**Communication**

**A Developmental Analysis of the Enolase Isozymes from *Ricinus communis*¹**

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**ABSTRACT**

Enolase activity was measured in clarified homogenates of various tissues during the life cycle of the castor oil plant (*Ricinus communis* L. cv Baker 296). The proportions of total activity due to the plastid and cytosolic isozymes were determined after separation by ion-exchange chromatography. The contribution of the plastid isozyme varied from more than 30% of the total at the midpoint of endosperm development to less than 1% in mature leaves and roots. During endosperm development, enolase activity increased to a peak coincident with the maximum rate of storage lipid accumulation, then decreased to nearly undetectable levels in the mature seed. Plastid enolase protein, measured using an enzyme-linked immunosorbent assay, increased in parallel with the increase in activity but decreased less rapidly and was still easily detectable in mature seeds.

The developing endosperm of the COS² has been used as a model system to study compartmentation of the glycolytic pathway in plants (reviewed in ref. 4). In this tissue, all of the glycolytic enzymes occur as distinct isozymes, present in both the cytosol and plastids. The results of surveys of other plant systems have, in essence, agreed with this model (7, 13). Most research on the control of glycolytic activity has addressed the acute, short-term regulatory properties of a few key enzymes: hexokinase, phosphofructokinase, and pyruvate kinase. There have been a few partial developmental analyses of glycolytic activity during COS maturation (3, 8, 17) or postgerminative catabolism (3), but there is not yet sufficient information to propose a long-term control mechanism. Enolase (EC 4.2.1.11) has been used as a probe with which to study various aspects of glycolysis in the developing COS endosperm (11–13). Herein we present a detailed developmental analysis of the activities of the enolase isozymes, and propose a possible control mechanism.

**MATERIALS AND METHODS**

Developing COS (*Ricinus communis* L. cv Baker 296) endosperm were selected, homogenized, and the homogenates clarified by centrifugation as previously described (11). In *Ricinus*, it is difficult to determine the precise date of pollination. Hence, a staging system was developed, based upon seed size, endosperm fresh and dry weights, percentage of total lipid, and seed coat morphology, similar to the system described for developing cotton seeds (14). For germinating endosperm, quiescent seeds were imbibed overnight in running tap water (day 0), then planted in trays containing vermiculite and placed in a glasshouse at 28°C and ambient light. To remove some pigments and to concentrate proteins, developing seedling tissues or organelle fractions were homogenized in 10 volumes of −20°C acetone, then centrifuged and the acetone decanted. The pellet was rehomogenized in 5 volumes of −20°C acetone and recentrifuged. The acetone powders were allowed to air dry in a fume hood and then stored desiccated at room temperature. Acetone powders were homogenized in a manner identical to that for native tissues, but in 10 volumes of buffer containing 2 mM DTT, 2 mM ascorbate, and 2% insoluble PVP. Low mol wt compounds were removed by centrifugation of samples of clarified homogenates through 2.5-mL bead volume columns of Sephadex G-25, previously equilibrated with homogenization buffer, set in conical Corex tubes. Enzyme assays, separation of the enolase isozymes, immunochemical analyses, and all other materials and methods have been previously described (11–13). The data for total endosperm enolase activity were analyzed using the curve-fitting capability of the Sigmaplot 3.0 graphics program from Jandel Scientific. The program employs the Marquardt-Levenberg algorithm to fit an equation to the data by iterative nonlinear regression.

**RESULTS AND DISCUSSION**

Ion-filtration chromatography using Sephadex A-25 at pH 6.7 will separate the plastid and cytosolic isozymes of enolase from developing COS endosperm (11, 12). Although this method is rapid and has high resolution, relatively large amounts of starting material are required. For this reason, ion-exchange chromatography using DEAE-Sephasel, equilibrated under identical conditions, was used to separate the isozymes from tissues of developing castor oil plants. A typical ion-exchange profile, from young, rapidly expanding leaves, is presented in Figure 1.

Total enolase activity, as a function of age during endo-
Figure 1. Anion-exchange chromatography, using DEAE-Sephacel, of a clarified homogenate (A), a purified chloroplast fraction (B), and a cytosol fraction (C) prepared from young, rapidly expanding Ricinus leaves and assayed for enolase activity. In each case, the acetone powder from approximately 5 g of tissue was used. The column was equilibrated with 20 mM imidazole, pH 6.7, containing 2 mM MgCl₂. Actual enzyme activities in nmol min⁻¹ fraction⁻¹ can be obtained by multiplying ordinate values by 30 (A), 2.7 (B), or 21 (C). Recoveries following chromatography were 107% (A), 93% (B), and 89%.

sperm development, maturation, quiescence, and subsequent germination, is presented in Figure 2. Over a period of 9 months, endosperm were dissected from 100 developing seeds of various ages, individually homogenized, and the clarified homogenates assayed for total enolase activity (Fig. 2, A and D) or plastid enolase protein (Fig. 2, C and F). During seed germination and postgerminative growth, endosperm from 10 seeds were selected daily and individually analyzed.

