

Processing of a Wheat Light-Harvesting Chlorophyll a/b Protein Precursor by a Soluble Enzyme from Higher Plant Chloroplasts

Gayle K. Lamppa and Mark S. Abad

Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

Abstract. A processing activity has been identified in higher plant chloroplasts that cleaves the precursor of the light-harvesting chlorophyll a/b-binding protein (LHCP). A wheat LHCP gene previously characterized (Lamppa, G. K., G. Morelli, and N.-H. Chua, 1985. *Mol. Cell Biol.* 5:1370-1378) was used to synthesize RNA and subsequently the labeled precursor polypeptide in vitro. Incubation of the LHCP precursors with a soluble extract from lysed chloroplasts, after removal of the thylakoids and membrane vesicles, resulted in the release of a single 25-kD peptide. In contrast, when the LHCP precursors were used in an import reaction with intact pea or wheat chloroplasts,

two forms (25 and 26 kD) of mature LHCP were found. The peptide released by the processing activity in the organelle-free assay comigrated with the lower molecular mass form of mature LHCP produced during import. Properties of the processing activity suggest that it is an endopeptidase. Chloroplasts from both pea and wheat, two divergent higher plants, contain the processing enzyme, suggesting its physiological importance in LHCP assembly into the thylakoids. We discuss the implications of LHCP precursor processing by a soluble enzyme that may be in the stromal compartment.

THE development of the chloroplast from an undifferentiated proplastid requires the cooperation of the nuclear and chloroplast genomes. Although the chloroplast contains its own DNA (23), and there can be thousands of copies per cell (26), most of its proteins are encoded by the nuclear genome and posttranslationally imported (8, 14, 32, 38, 41; for review see reference 37). The nuclear-encoded proteins localized to the stroma and thylakoids are synthesized as precursor polypeptides, and are processed to their mature, functional forms within the organelle.

We are investigating the import and processing of the precursor for the light-harvesting chlorophyll a/b-binding protein (LHCP),¹ an abundant thylakoid membrane protein (14, 38, 47). LHCP plays a major role in gathering and distributing light energy between photosystems I and II, and thereby influences overall photosynthetic efficiency under different light regimes (4, 44). LHCP is encoded by a multigene family in higher plants (12, 16, 27, 45) and is synthesized with a 34-37-amino acid NH₂-terminal extension (6, 7, 16, 27, 29, 45), a transit peptide which is cleaved from the mature protein at some point during its incorporation into the thylakoids and binding of chlorophylls a and b (14, 38).

Our current understanding of the processing enzymes involved in the maturation of the large number of proteins imported into the chloroplast is limited, although proteases play an essential role in the assembly of the organelle and the

reactions it carries out. One type of chloroplast processing enzyme has been partially purified from pea (35, 36) and *Chlamydomonas* (39); it processes the precursor of the small subunit of ribulose 1,5-bisphosphate carboxylase (pre-rbcS) in two steps (31). It is not known if the same enzyme processes the LHCP precursor. The partially purified enzyme cleaves the precursor of plastocyanin to an intermediate form that is then cleaved by a thylakoid protease releasing the mature protein (19). The pre-rbcS protease has not yet been purified to homogeneity, however, and a different enzyme may be responsible for processing pre-plastocyanin to the intermediate form. The amino acids at the junction of the transit and mature peptides are not the same for the various proteins imported into the chloroplast (for review see reference 39) suggesting that proteases may be precursor specific. On the other hand, a common amino acid framework of a number of transit peptides has been identified (21) that may contain critical information for processing. In support of this possibility is the fact that upon import, transit peptides are at least partially cleaved from several hybrid proteins (43, 46).

We have initiated a study to isolate the processing enzyme for the LHCP precursor (pre-LHCP). Because the precursor does not accumulate in vivo (14, 38), we have placed a wheat LHCP gene, previously characterized and shown to be active in wheat and transgenic plants (27, 28), downstream of the SP6 promoter (24) to generate RNA for in vitro synthesis of radiolabeled precursor. We show that the in vitro-synthesized wheat LHCP precursor is imported and processed to

1. **Abbreviations used in this paper:** HSM, 50 mM Hepes-KOH, pH 8, 0.33 M sorbitol, 8 mM methionine; LHCP, light-harvesting chlorophyll a/b-binding protein; rbcS, ribulose 1,5-bisphosphate carboxylase.

two forms by both pea and wheat chloroplasts. Furthermore, we demonstrate that both wheat and pea chloroplasts contain a soluble processing enzyme that cleaves the precursor, producing a peptide that comigrates with the lower molecular form of LHCP obtained *in vitro* during uptake of radiolabeled LHCP into isolated chloroplasts.

Materials and Methods

Plant Growth

Peas (*Pisum sativum*, Laxton's Progress) were grown at 26°C under cool white fluorescent lights on a 12-h light/12-h dark daily cycle, and harvested 7 d after germination. Wheat (*Triticum aestivum*, Era) was grown in a green house, and introduced to a controlled environment, as described above, 6 d after germination, 2 d before harvesting.

