII N-terminal peptide and proteolytic breakdown products. The HPLC chromatogram shows (A) the LHCII N-terminal peptide; (B) LHCII N-terminal peptide and breakdown products (BRP), products from incubation of the peptide with the partly purified stromal protease; (C) LHCII N-terminal peptide and the breakdown product $\Delta 9$, the peptide lacking the 9 N-terminal amino acids, obtained when incubating the peptide with washed thylakoids.

concentration of about 180 mM. To identify the protease, the protease activity of individual fractions was compared with the protein patterns of the corresponding fractions, after separation by SDS-PAGE and Coomassie or silver-staining of the gels. In this way we could

Table 1. Localization of the two proteases. The LHCII N-terminal peptide was incubated with different chloroplast fractions whereafter the peptide and the breakdown products were separated by HPLC, all as described in 'Materials and Methods'. The value for each peak in the table is expressed as the percentage of total peak area.

	LHCII N-terminal	BRP	$\Delta 9$
Peptide only (LHCII N-terminal)	97	0	0
Intact chloroplasts + peptide	85	10	0
Broken chloroplasts + peptide	38	53	0
Stroma + peptide	23	73	0
Thylakoids + peptide	90	0	9

correlate the protease activity to the presence of a 95-kDa band. No other protein with the same elution profile was detected (Fig. 2). N-terminal sequencing attempts have so far failed, indicating that the protease has a blocked N-terminus, a feature of many chloroplast proteins. However, MS-MS sequencing of several peptides obtained after SDS-PAGE and *in gel* digestion of the excised 95 kDa band was successful. The sequenced peptides showed high similarity to sequences in a protein of *A. thaliana* (Fig. 3). The *A. thaliana* protein (locus ID: At2g47390) showed similarity to several proteases in the NCBI public database. According to CHLORO P, TARGET P, PREDOTAR and P-SORT, computer programs for prediction of subcellular localization of proteins (Emanuelsson et al. 1999, Nakai and Horton 1999, Emanuelsson et al. 2000), the *A. thaliana* protein is predicted to be targeted to the chloroplast, chloroplast, mitochondria and mitochondria/chloroplast, respectively. The calculated theoretical molecular weight of the mature form of the *A. thaliana* protein, 99 kDa, corresponds well with the 95 kDa band in SDS-PAGE, observed for the pea stromal protease described herein.

In order to further characterize the protease, the proteolytic activity was measured in the presence of different

Table 2. Identification of proteolytic breakdown products of the N-terminal Lhcb1 peptide by cGEP (stroma localized protease). Analyses were carried out by MALDI-TOF mass spectrometry (MS) and/or with N-terminal sequencing (Edman). The mass values given are the experimental masses (monoisotopic, $[M+H]^+$) of the breakdown products.

Position in N-ter	Sequence	Mass	Method
1-30	NH ₂ -KSATTKKVAS SGSPWYGPDR VKYLGPFSGE	3200.8	MS/Edman
1-38	NH ₂ -KSATTKKVAS SGSPWYGPDR VKYLGPFSGE SPSYLTGE	4035.0	MS
31-62	(E)SPSYLTGEFP GDYGWDTAGL SADPETFSKN RE	3494.8	MS
39-55	(E)FPGDYGWDTA GLSADPE	-	Edman
39-62	(E)FPGDYGWDTA GLSADPETFS KNRE	2660.3	MS/Edman
56-69	(E)TFSKNRELEV IHSR-COOH	1715.9	MS/Edman