At regular developmental intervals, 3 to 5 g of dissected endosperm were homogenized and total enolase activity resolved into the plastid and cytosolic isozymes by ion-exchange chromatography (Fig. 2, B and E). The peak endosperm activities of both total and plastid enolase occurred around 30 days after anthesis, during seed development, and after 3 to 4 d of postgerminative growth, coincident with peak levels of storage lipid accumulation and organelle membrane biogenesis, respectively.

Total enzyme activity per g fresh weight was determined for various tissues from Ricinus seedlings (Fig. 3). The highest

Figure 2. Endosperm enolase during development, maturation, quiescence, and subsequent germination of castor oil seeds. Panels A and D are total enolase activity; panels B and E are the proportion of total activity that can be attributed to the plastid isozyme; panels C and F are plastid enolase protein in the same samples as presented in panel A, as determined by ELISA. Values during germination are means ± se for 10 separate experiments.

Figure 3. Total enolase activity and the percentage distribution of the plastid and cytosolic isozymes in the various tissues of the castor oil plant. Tissues are: 1, mature leaves; 2, young, expanding leaves; 3, fully expanded green cotyledons; 4, stems; 5, roots.
total activity and the greatest proportion of plastid activity were found in young, rapidly expanding leaves. No plastid enolase activity was detectable in stems or mature leaves (Fig. 3), nor was plastid enolase protein detectable in these tissues by ELISA (data not presented). The highest plastid enolase activity in the seedlings was found in the cotyledons (18%), followed by young leaves (12%) and roots (7%).

We have previously demonstrated that plastid glycolysis can provide pyruvate that is subsequently converted to acetyl-CoA by the plastid pyruvate dehydrogenase complex (10, 16). This acetyl-CoA is the substrate for de novo fatty acid synthesis, which occurs exclusively within the plastids of higher plant cells. In agreement with this model, plastid enolase activity is highest during the periods of most rapid storage lipid accumulation during endosperm development (Fig. 2), or during periods of elevated membrane lipid synthesis (e.g. postgerminative growth and leaf expansion) (Figs. 2 and 3). Using plastid enolase as the reporter, it appears that there is little if any plastid glycolysis in mature or fully differentiated tissues. Acetyl-CoA is still necessary for membrane maintenance at these developmental stages, but could be provided by alternative pathways (e.g. 9, 19).

Whereas plastid enolase followed a developmental program parallel to that of storage lipid accumulation (Fig. 2B), plastid enolase protein did not (Fig. 2C). Activity declined rapidly to near zero levels in the mature, quiescent seed, whereas plastid enolase protein declined more slowly and was still detectable in the mature seed. It is possible that the loss of activity was due to some sort of posttranslational modification.

It has been reported that enolases from various sources can be phosphorylated, and that phosphorylation reduces catalytic activity (5). Alternatively, phosphorylation could be a signal for proteolysis (10). Proteolytic modification of plastid glycolytic isozymes has been demonstrated (3, 15), and a modified protein could give a positive ELISA signal, whereas catalytic activity was greatly reduced or absent. More detailed analyses than those reported here will be necessary to resolve the various possibilities.

Recently, enolase from higher plants has been addressed at the molecular level (8, 18). The results of Southern analyses of plant genomic DNA suggest that in most instances enolase is encoded by a small multigene family (18), consistent with the multiple enolase isozymes typically observed at the protein level (13). Arabidopsis and maize were exceptions in that when genomic Southern blots were probed with cytoplasmic enolase cDNAs, only a single gene was detected (8, 18).

Based upon the results of Southern analyses plus an inability to measure enolase in purified chloroplasts, Van Der Straeten et al. (18) suggested that Arabidopsis plastids lack at least part (enolase) of the glycolytic pathway. Although this could be true, such a conclusion is at this point premature. As we report herein, the ability to measure plastid enolase activity is dependent upon plant developmental state. Furthermore, there is not always immunochromial cross-reactivity between cytoplasmic and plastid isozymes of enolase (12) or other glycolytic enzymes (1, 2). Finally, there are instances in which cDNAs for plastid and cytoplasmic glycolytic isozymes have too little homology to generate positive signals on reciprocal Southern blots (2). Given the widespread occurrences of plastid enolase isozymes (13), it seems less likely that Arabidopsis is exceptional than that in this case inappropriate methods of detection were employed.

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LITERATURE CITED

2. Blakeley SD, Plaxton WC, Dennis DT (1991) Relationship between the subunits of leucoplast pyruvate kinase from Ricinus communis and a comparison with the enzyme from other sources. Plant Physiol 96: 1283–1289