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Leucoplast Pyruvate Kinase from Developing Castor Oil Seeds[1]

Characterization of the Enzyme's Degradation by a Cysteine Endopeptidase

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ABSTRACT

Leucoplast pyruvate kinase (PK_p; EC 2.7.1.40) from endosperm of developing castor oil seeds (*Ricinus communis* L. cv Baker 296) appears to be highly susceptible to limited degradation by a cysteine endopeptidase during the purification of the enzyme or incubation of clarified homogenates at 4°C. Purified castor seed PK_p was previously reported to consist of immunologically related 57.5 and 44 kilodalton subunits (Plaxton WC, Dennis DT, Knowles VL [1990] Plant Physiol 94: 1528–1534). By contrast, immunoreactive polypeptides of about 63.5 and 54 kilodaltons were observed when a western blot of an extract prepared under denaturing conditions was probed with affinity purified rabbit anti-(castor seed PK_p) immunoglobulin G. Proteolytic activity against PK_p was estimated by the disappearance of the 63.5 and 54 kilodalton subunits and the concomitant appearance of lower molecular mass immunoreactive degradation products during the incubation of clarified homogenates at 4°C. The presence of 2 millimolar dithiothreitol accelerated the degradation of PK_p. The conservation of the 63.5 and 54 kilodalton subunits was observed after extraction of the enzyme in the presence of 1 millimolar *p*-hydroxymecuribenzoate, or 1 millimolar *Nα-p*-tosyl-L-lysine chloromethyl ketone, or 10 millimolar iodoacetate. These results reveal that a cysteine endopeptidase was responsible for the *in vitro* proteolysis of PK_p. This endopeptidase is present throughout all stages of endosperm development. Its PK_p-degrading activity, however, appears to be most pronounced in preparations from older endosperm. When lysates of purified leucoplasts were incubated at 4°C for up to 21 hours, no degradation of PK_p was observed; this indicated an extra-leucoplastic localization for the cysteine endopeptidase. Although the *in vivo* subunit structure of PK_p remains uniform throughout all stages of endosperm development, the large decrease in PK activity that accompanies castor seed maturation coincides with a marked reduction in the concentration of PK_p.

PK[2] (ATP:pyruvate phosphotransferase) is a key regulatory enzyme of the glycolytic pathway that catalyzes the irreversible conversion of PEP and ADP to pyruvate and ATP. The physical, immunological, and/or kinetic properties of several purified plant cytosolic and plastid PKs (PK_c and PK_p, respectively) have been studied in detail (1, 9, 11, 13–15, 17–19, 21). As well, cDNAs encoding higher plant cytosolic and plastid PKs have recently been cloned and sequenced (2–3).

Like most nonplant PKs, the cytosolic enzyme from developing endosperm and leaf tissue of the castor oil plant appear to be 230 to 240 kD homotetramers (18). By contrast, PK_c from germinating COS endosperm is a heterotetramer composed of two types of closely related, but nonidentical, 57 and 56 kD subunits (17–18), whereas chloroplast PK from the green alga *Selenastrum minutum* was shown to be a 210 kD monomer (13).

The subunit composition for leucoplast PK from developing oil seeds has not yet been resolved. Developing COS endosperm leucoplast PK was recently purified to near homogeneity, and had a native molecular mass of about 305 kD (19). SDS-PAGE and western blot analysis of the final preparation revealed two major protein staining bands of 57.5 and 44 kD, that were immunologically related and occurred in an approximate 2:1 ratio, respectively. It was tentatively proposed that either there was proteolytic degradation of the 57.5 kD subunit yielding the 44 kD subunit, or that the enzyme might be a heterohexamer composed of two different types of related subunits (19). The present study was initiated to verify the *in vivo* subunit structure of developing COS PK_p. Evidence is presented that demonstrates that: (a) throughout the course of endosperm development, PK_p is composed *in vivo* of equal amounts of homologous 63.5 and 54 kD subunits; and (b) both subunits are highly susceptible to limited *in vitro* degradation by a nonleucoplast localized cysteine endopeptidase.

MATERIALS AND METHODS

Chemicals and Plant Material

All biochemicals and chemicals, purified developing COS PK_p, and affinity purified rabbit anti-(developing COS endosperm PK_p) IgG were procurred as in ref. 19.

Developing seeds were harvested from castor oil plants (*Ricinus communis* L. cv Baker 296) grown in a greenhouse under natural light, supplemented with 16 h fluorescent light.