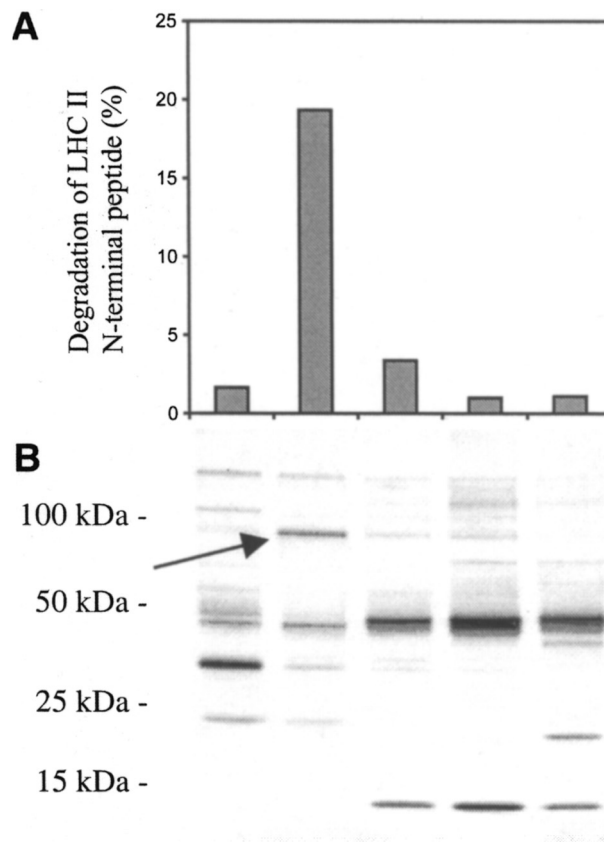


Fig. 2. Partial purification of the stromal protease. The stromal protease was partially purified using a combination of molecular weight cut-off filters, gel filtration chromatography and finally ion exchange chromatography. All fractions were assayed for protease activity and protein content was analysed by SDS-PAGE. (A) Five fractions, eluted at around 180 mM NaCl in the ion exchange chromatography, were shown to contain protease activity. The protease activity in these fractions are expressed as percentage, of the LHCII N-terminal peptide degraded. (B) Silver-stained polyacrylamide gel of proteins in these five fractions containing protease activity. A protein migrating in SDS-PAGE with an apparent molecular weight of 95 kDa (indicated with an arrow) was correlated to the protease activity in gel filtration and in ion exchange chromatography.

protease inhibitors. Maximum inhibition was obtained with serine-type protease inhibitors such as Pefabloc SC and antipain (Table 3). The protease activity was neither ATP-dependent nor GTP-dependent and was not affected by the presence of the reducing agent DTT (results not shown).

Another protease activity was observed in the thylakoid fraction (Fig. 1C), using the same peptide substrate (recombinant peptide corresponding to the LHCII N terminus). The degradation product in this case was slightly retarded in the reversed phase HPLC compared with the original peptide. This protease activity was activated about four-fold in the presence of 10 mM DTT, but unaffected by ATP, GTP or light (results not shown). Analysis of the only breakdown product found, by N-terminal sequencing and MALDI-TOF

Identities = 14/15 (93%), Positives = 15/15 (99%)

P. sativum - cGEP: VDAPPVPALTFSPHR
 VDAPPVPAL+FSFPHR
A. thaliana - NP_850473: 121 VDAPPVPALSFSFPHR 135

Identities = 8/10 (80%), Positives = 8/10 (80%)

P. sativum - cGEP: NFVTWSPDVK
 NFVTWS D K
A. thaliana - NP_850473: 207 NFVTWSNDGK 216

Identities = 8/9 (88%), Positives = 8/9 (88%)

P. sativum - cGEP: ADVETGNAR
 ADVETG AR
A. thaliana - NP_850473: 238 ADVETGVAR 246

Identities = 12/19 (63%), Positives = 13/19 (68%), Gaps = 6/19 (31%)

P. sativum - cGEP: VTNF---YAMEPQLASLQK
 +TNF Y PQLASLQK
A. thaliana - NP_850473: 638 ITNFPHPY---PQLASLQK 653

Identities = 7/9 (77%), Positives = 9/9 (99%)

P. sativum - cGEP: LVLLPYESH
 LV+LP+ESH
A. thaliana - NP_850473: 881 LVVLPHEHSH 889

Fig.3. Internal amino acid sequence alignment of the pea chloroplast localised glutamyl endopeptidase (cGEP) and its homologue in *A. thaliana* (NP_850473). Internal sequences from the stromal protease (cGEP) were obtained by MS-MS of tryptic fragments.

MS revealed that the protease cleaves the peptide once, namely after the second A in the sequence KSATTKK-VASSG (i.e. after residue 9 in the peptide). This protease activity appears to be primarily associated with the thylakoid membranes, since protease activity was only partially removed from the thylakoid membranes after washes with either 200 mM KCl or 200 mM (NH₄)₂SO₄ (results not shown). Also after treatments with Triton X-100 in (final) concentrations ranging from 0.1 to 1% (w/v) substantial amounts of the protease activity remained associated with the thylakoid membranes (results not shown). The activity of this protease was inhibited by addition of several inhibitors of serine/cysteine-type proteases. E-64, an inhibitor of cysteine-type proteases almost completely inhibited this protease activity (Table 3).

Discussion

The N-terminus of LHCII is important for its proteolysis (Yang et al. 2000). Using a peptide corresponding to the pea Lhcb1 N terminus as a substrate we found two

Table 3. Protease inhibitor studies. Thylakoid membranes were treated with various protease inhibitors and subsequently incubated with the LHCII N-terminal peptide in protease assays. The peptide and the breakdown products were separated by HPLC. In the column 'Thylakoid protease' the values shown are based on the occurrence of the Δ9 breakdown product. In the column 'Stromal protease' the values shown are based on the disappearance of the substrate. All values are normalized to the value for the control, in which no inhibitor was present.