Chloroplast Isolation

Plants were homogenized with a Polytron at 4°C in 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 50 mM Hepes-KOH, pH 7.5, 0.33 M sorbitol, 5 mM sodium ascorbate, 0.25% BSA at a ratio of 10 vol/g fresh weight tissue. Procedures described by Bartlett et al. (2) were used to purify intact chloroplasts on Percoll gradients.

Plasmid Construction

A wheat LHCP gene, whAB1.6, previously described (27, 28) was subcloned into SP65 by directed ligation using the Bam HI and Hind III sites in the polylinker. The final construct, SP6-whAB1.6, contained ~200 nucleotides between the SP6 promoter and the first AUG of the gene, and 550 nucleotides 3' to the stop codon.

In Vitro Transcription, Translation, and Import

The SP6-whAB1.6 plasmid construct was linearized and transcribed by SP6 polymerase (Promega Biotec, Madison, WI) in the presence of either 500 μM m⁷GpppG or GpppG. No differences in the translation products were observed using either of the two cap structures, or none at all. The RNA generated from a 50-μl reaction was resuspended in 20 μl sterile water, and 1–5 μl (estimated 0.2–1.0 μg) was used in a standard 30-μl reticulocyte (Bethesda Research Laboratories, Bethesda, MD) or wheat germ (Bethesda Research Laboratories or Amersham Corp., Arlington Heights, IL) translation reaction, as prescribed by the vendor, using [³⁵S]methionine (1,000 Ci/mmol) as the labeled amino acid. Procedures for the import reaction were essentially as described (2) and optimized (17, 18). Chloroplasts were resuspended in 50 mM Hepes-KOH, pH 8, 0.33 M sorbitol, 8 mM methionine (HSM) at a concentration of 600–900 μg chlorophyll/ml, and 50 μl was added to a 300-μl sample containing HSM, plus 10 mM ATP, 10 mM MgCl₂, and one translation reaction. The chloroplasts were incubated with the translation products at 26°C with gentle shaking for 45 min, and then were diluted 5–10-fold with HSM, spun at 2,500 g for 10 s, and immediately resuspended in cold 1 mM phenylmethylsulfonyl fluoride (PMSF) for lysis. When the chloroplasts were treated with thermolysin, half of the incubation reaction was mixed with an equal volume of 200 μg/ml thermolysin in HSM, 4 mM CaCl₂. The reaction was stopped by making it 40 mM EGTA, 2 mM PMSF, and diluted threefold with HSM; the chloroplasts were pelleted at 2,500 g for 10 s, and immediately lysed in 1 mM PMSF. To separate the membrane and soluble phases of the chloroplast, the sample was spun for 20 min in a microfuge at 16,000 g. The pellet was resuspended in 0.1 M NaCO₃, 0.1 M DTT, whereas the protein from the supernatant was precipitated with 10% TCA, washed with 80% acetone, and resuspended in the same buffer. The samples were brought to 2% SDS, 12% sucrose, and 0.04% bromophenol blue for gel electrophoresis.

Gel Analysis

The samples were boiled for 1 min, and immediately analyzed on SDS-polyacrylamide gels as described (25, 34). After electrophoresis, gels were stained with Coomassie Blue, destained, and prepared for autoradiography by soaking in DMSO for 1 h, 2,5-diphenyl oxazole/DMSO for 3 h, water for 30 min, and then dried.

Immunoprecipitation

IgG prepared against *Chlamydomonas* LHCP was a gift from Dr. Nam-Hai Chua (The Rockefeller University, New York). The antibody has previously been shown to be LHCP specific and to cross-react with higher plant LHCP (38). Reactions were essentially as described (38). The translation reaction products (5 μl) were boiled in 2% SDS, then raised to 1% Triton X-100, 0.5 M NaCl, 12.5 mM methionine, 10 mM EDTA, and incubated overnight with the antibody. Protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 15 mg/ml to adsorb the antigen-antibody complexes. The samples were spun at 16,000 g for 3 min, washed twice with 0.5 M NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.2% SDS, 1% Triton X-100, and twice with 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% SDS before release of the complexes by incubation in 20 mM Tris-HCl, pH 7.5, 2% SDS at 37° for 15 min.

Organelle-free Processing Assay

Intact chloroplasts were isolated from Percoll gradients according to the method of Bartlett et al. (2), resuspended in HSM, and centrifuged for 1 min at 1,600 g. The pellet was resuspended in 5 mM Hepes-KOH, pH 8, to lyse the chloroplasts. The membrane fraction was pelleted by centrifugation for 10 min at 16,000 g, and resuspended in 5 mM Hepes and assayed for activity; no processing activity was found in the suspension. The supernatant, which we refer to as the soluble fraction, was either immediately used in a processing assay, or stored at –20°C. The assay contained [³⁵S]-methionine-labeled pre-LHCP from a translation reaction, 20 mM Hepes, pH 8, and 2 μg/ml chloramphenicol. The reaction was stopped by bringing the sample to 60 mM DTT, 60 mM sodium carbonate, 12% sucrose, 0.04% bromophenol blue, and 2% SDS, and analyzed by SDS-PAGE as described above. Optimization of the processing activity is the subject of a separate report (Abad, M. S., and G. K. Lamppa, manuscript in preparation). Vesicles were removed from the soluble fraction by centrifugation at 137,000 g in a TLS-55 rotor (Beckman Instruments Inc., Palo Alto, CA) for 1 h.