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[2] Abbreviations: PK, pyruvate kinase; PK_p and PK_c, plastid and cytosolic pyruvate kinase isozymes, respectively; COS, castor oil seed; *p*HMB, *p*-hydroxymecuribenzoate; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; IA, iodoacetate; IgG, immunoglobulin G.

Preparation of Clarified Homogenates

Endosperm was collected at different stages of seed development, the exact stage being determined as described by Greenwood and Bewley (7). Harvested endosperm was rinsed in distilled water and the fresh weight determined. The tissue was homogenized (1:4; w/v) with a Polytron in 25 mM KPi (pH 7.6) containing 1 mM EDTA, 2 mM $MgCl_2$, and 10% (v/v) glycerol. In some experiments, the homogenization buffer was: (a) supplemented with 2 mM DTT or various protease inhibitors; or (b) replaced with 50 mM Bis-Tris-propane-HCl (pH 9.5) containing 1 mM EDTA, 2 mM $MgCl_2$, 10% (v/v) glycerol, and 2 mM DTT. Homogenates were filtered through two layers of Miracloth, and the filtrates centrifuged at 15,600*g* and 4°C in an Eppendorf microcentrifuge. Protease inhibitor stock solutions were prepared according to Gray (8) or Storey and Wagner (24). Aliquots (0.5 mL) of clarified homogenates were incubated at 4°C for up to 21 h. At various times, 50 μL aliquots were removed, immediately mixed with an equal volume of SDS-PAGE sample buffer, and boiled for 2 min (for later analysis via SDS-PAGE/western blotting). At least three extracts of each developmental stage were made and analyzed.

Western Blotting

Western blotting was performed as described previously using the Bio-Rad mini-gel apparatus and rabbit anti-(developing COS PK_p) IgG that had been affinity purified against the larger (57.5 kD) PK_p polypeptide (19). The affinity purified IgG was diluted 12-fold with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.05% (v/v) Nonidet P40, and 0.3% (w/v) BSA (fraction V). Antigenic polypeptides were visualized using an alkaline phosphatase-conjugated secondary antibody as in ref. 19. Phosphatase staining was for 5 to 10 min at 30°C. Densitometric scanning of western blots was performed using an LKB Ultroscan XL Enhanced Laser Densitometer. Densitometric data were analyzed and molecular mass estimates were made using the LKB Gelscan XL software (version 2.1). Molecular masses of immunoreactive polypeptides were estimated by comparing the mobility of the various bands with that of the following prestained standard proteins: myosin (205 kD); phosphorylase b (97.4 kD); BSA (66 kD); ovalbumin (45 kD); carbonic anhydrase (29 kD); trypsin inhibitor (20.1 kD).

Protein Determination

Protein concentrations were determined by the method of Bradford (6) using the Bio-Rad prepared reagent and bovine γ-globulin as standard.

RESULTS AND DISCUSSION

Subunit Composition of Leucoplast Pyruvate Kinase from Developing Castor Seed Endosperm

When purified from developing COS endosperm, PK_p was found to contain immunologically related 57.5 and 44 kD subunits that existed in a ratio of approximately 2:1 (Fig. 1, lane 1) (19). By contrast, two immunoreactive polypeptides

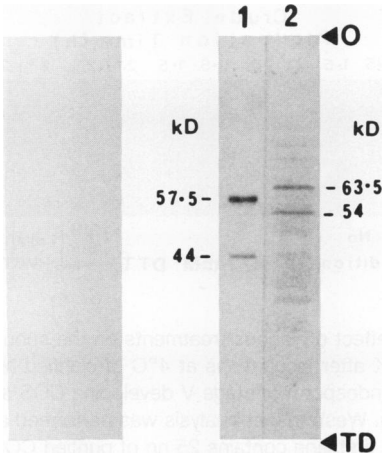

Figure 1. Immunological detection of leucoplast PK from developing COS. Samples were subjected to SDS-PAGE and blot-transferred to a polyvinyledene difluoride membrane. Western analysis was performed using anti-(developing COS PK_p) IgG that had been affinity-purified against 25 μg of the 57.5 kD polypeptide of purified developing COS PK_p (19). Lane 1 contains 25 ng of purified COS leucoplast PK. Lane 2 contains 50 μg of protein of a clarified homogenate from endosperm of stage V (or midcotyledon stage) developing COS that was prepared according to Wu and Wang (26) so as to eliminate potential protease activity during enzyme extraction and sample processing. This procedure involves homogenization of the tissue in 10% (w/v) TCA, followed by resolubilization of precipitated proteins in SDS-PAGE sample buffer. O, origin; TD, tracker dye front.

that stained with approximately equal intensities and migrated with molecular masses of 63.5 and 54 kD were observed when a western blot of a developing COS extract prepared under denaturing conditions was probed with the affinity purified anti-(PK_p) IgG (Fig. 1, lane 2). These data indicate that: (a) developing COS PK_p is composed *in vivo* of equal amounts of closely related 63.5 kD (PK_p-α) and 54 kD (PK_p-β) subunits; and (b) the novel subunit composition initially observed for purified COS PK_p (19) artifactually arose after tissue homogenization via proteolysis of both the α- and β-subunits.