Inhibitors	Thylakoid protease inhibition (%)	Stromal protease inhibition (%)
Control	0	0
Antipain	93	87
Bestatin	0	26
E-64	93	50
Leupeptin	92	15
Pepstatin	0	15
Phosphoramidon	25	0
Pefabloc SC	100	100
EDTA-Na ₂	0	30
Aprotinin	18	37
Complete	81	80

endoprotease activities in the chloroplast, one in the stroma and one associated with the thylakoid membrane. The stroma protease was purified at least 100-fold and the activity was correlated with the most abundant protein in the fraction. In SDS-PAGE this protein had an apparent molecular weight of 95 kDa. Amino acid sequence obtained from tryptic fragments of this protein showed significant sequence similarity to an 'unknown protein' of *A. thaliana* (locus ID: At2g47390) in the NCBI public database. The *A. thaliana* sequence showed similarity to several proteases in the NCBI public database and is annotated as a glutamyl endopeptidase (GEP) in the MEROPS database (Barrett et al. 2001). Glutamyl endopeptidases are serine-type proteases of the S9 family (Barrett and Rawlings 1995) with a Ser/Asp/His catalytic triad. Our inhibitor studies indicate that the pea chloroplast stroma localized protease is a serine-type protease and sequence comparisons indicate that the active-site serine in the *A. thaliana* sequence is Ser⁷⁸¹ and the catalytic triad, Ser⁷⁸¹/Asp⁸⁵⁴/His⁸⁷⁹ (see also MEROPS database – <http://merops.sanger.ac.uk>). Glutamyl endopeptidases are known to cleave their substrate on the carboxy-side of glutamic acid residues as the chloroplast stroma protease described in the present study.

We conclude that the soluble protease, active against the LHCII N-terminal, found in our work is a chloroplast glutamyl endopeptidase (cGEP). We also suggest that the unannotated homologue in *A. thaliana* also is a cGEP.

A glutamyl endopeptidase from the stroma of spinach chloroplasts has previously been partially purified (Laing and Christeller 1997). The authors could not then determine which of the Coomassie-stained proteins, one major (112 kDa) and three minor (90, 51 and 34 kDa), in the SDS-polyacrylamide gel that was the glutamyl endopeptidase. Most consistent with our findings would be that the 90 kDa band in their experiments was the

cGEP. Yamauchi et al. (2001) have recently purified a plant glutamyl endopeptidase from crude extracts of cucumber leaves. An internal amino acid sequence from this protein showed high similarity to the same *A. thaliana* sequence as the pea cGEP did. We suggest that the chloroplast is the *in vivo* subcellular localization of the cucumber GEP purified by Yamauchi et al. (2001).

A BLAST search through the annotated Arabidopsis genome at TIGR (available on-line at <http://www.tigr.org>) produced the At4g14750 and At5g36210 gene products that shared a moderate sequence similarity with the C-terminal domain of the At2g47390 gene product. A BLAST search through the current version of the rice genome at TIGR revealed the presence of three highly homologous sequences, which indicates that homologues of the At2g47390 gene product are a common feature in higher plants.

Although cGEP is active on the LHCII N-terminal peptide, attempts to degrade native LHCII with cGEP has so far failed. This suggests that the structure or availability of the substrate is a determining factor for protease activity *in vivo*. However, an *A. thaliana* mutant (*cchl1*) showed, in difference to wild type, a loss of Lhcb proteins when transferred from low-light conditions to moderate-light conditions (Harper et al. 2004). The *cchl1* mutant was used in an EST micro-array study in the search for genes that show different expression in comparison with wild type, when transferred from low-light conditions to moderate-light conditions. Gene products of the genes that are differently expressed are potentially involved in the disassembly of LHC (Arabidopsis Information Resource (TAIR) on <http://www.arabidopsis.org>; Submission number: 35, Experimenter: Judy Brusslan). The gene (At2g47390) encoding cGEP was found to be roughly 2.5-fold higher expressed in the *cchl1* mutant compared to wild type. This suggests that cGEP might, *in vivo*, be involved in the degradation of Lhcb proteins.

In gel filtration chromatography, the pea cGEP eluted with an apparent molecular weight of 130 kDa. This could indicate that the native protease is a heterodimer, or that the protease behaves in a non-ideal manner during the gel filtration. The apparent molecular weight of 130 kDa in gel filtration of the pea cGEP is at difference with the results obtained in the studies of spinach cGEP and cucumber GEP. In these studies it was reported that the protease eluted with an apparent molecular weight of 350–380 kDa and 400 kDa, respectively, indicating that the native protease is a homotetramer (Laing and Christeller 1997, Yamauchi et al. 2001).