For ammonium sulfate fractionation, precipitates from three ranges of percent saturation were collected: 0–40, 40–70, and 70–100%. Solid ammonium sulfate was added gradually to the soluble extract at 4°C, and the sample was centrifuged at 10,800 g. The precipitate was dissolved in 5 mM Hepes-KOH, pH 8, and dialyzed. Additional ammonium sulfate was added to the supernatant for the next cut. An aliquot of the resuspended 40–70% ammonium sulfate fraction was spun at 137,000 g to remove particulate matter.

Results

Import of Wheat LHCP Precursors by Pea Chloroplasts

Before initiating a search for a processing enzyme that recognizes the wheat light-harvesting chlorophyll a/b-binding protein precursor (pre-LHCP) it was necessary to synthesize the precursor *in vitro* and demonstrate its successful import and processing by isolated chloroplasts. A wheat gene (whAB1.6) coding for LHCP, characterized by Lamppa et al. (27) was inserted 3' to the SP6 promoter (24). The resulting construct, SP6-whAB1.6 (see Fig. 1) was linearized with Hind III and used as a template to synthesize LHCP RNA *in vitro*. As shown by SDS-PAGE (Fig. 2 A, lane 1), translation of this RNA in a reticulocyte lysate in the presence of [³⁵S]methionine produced one major precursor form (31 kD) and two minor species (32.5 and 33.5 kD) slightly larger than expected for the LHCP precursor (29 kD) deduced from its nucleotide sequence (27), but in agreement with previous SDS-PAGE size estimates (38). RNA capped with m⁷GpppG or GpppG, or translated in the wheat germ extract, gave the same results (not shown). The 31-kD polypeptide was always the most prominent translation product, and appeared first during translation (see Fig. 3 A). Although all three polypeptides cross-reacted with antibody specific to LHCP (Fig. 2 B, lane 1), the 31-kD polypeptide comigrates with the major

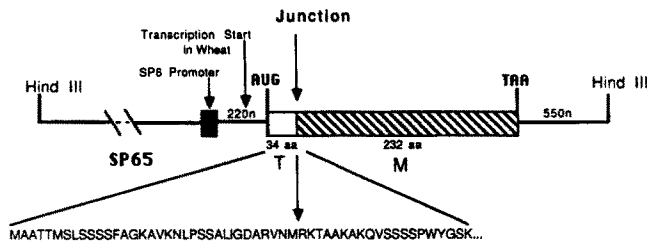


Figure 1. Structure of the SP6-whAB1.6 plasmid construct used for *in vitro* RNA synthesis. The wheat LHCP gene (whAB1.6) was placed 3' to the SP6 promoter as diagrammed. The plasmid was linearized with Hind III. The number of nucleotides between the promoter and first AUG and after the TAA stop codon are given. The transcription initiation site in wheat occurs 70 nucleotides upstream of the AUG (27). The sequence of the transit peptide (T, unshaded) and the first 20 amino acids of mature LHCP (M, diagonal lines) is given (see reference 27) in the single-letter amino acid code, and the putative cleavage site at their junction is indicated.

polypeptide synthesized from wheat poly A⁺ RNA and precipitated with the LHCP-specific antibody (Fig. 2 B, lanes 2–4).

Pea chloroplasts have been shown to be capable of importing the precursors for *rbcS* and other stromal polypeptides (13). Because of the ease of isolating and working with pea chloroplasts, we investigated if they would also successfully import and process the wheat LHCP precursor forms that