The above findings are in accord with those of a recent investigation in which the affinity purified anti-(COS PK_p) IgG was used to immunoselect two similar, but nonidentical, full length clones from a developing COS cDNA library; the clones were designated as $PK_p\alpha$ and $PK_p\beta$ and were shown to contain open reading frames encoding proteins with predicted molecular masses of 64.1 and 54.5 kD, respectively (3). The deduced NH_2-terminal sequence of the $PK_p\alpha$ open reading frame contains a transit peptide-like hydrophobic region of 78 amino acid residues followed by a sequence of 8 amino acids that is identical to the NH_2-terminus determined for the 57.5 kD subunit of purified, degraded, COS PK_p (3). Together with the findings of Figure 1, these data suggest that: (a) the 57.5 and 44 kD polypeptides initially observed for purified COS PK_p (19) were derived from the 63.5 kD (α-) and 54 kD (β-) subunits, respectively; and (b) the putative transit peptide sequence for the α-subunit (3) is not cleaved following import of PK_p into the leucoplast.

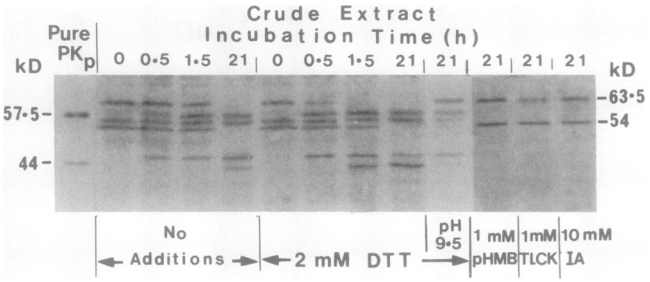

Figure 2. The effect of various treatments on the subunit structure of leucoplast PK after incubations at 4°C of clarified homogenates obtained from endosperm of stage V developing COS as judged by western blotting. Western blot analysis was performed as described in Figure 1. The first lane contains 25 ng of purified COS leucoplast PK. All other lanes contain 60 μg of protein from a developing COS extract.

Enzyme Degradation

A clarified homogenate was prepared from developing COS endosperm (stage V, or midcotyledon stage) in the absence of added protease inhibitors, with and without 2 mM DTT, and incubated at 4°C for up to 21 h. Aliquots were removed at various times and subjected to western blot analysis using the affinity-purified anti-(COS PK_p) IgG (Fig. 2). At $t = 0$ h, major immunoreactive polypeptides of about 63.5 and 54 kD were observed. Incubation at 4°C caused the progressive appearance of smaller antigenic polypeptides that by 21 h were very similar in size to those originally observed for the purified, degraded, COS PK_p (Fig. 2). Comparison of western blots of COS clarified homogenates prepared in the presence and absence of 2 mM DTT indicated that this sulfhydryl reducing agent can accelerate the degradation of COS PK_p over the initial several hours of incubation at 4°C (see Fig. 2, $t = 1.5$ h). This indicated that a cysteine endopeptidase might be responsible for the *in vitro* degradation of COS PK_p.

A variety of protease inhibitors were tested for their ability to prevent the *in vitro* degradation of developing COS PK_p. The following substances had no effect as judged by western blot analyses of extracts incubated for 21 h at 4°C (results not shown): EDTA, EGTA, bipyridyl, iodoacetamide, *N*-ethylmaleimide (all 10 mM); 1,1-phenanthroline and Cu^{2+}-diazoacetyl nor-leucine methyl ester (both 5 mM); 3 mM 1,2-epoxy-3-(*p*-nitrophenoxy)-propane; 2 mM PMSF; 1 mM diphenylcarbamylchloride; *p*-aminobenzamidine and benzamidine (both 1 mM); 10 μM L-(*trans*)-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64); aprotinin, pepstatin, antipain, cystatin, and soybean trypsin inhibitor (all 100 μg/mL); chymostatin and leupeptin (both 25 μg/mL). Extraction of developing COS endosperm in the presence of 1 mM IA, or 1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, or 1 mM dithionitrobenzoate, or 10 mM 2,2′-dipyridyl disulfide was partially effective in ameliorating PK_p's subsequent degradation during incubation of the respective extracts for 21 h at 4°C (data not shown). Similarly, extraction at pH 9.5 in the presence of 2 mM DTT afforded only partial protection to the enzyme from the PK_p-degrading activity (Fig. 2). However, when tested separately, 1 mM *p*HMB, 1 mM TLCK, or 10 mM IA completely prevented any degradation of the α- or β-subunits after incubation of the respective extracts at 4°C for 21 h (Fig. 2).