The protease activity found associated to the thylakoid membrane is probably a cysteine-type protease as it was inhibited both by inhibitors of serine/cysteine-type proteases and by E-64, which is a specific inhibitor of cysteine-type proteases. The protease activity was drastically increased in the presence of the reducing agent DTT and is concluded to be ATP- and GTP-independent as its activity was not affected by the addition of any of these

nucleotides. The energy seems, however, to be used for unfolding of the substrate and to be strictly necessary for degradation of larger proteins. Peptides and smaller disordered proteins can be cleaved by for example Lon and Clp, without ATP hydrolysis, but at a lower rate (Gottesman and Maurizi 1992, van Melderen and Gottesman 1999). The activity of this protease is probably not regulated directly by the redox state of the plastoquinone pool, since illumination by white light ($80 \mu\text{mol s}^{-1} \text{m}^{-2}$) did not affect the protease activity. In the absence of an electron acceptor, the thylakoid plastoquinone pool is assumed to be reduced under these light conditions.

In the thylakoid membrane, isoforms of FtsH (Lindahl et al. 1996, Chen et al. 2000) and DegP (Itzhaki et al. 1998, Haußühl et al. 2001) have been found. However, FtsH proteases are ATP-dependent Zn-metallo-proteases. DegP are ATP-independent serine-type proteases but they are not inhibited by inhibitors of cysteine-type proteases and are also not activated by DTT (Lipinska et al. 1990). These differences lead us to conclude that neither of these families of proteases are responsible for the proteolytic cleavage of the peptide substrate that we observe.

A membrane-bound protease from *Chlamydomonas reinhardtii* which showed ability to degrade light-harvesting proteins, not ligated to chlorophylls, has been identified (Hooper and Hughes 1992). Most conventional protease inhibitors do not inhibit this protease, which is at variance with how inhibitors act on the protease described herein. Furthermore, DTT does not affect its activity.

A protease activity peripherally associated with the thylakoid membrane and able to cleave LHCII has been described (Anastassiou and Argyroudi-Akoyunoglou 1995). It is at present not possible to make any conclusions regarding potential connections between that protease and the protease reported here.

With regard to the fact that substantial amounts of the membrane bound protease activity remained associated with the thylakoid membranes after Triton X-100 treatment it is interesting to note that protease activity co-purifying with LHCII, isolated even from Triton X-100 solubilized thylakoids, recently has been reported. The protease is suggested to be part of the PSII super-complex and is capable of degrading LHCII as well as the D1 and D2 proteins (Georgakopoulos et al. 2002).

It has been shown that the acclimative degradation of LHCII under high light is carried out by a serine- or cysteine-type protease and that this protease activity is strictly ATP dependent (Lindahl et al. 1995). The effects of inhibitors on the acclimative degradation of LHCII (Lindahl et al. 1995) and on the membrane-associated proteolytic activity we report here are similar. The differences in ATP dependency could be explained if the ATP required for the acclimative degradation of LHCII, is used to activate the substrate (Yang et al. 1998). However, the protease involved in the acclimative degradation of LHCII requires induction of expression or activation. This occurs over a period of 2 days in high light (Lindahl et al. 1995, Yang et al. 1998).

Although it is not yet established whether LHCII is the physiological substrate for the two proteases reported here, it is reasonable to assume that LHCII can be the target under specific circumstances. Of special interest in this respect are the recent results of Stauber et al. (2003). By a proteomic approach on light-harvesting proteins of *Chlamydomonas reinhardtii* they found N-terminal fragments (SSGIEFYGPNR and SSGVEFYGPNR) of the gene products of lhcbm3 (LhcII-1.3) and lhcbm6 (Lhcb1, CabII-1), respectively, which was concluded to be the result of an endogenous protease. The cleavage site in that study was exactly the same as that found for the thylakoid bound protease in our study, thus supporting the view that the protease may well be active against LHC *in vivo*. What could the physiological role of the thylakoid-associated protease be? The removal of the N-terminus might be one of the initial steps of adaptation to high light. Under strongly reducing conditions the protease become active, participate in degradation of LHC and thus reduce the antenna system. It is also notable that the N-terminal part of LHC removed by the protease, carries the regulatory phosphorylation site(s) required for state transitions. Proteolytic removal of the phosphorylation site would be an efficient way to abolish state transitions and ensures that LHCII remains associated with photosystem II. The removal of the N-terminal part of Lhcb1 makes the protein similar in sequence to Lhcb3, an LHC species constantly associated with photosystem II (Jansson 1994). This would direct photo-induced damage to photosystem II, for which an efficient repair system exists, and relieve photosystem I of some excitation pressure.

Conclusive identification and elucidation of the physiological role of this protease is currently under study.

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