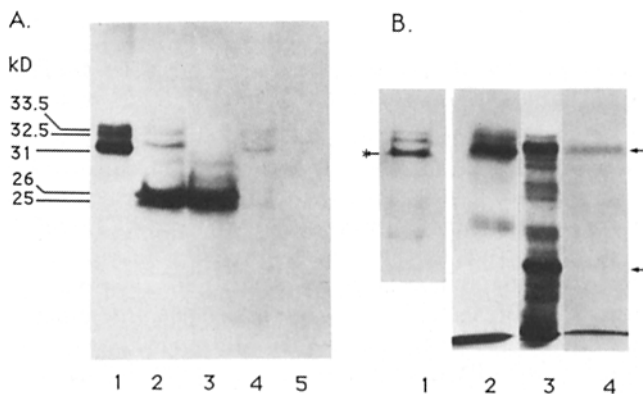


Figure 2. (A) *In vitro* translation of synthetic LHCP RNA and import of labeled precursors by pea chloroplasts. RNA generated from the SP6-whAB1.6 plasmid was translated in a reticulocyte lysate in the presence of [³⁵S]methionine, and the products (lane 1) were incubated with chloroplasts for 1 h. The chloroplasts were either untreated (lanes 2 and 4) or treated (lanes 3 and 5) with thermolysin before lysis and separation of the membrane (lanes 2 and 3) and soluble (lanes 4 and 5) fractions. The products were analyzed by SDS-PAGE on a 12% gel. Size estimates are given in kilodaltons (kD). (B) Immunoprecipitation of LHCP precursors with LHCP-specific antibody. Translation products of synthetic LHCP RNA (lane 2) and wheat poly A⁺ (lane 3) made in a wheat germ extract were analyzed together on a 12% gel. An aliquot of each reaction was incubated with LHCP antibody as described in Materials and Methods. The antibody precipitated the 33.5, 32.5, and 31 kD (*) forms made from the synthetic RNA (lane 1) and only a 31-kD polypeptide made from the poly A⁺ RNA (lane 4). The arrows on the right point to pre-LHCP and pre-*rbcS*, respectively, in the poly A⁺ translation profile.

were made *in vitro* and were destined for the thylakoids. Labeled polypeptides synthesized in the reticulocyte lysate using RNA transcribed from the SP6-whAB1.6 construct were incubated with pea chloroplasts, which were then either treated or not treated with thermolysin before lysis. Thermolysin has been shown to preferentially digest outer membrane proteins (10, 20) that have not entered the organelle. The membrane and soluble fractions of the chloroplast were separated by centrifugation and analyzed by SDS-PAGE. Fig. 2 A (lane 2) shows that the 32.5- and 31-kD precursor forms were associated with the membrane fraction after incubation with the chloroplasts. Both were thermolysin sensitive (Fig. 2 A, lane 3) indicating that they were attached to the outside of the chloroplast. In addition, two new bands that were thermolysin resistant (Fig. 2 A, lane 3) appeared at lower molecular mass (26 and 25 kD). The two new bands comigrated during SDS-PAGE with Coomassie Blue-stained LHCP isolated in the membrane fraction (not shown), and thus, we conclude that they correspond to processed, mature LHCP. The soluble fraction isolated from chloroplasts before thermolysin treatment contained low levels of all three precursor forms (lane 4), but all were sensitive to thermolysin (lane 5), indicating that they had not entered the organelle. It is possible that the soluble fraction contained the components of the outer-inner intermembrane space, or that alternatively, outer envelope vesicles remained in the soluble fraction. Neither the thermolysin-sensitive or -resistant soluble fraction contained the two processed forms of LHCP, which were restricted to the pelleted membrane fraction. Taken together, these results indicate that the *in vitro*-synthesized wheat LHCP precursor forms are successfully imported, localized to an internal membrane compartment, presumably the thylakoids, and processed by pea chloroplasts.

Initially, we thought that the two mature forms of LHCP, which sometimes resolved into three bands (data not shown), might be derived from the three precursor forms in a direct precursor-product relationship. To test this, we took advantage of the observation that the 31-kD polypeptide appeared first during translation (Fig. 3 A) and, thus, were able to synthesize this component alone. The 31-kD protein was then incubated with pea chloroplasts, and the products were analyzed after import. As shown in Fig. 3 B, two thermolysin-resistant bands the size of mature LHCP were again obtained. This suggests that the 31-kD polypeptide is processed at two different sites by chloroplasts, or alternatively, one cleavage occurs, and the 25-kD product is covalently modified to produce the higher molecular mass form. Based on these results, in addition to the abundance of the 31-kD form in the translation products, its cross-reactivity with the LHCP-specific antibody, and its preferential processing as described below in a later section, we conclude that the 31-kD polypeptide represents the major physiologically active form of the LHCP precursor synthesized *in vitro* from the whAB1.6 gene. The origin of the 33.5- and 32.5-kD polypeptides remains to be resolved, and is currently under investigation.

Import of Wheat LHCP Precursors by Wheat Chloroplasts

Incomplete processing of precursors has been observed previously using a heterologous import system comprised of