Whereas *p*HMB and IA are nonspecific cysteinyl modifying reagents, TLCK is an active site-directed covalent affinity label that was principally designed for the inhibition of trypsin- or chymotrypsin-like serine endopeptidases (8). TLCK, however, has also been shown to inhibit plant cysteine endopeptidases such as papain (8). Furthermore, 2 mM DTT afforded complete protection of the PK_p-degrading protease from inactivation by 1 mM TLCK or 1 mM *p*HMB (results not shown). Therefore, this protease appears to be a cysteine endopeptidase. It could be the same cysteine endopeptidase of dry and germinating COS that was also activated by DTT, inactivated by *p*HMB and IA, but unaffected by *N*-ethylmaleimide or iodoacetamide (25).

Developmental Profiles of Protease Activity against PK_p, and PK_p Subunit Composition, during Castor Seed Formation

Endosperm from developing COS was collected at various stages of seed development, extracted in the presence of 2 mM DTT, and the resulting extracts incubated at 4°C. Western blot analyses of aliquots that were removed at 0.5, 1.5, and 21 h indicated that although the cysteine endopeptidase that degrades PK_p *in vitro* is present throughout all stages of seed development, its PK_p-degrading activity appears to be more pronounced in extracts prepared from older endosperm (Fig. 3A). PK_p degradation in 21 h-incubated extracts prepared

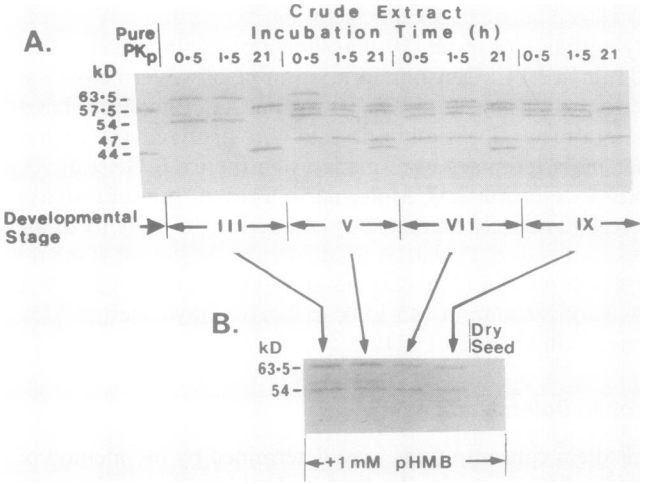

Figure 3. Developmental profiles of protease activity against leucoplast PK (A), and *in vivo* leucoplast PK subunit structure (B), during COS endosperm development as judged by western blotting. Western blot analysis was performed as described in Figure 1. The lane labeled Pure PK_p contains 25 ng of purified COS PK_p. The lane corresponding to developmental stage III contains 15 μg of protein; all other lanes contain 60 μg of protein. Developmental stages III, V, VII, and IX correspond to the heart-shaped embryo, midcotyledon, full cotyledon, and maturation stages of endosperm development, respectively (7). The lane labeled Dry Seed designates a fully mature castor oil seed.

from the youngest developmental stage that was analyzed (stage III, or heart-shaped embryo stage) was manifested mainly by the generation of 57.5 and 44 kD fragments. By contrast, an identical incubation of extracts prepared from stage IX (or maturation stage) developing endosperm caused the production of 56 and 47 kD fragments, whereas 57.5, 56, 47, and 44 kD immunoreactive polypeptides were observed after a 21 h incubation of extracts prepared from intermediate stages of seed development (Fig. 3A, stages V and VII; see also Fig. 2).

To determine if the *in vivo* subunit structure of PK_p is altered during seed formation, extracts of endosperm at various developmental stages were prepared in the presence of 1 mM *p*HMB and analyzed by western blotting. As shown in Figure 3B, endosperm PK_p from seeds at developmental stages III through IX appears to be composed of equal proportions of the 63.5 and 54 kD subunits. No antigenic polypeptides were detected on a western blot of an extract prepared from a fully mature (dry) seed (Fig. 3B). Thus, as is the case with COS phosphoglyceromutase (4), the large decrease in leucoplast PK activity that accompanies COS maturation (10, 23) coincides with a considerable reduction in the concentration of PK_p.