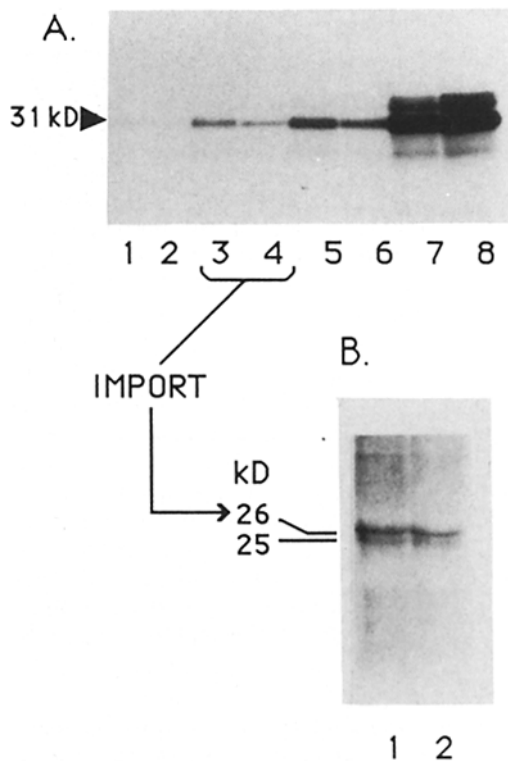


Figure 3. (A) Time course of in vitro translation in the reticulocyte lysate. Translation reactions containing the SP6-whAB1.6 generated RNA were carried out for 15 (lanes 1 and 2), 17 (lane 3 and 4), 20 (lanes 5 and 6), 25 (lane 7), and 35 (lane 8) min, and immediately stopped by addition of gel loading buffer to a final concentration of 0.1 M NaCO₃, 0.1 M DTT, 2% SDS, 12% sucrose, and 0.4% bromophenol blue. The products were analyzed on a 12% acrylamide gel. (B) LHCP translation products synthesized during the first 17 min in the reticulocyte lysate (see bracket in A) were incubated with pea chloroplasts in an import reaction. The membrane fraction was isolated from both untreated (lane 1) and thermolysin-treated (lane 2) organelles. The positions of the 31-, 26-, and 25-kD polypeptides are indicated.

higher plant chloroplasts and the precursor for *rbcS* from the green alga *Chlamydomonas* (31). This has been attributed to a lack of conservation of the *rbcS* transit peptide. A comparison of the amino acid sequences of pre-LHCP from wheat and pea revealed a number of differences in their transit peptides and the NH₂-termini of the mature proteins (27). To determine if the multiple forms of mature LHCP observed in the present study are the result of using a heterologous system (i.e., incubating precursors from a monocotyledon [wheat] with chloroplasts from a dicotyledon [pea]) a homologous import reaction using wheat chloroplasts was performed. Wheat chloroplasts were not used routinely because recovery of intact chloroplasts is complicated by the apparent high levels of endogenous degradative proteases that rapidly destroy the integrity of the chloroplast envelope (Lamppa, G., unpublished observation). However, intact organelles competent for import have been obtained using young plants (6 d from germination). Wheat chloroplasts imported and processed the wheat LHCP precursors, again yielding two forms of mature LHCP associated with the thermolysin-resistant membrane fraction (Fig. 4 A). We conclude that the two

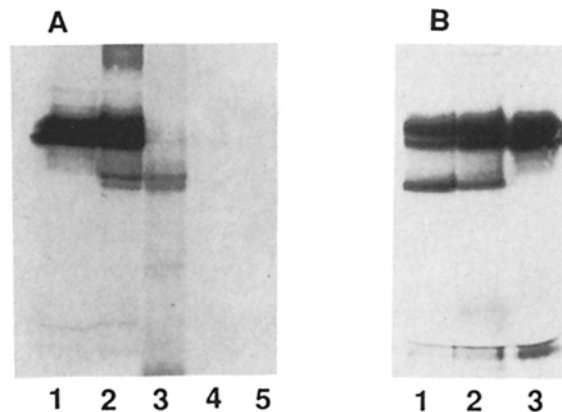


Figure 4. (A) Import of in vitro-synthesized wheat LHCP precursors using wheat chloroplasts. LHCP RNA was translated in the reticulocyte system, and the products (lane 1) were incubated with wheat chloroplasts as described in the legend to Fig. 1 and in Materials and Methods. The membrane (lanes 2 and 3) and soluble (lanes 4 and 5) fractions were analyzed from thermolysin untreated (lanes 2 and 4) and treated (lanes 3 and 5) chloroplasts. (B) Processing of wheat LHCP precursors using a wheat chloroplast-soluble fraction in an organelle-free assay. Labeled translation products (lane 3) were incubated with a soluble extract from wheat chloroplasts lysed in 5 mM Hepes-KOH, pH 8, in the presence (lane 2) and absence (lane 1) of added 10 mM MgCl₂.

forms are produced as part of normal import, maturation, and assembly of LHCP into the thylakoids of both wheat and pea chloroplasts.

Identification of a Soluble Processing Activity from Pea Chloroplasts that Cleaves the Wheat LHCP Precursor

LHCP RNA synthesized in vitro was translated in the reticulocyte lysate to obtain relatively pure labeled precursor to use as a substrate in a processing assay. Intact pea chloroplasts were isolated and then lysed in 5 mM Hepes-KOH, pH 8. The lysed organelles were centrifuged to separate the membranes and the soluble fractions, which were independently used in an organelle-free assay to investigate processing of pre-LHCP. After a typical reaction, the products were analyzed by SDS-PAGE to determine if a peptide had been released from the precursor. To date, no processing activity has been found in a 5-mM Hepes-KOH, pH 8, suspension of the membrane fraction from lysed chloroplasts (not shown). Our study has not been exhaustive, however, and an enzyme may be associated with the membranes, and be released and active under different conditions. In contrast, when the soluble fraction from lysed chloroplasts was assayed, processing activity was found. Incubation of labeled pre-LHCP with the soluble extract resulted in the release of a single 25-kD peptide (Fig. 5 A, lane 4). This peptide comigrates with the lower form of mature LHCP produced during in vitro import using intact chloroplasts (see below; Fig. 6). To establish that processing of pre-LHCP was dependent on the addition of the soluble extract, and not due to lability and breakdown of the precursor, pre-LHCP was incubated with and without the extract under identical conditions. Only when the extract was added to the reaction was the 25-kD peptide released (Fig. 5 A, compare lanes 1 and 4). When the soluble extract was boiled for 1 min before addition to the assay, no processing

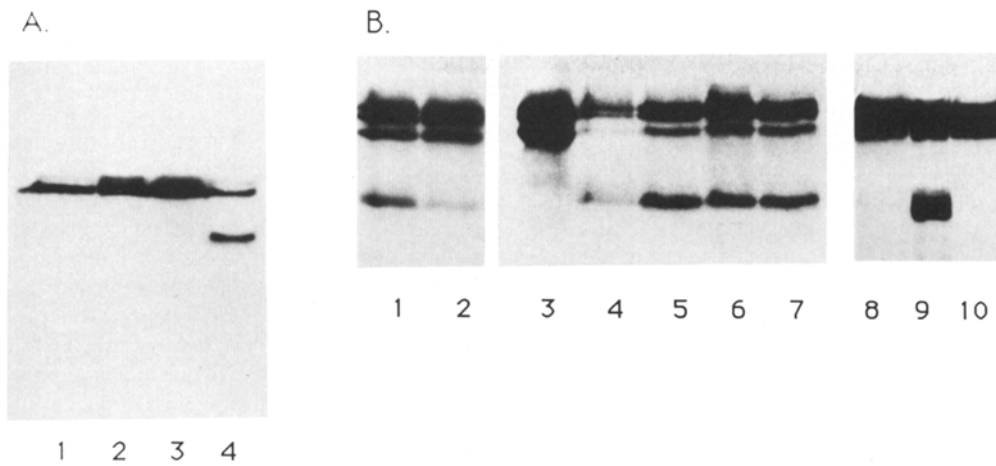


Figure 5. Processing of the wheat LHCP precursors in an organelle-free assay using a soluble fraction prepared from pea chloroplasts. (A) The SP6-whAB1.6 generated RNA was translated in the reticulocyte lysate and the products (lane 3) were incubated in a mock processing reaction in which 5 mM Hepes-KOH, pH 8, was substituted for the extract (lane 1); after boiling of the extract for 1 min before adding (lane 2); with the soluble extract from chloroplasts lysed in 5 mM Hepes-KOH, pH 8 (lane 4). (B) Labeled LHCP precursors (lane 3) were incubated with the soluble extract without (lane 1) and with (lane 2) $MgCl_2$ added to 10 mM. The soluble extract was assayed before (lane 4) and after (lane 5) ultracentrifugation to remove membrane vesicles. In separate experiments, ammonium sulfate was added to the soluble extract to obtain concentration cuts of 0–40, 40–70, and 70–100% saturated. The precipitates were then dialyzed against 5 mM Hepes-KOH, pH 8, and used in the organelle-free assay. No processing activity was found in the 0–40 (lane 8) or 70–100% (lane 10) fractions, whereas the 25-kD peptide was released in the 40–70% cut (lane 9). Processing activity was retained in the supernatant of the 40–70% cut after further centrifugation to remove particles, as shown before (lane 6) and after (lane 7) the spin.

occurred (Fig. 5 A, lane 2), demonstrating that a denaturable molecule is responsible for the release of the 25-kD peptide. In the initial assays 10 mM Mg^{++} was added to the reactions because at this concentration Mg^{++} has been shown to promote import of pre-LHCP into intact chloroplasts (9, 11). However, Mg^{++} proved to have a slight inhibitory effect on processing of pre-LHCP in the organelle-free assay (Fig. 5 B, compare lanes 1 and 2).

The chloroplast soluble fraction could have contained outer envelope vesicles formed during organelle lysis and not pelleted by the fractionation protocol. To determine if the processing activity was associated with membrane vesicles, the soluble fraction was clarified by ultracentrifugation at 137,000 g and again assayed. The supernatant contained at least the same level of processing activity as the original, uncentrifuged material (Fig. 5 B, lanes 4 and 5). Starting with the total soluble extract, the activity was selectively precipitated in a 40–70% ammonium sulfate fraction, and was retained upon dialysis in 5 mM Hepes-KOH, pH 8. No activity was found in 0–40 and 70–100% ammonium sulfate fractions (Fig. 5 B, lanes 8–10). The 40–70% fraction was further

clarified by centrifugation at 137,000 g without losing activity (Fig. 5 B, lanes 6 and 7). These results provide strong evidence that pea chloroplasts contain a soluble proteolytic enzyme that cleaves pre-LHCP. In addition, because of the gentle conditions used to lyse the chloroplasts, it is most probable that the enzyme is located in the stroma and not the lumen of the thylakoids.