Evidence against a Leucoplast Localization for the Protease that Degrades Castor Seed Leucoplast Pyruvate Kinase

Leucoplasts from endosperm of stage V developing COS were purified on a discontinuous Percoll gradient, and lysed in the presence of 2 mM DTT. Western blotting demonstrated that no degradation of PK_p occurred after a 21 h incubation of the leucoplast lysate at 4°C (Fig. 4, lanes 1 and 2). This indicates a nonleucoplastic location for the protease that degrades COS PK_p in total endosperm extracts. Nonetheless, this cysteine endopeptidase could play a role in the *in vivo* proteolytic removal of COS PK_p during the final stages of seed maturation, when the leucoplasts may lose their integrity. Although few data are at present available concerning the substrate specificity of this endopeptidase, it is intriguing that, under extraction conditions that cause substantial proteolysis of PK_p, developing COS PK_c (18), phosphoglyceromutase (4), and phospho*enol*pyruvate carboxylase (RS Sangwan, N Singh, WC Plaxton, unpublished data) appear to show no degradation. Moreover, the generation of the 57.5 kD degradation product from the 63.5 kD α-subunit (Fig. 1) appears to represent a specific endoproteolytic cleavage event. The site of cleavage is immediately adjacent to a unique sequence of four asparagine residues that link the putative transit peptide to the remainder of the α-subunit (3).

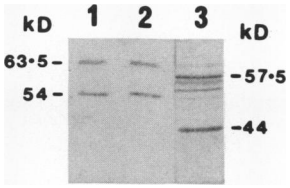

Figure 4. Immunological detection of COS leucoplast PK. Intact leucoplasts were purified from endosperm of stage V (or midcotyledon stage) developing COS on a discontinuous Percoll step gradient according to Boyle *et al.* (5). Leucoplast lysis was accomplished by diluting the final leucoplast suspension with an equal volume of 25 mM KPi (pH 7.6) containing 10% (v/v) glycerol, 1 mM EDTA, 1 mM $MgCl_2$, 4 mM DTT, and 0.3% (v/v) Triton X-100. Western blot analysis was performed as described in Figure 1. Lanes 1 and 2 contain 20 μg of protein from a leucoplast lysate that had been incubated at 4°C for 0 and 21 h, respectively. Lane 3 contains 10 μg of protein of partially purified leucoplast PK that had been prepared from a developing COS 10,000*g* crude leucoplast pellet (18).

The results reported above indicate that previous workers who have examined various *in vitro* properties of developing COS PK_p may have been studying an enzyme partially degraded by protease. For instance, Ireland and coworkers (11) reported kinetic properties for developing COS PK_p that had been partially purified from a 10,000*g* "crude leucoplast pellet." However, extensive proteolysis of the enzyme occurred even when it was partially purified at 4°C from a developing COS 10,000*g* "crude leucoplast pellet" (Fig. 4, lane 3). Hence, it is highly probable that Ireland *et al.* (11) were inadvertantly studying the catalytic characteristics of degraded PK_p. Further work is needed to determine the catalytic and regulatory properties of purified, nondegraded COS PK_p. Elucidation of the respective biochemical function(s) and genetic origin for the α- and β-subunits of COS PK_p is another fundamental area for future research.

CONCLUSIONS

1. The findings of the present and an associated study (3) are consistent with leucoplast PK from developing COS being composed *in vivo* of equal amounts of homologous, but nonidentical, α- and β-subunits having molecular masses of approximately 63.5 and 54 kD, respectively. These results also reveal that the α-subunit's NH_4-terminal putative transit peptide sequence remains associated with the enzyme following its import into the leucoplast.

2. When incubated in a clarified homogenate or purified at 4°C, both PK_p subunits are extremely susceptible to limited *in vitro* degradation by a nonleucoplast-localized cysteine endopeptidase. COS PK_p thus joins the likes of ribulose bisP carboxylase, glutamate synthase, Cyt *f*, inorganic pyrophosphatase (8), ADPglucose pyrophosphorylase (20), NADP-malate dehydrogenase (12), and ferredoxin-NADP reductase (22) as examples of plastid-localized proteins that appear to be highly vulnerable to proteolytic degradation during their extraction and/or purification at 4°C. Obviously, there should now be a universal awareness of the significant potential for a "protease problem" as it pertains to the isolation and study of any plant protein.

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