Time Course of Processing the Wheat LHCP Precursor

To determine if any intermediates were made during the release of the 25-kD peptide from pre-LHCP, a time course of processing by a soluble extract from pea chloroplasts was performed. The 25-kD peptide began to appear at 5 min, and continued to accumulate over an hour (Fig. 6 A). The faint band (26 kD) migrating above the 25-kD peptide was also seen in the profile of the translation products (Fig. 6 A, lane 1), and may reflect translation initiation at an internal AUG at the transit peptide-mature protein junction (see Fig. 1). Because this band did not change significantly in intensity during the course of the reactions, it probably does not repre-

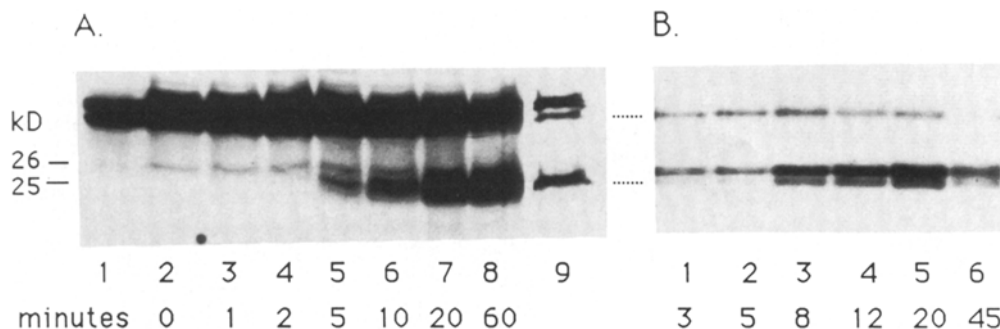


Figure 6. (A) Time course of a processing reaction. Reticulocyte translation products (lane 1) of LHCP RNA were incubated with a soluble extract from pea chloroplasts for 0, 1, 2, 5, 10, 20, and 60 min (lanes 2–8, respectively), and for 18 h (lane 9). The positions of the 25- and 26-kD peptides are indicated. (B) Time course of import using pea chloroplasts. Translation products were in-

cubated with chloroplasts for 3, 5, 8, 12, 20, and 45 min (lanes 1–6, respectively). To stop the reactions, chloroplasts were diluted fivefold on ice, pelleted, and lysed in 1 mM PMSF. No thermolysin treatment was included. The membrane fraction was pelleted and prepared for gel analysis. The upper and lower dashed lines align either the 31- or 25-kD proteins, respectively, in the processing and import reactions.

sent a processing intermediate. To establish this with certainty, we are currently preparing gene constructs lacking the internal AUG site. In contrast, a time course of import using intact pea chloroplasts showed that both the 26- and 25-kD peptides appeared within 3 min, and then accumulated in parallel (Fig. 6 B). In all the organelle-free processing reactions, the 31-kD polypeptide appeared to be preferentially cleaved, which was most evident after an overnight (18-h) incubation (Fig. 6 A, lane 9). Complete processing of the precursor has not yet been observed, probably because the processing enzyme, or another factor in the crude extract, becomes limiting.

Wheat Chloroplasts Also Contain a Soluble Processing Activity

Wheat chloroplasts were isolated to determine if they contain a processing activity that recognizes the wheat LHCP precursor, comparable to that identified in pea. The chloroplasts were lysed, and the soluble and membrane fractions separated; only the soluble fraction was assayed. As shown in Fig. 4 B, the soluble fraction from wheat chloroplasts also cleaved pre-LHCP, releasing only a single peptide. The peptide comigrates with the lower form of mature LHCP produced during an import reaction using wheat chloroplasts (Fig. 4 A). Addition of 10 mM Mg⁺⁺ to the wheat-soluble extract resulted in a minor inhibition of processing (Fig. 4 B, lanes 1 and 2). These observations indicate that both wheat and pea chloroplasts contain a similar processing enzyme for pre-LHCP, underscoring the likelihood that the enzyme may be a universal component of the chloroplast import and processing machinery.

Discussion

In these experiments, we have identified a soluble processing enzyme from chloroplasts that cleaves wheat LHCP precursor to release a peptide which comigrates with mature LHCP. The processing enzyme was found in chloroplasts from two higher plants, pea and wheat, representatives of the dicotyledons and monocotyledons, respectively. At least two forms (25 and 26 kD) of mature LHCP were produced during in vitro import of the LHCP precursor using intact chloroplasts from both pea and wheat. The processing enzyme extracted from lysed chloroplasts produced only the lower molecular mass form in our organelle-free assay. Multiple forms of LHCP are found in vivo (38, 47), and are commonly observed after incubation of higher plant chloroplasts with pre-LHCP synthesized in vitro from hybrid-selected RNA (9, 11, 38, 42, 47). These results have usually been interpreted to reflect the LHCP multi-gene family, and the ability of a heterogeneous population of LHCP mRNA, derived from those genes, to hybridize with a single LHCP DNA clone. Recently, however, Kohorn et al. (22) have also observed several forms of mature LHCP in an in vitro import assay for *Lemna*, analogous to the one used here, in which a single gene is used for the synthesis of LHCP RNA. Thus, the occurrence of multiple LHCP forms need not only reflect differences in gene structure.

One explanation for the appearance of multiple forms of mature LHCP is that, besides the removal of the transit peptide during import and assembly into the thylakoids, a subpopulation of the LHCP precursor is further processed to a

smaller peptide. Darr et al. (15) have characterized a monoclonal antibody that recognizes only the NH₂ terminus of the 26-kD polypeptide, which they propose is proteolytically removed to produce the smaller forms of LHCP in pea. In support of this hypothesis is our identification of a protease that produces only the 25-kD peptide from pre-LHCP. The functional significance of the different structural forms of LHCP is not yet clear, although it is interesting to speculate that the selective removal of a region of LHCP will determine its location in the thylakoids, and involvement in the distribution of light energy between the photosystems. The NH₂ terminus of LHCP, for example, has been implicated in promoting thylakoid appression (33, 44), and upon phosphorylation, the diffusion of a subgroup of LHCP from the appressed membranes where it is associated with photosystem II to the stromal thylakoids (1, 4, 44). We are currently investigating if it is indeed the NH₂ terminus of the LHCP precursor which is removed by the soluble protease described here.

During a time course of the in vitro processing reaction, only the 25-kD peptide was produced. The 26-kD form of LHCP, which was a major product in the import assays, was present only at low levels that were also observed in the translation profiles (probably due to initiation at an internal AUG), and did not change in intensity during organelle-free processing. No other intermediates were observed. This suggests that the soluble enzyme may act as an endopeptidase. Furthermore, it raises the possibility that during import a different protease produces the more predominant 26-kD form of LHCP, presumably by cleavage at either the Asn-Met or Met-Arg junction (see Fig. 1), to remove the transit peptide alone. However, our organelle-free reactions are carried out without a membrane component that could influence the nature of the processing reaction and the sites available for cleavage. The fact that a discrete peptide is released from the precursor, and not a continuum of smaller peptides, indicates that the protease is not a general, degradative enzyme as described for LHCP turnover (3), but instead is highly specific. To understand the role of the protease, it will be essential to determine the precise site of proteolytic cleavage.

Of equal importance is an understanding of the temporal relationship between precursor processing and insertion into the thylakoids. It has been found using isolated thylakoids that pre-LHCP can insert into the membranes without processing (9). If this is part of the normal in vivo pathway of localization within the organelle, the precursor must be protected as it traverses the stroma from the soluble protease, which our data predicts is in the stromal compartment. Alternatively, pre-LHCP may effectively insert in vitro when an active protease is absent, but in vivo processing occurs before or concomitant with incorporation into the thylakoids. Inconsistent with the latter model is the observation that, in addition to the processed form, low levels of the LHCP precursor have been found associated with the thylakoid membranes after in vitro uptake by barley chloroplasts (7).

The diversity of proteases required to process the large number of polypeptides imported into the chloroplast during its biogenesis is unknown. Only two such proteases have been previously identified in higher plants. One is a soluble, stromal enzyme that processes the precursor of rbcS to its mature form (35, 36), as well as pre-plastocyanin to an intermediate size (19). The other is a thylakoid protease that

processes the pre-plastocyanin intermediate to the mature form found in the lumen (19). We do not yet know the relationship between the previously characterized pea stromal protease and the soluble processing enzyme identified in this study. A whole cell soluble extract of *Chlamydomonas* will process both pre-rbcS and pre-LHCP (30, 31), and also precursors of several ribosomal proteins (40, 41), but it is unknown if the same enzyme is responsible for these different activities. In yeast mitochondria, a general protease has been described that cleaves imported precursors which reside in the matrix, or become incorporated into the inner membrane (5). Our studies are now focused on characterizing the processing enzyme that cleaves pre-LHCP to determine its cleavage site, substrate specificity, and biochemical properties.

We wish to thank Dr. Nam-Hai Chua for kindly providing us with the LHCP-specific antibody, and Dr. Laurens Mets for critically reading the manuscript. We thank Thomas Dorman for his excellent technical assistance.

This study was supported in part by a United States Department of Agriculture grant (No. 85-CRCR-1-1569) and a National Institutes of Health grant (No. GM36419-02) awarded to G. K. Lamppa, and a Sigma XI Grants-in-Aid awarded to M. S. Abad.

Received for publication 16 May 1987, and in revised form 25 August 1987.

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