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Purification, Properties and Phosphorylation of Anaerobically Induced Enolase in *Echinochloa phyllopogon* and *E. crus-pavonis*

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Enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) activity is differentially induced by anoxia in the flood-tolerant species *E. phyllopogon* (Stev.) Koss and the flood-intolerant species *E. crus-pavonis* (H.B.K.) Schult. To examine the regulation of enolase at the protein level, we purified the enzyme from both species to near homogeneity and compared their physico-chemical and catalytic properties. Enolase purified from *E. phyllopogon* exhibits optimal activity at pH 7.0, a K_m of 80 μM for 2-PGA, a Q_{10} of 1.97 and an E_a of 12.3 kcal mol^{-1}. Similarly, enolase from *E. crus-pavonis* exhibits optimal activity at pH 7.0, a K_m of 50 μM for 2-PGA, a Q_{10} of 2.04 and an E_a of 12.9 kcal mol^{-1}. The enzyme from both species is thermostable (100% active after 15 min, 50°C) and is a homodimer of 52.5 kDa subunits as resolved by SDS-PAGE and immunoblotting. *E. phyllopogon* enolase was phosphorylated in vitro using either [γ-^{32}P]ATP or [γ-^{32}P]GTP; however, enolase activity was neither stimulated nor inhibited by phosphorylation. Furthermore, addition of alkaline phosphatase had no effect on enolase activity. These findings suggest that factors other than phosphorylation regulate enolase activity under anaerobic stress. Likewise, since the properties of purified enolase from the two species are almost identical, the differential induction of activity under anoxia cannot be ascribed to possible differences in catalytic functions between the two enzymes.

Key words: Anaerobic stress — *Echinochloa crus-pavonis* — *Echinochloa phyllopogon* — Enolase (EC 4.2.1.11) — Phosphorylation — Rice weeds.

The anaerobic response in several species of *Echinochloa* and rice has been categorized into five classes based on their patterns of gene expression (Mujer et al. 1993). Of particular interest are the ASPs, the class of proteins induced during anoxia. Nine to 13 major ASPs were observed depending upon species. Some of the *Echinochloa* ASPs were identified as alcohol dehydrogenase, aldolase and pyruvate decarboxylase by enzyme activity measurement and immunoblotting (Rumpho and Kennedy 1981, 1983, Mujer et al. 1993). Through internal amino acid microsequencing Fox et al. (1995) identified ASP55 as enolase (2-phospho-D-glycerate hydrolyase, E.C. 4.2.1.11); a glycolytic enzyme that catalyses the dehydration of 2-PGA to PEP (Wold 1971).

Further investigation of the induction and regulation of enolase activity following the imposition of anaerobic stress revealed that the activity did not necessarily correlate with enolase protein levels, particularly in the intolerant species, *E. crus-pavonis*. In this species, enolase protein increased within 4 h of exposure to anaerobiosis (Zhang et al. 1994), whereas, enolase activity was only transiently induced, starting after 12 to 15 h of anoxia, afterwhich it declined (Fox et al. 1995). In contrast, in *E. phyllopogon*, an anaerobic-tolerant species, enolase activity and protein levels were immediately induced under anoxia and remain-

Abbreviations: ASP, anaerobic stress protein; ASP55, 55 kDa anaerobic stress protein; E_a, Arrhenius activation energy; Glygly, glycylglycine; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MOPS, 3-morpholinopropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; 2-PGA, 2-phosphoglycerate; PEP, phospho*enol*pyruvate; Q_{10}, temperature coefficient; STAT, signal transducer and activator of transcription.

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ed high after 12 h (Fox et al. 1995). One possible explanation for the discrepancy between enolase activity and protein level is posttranslational modification of the enzyme (Eigenbrodt et al. 1983, Cooper et al. 1984). Similar observations were reported for the plastid enolase from castor oil seed (Miernyk and Dennis 1992) and the salt stress-induced enolase from the ice plant, *Mesembryanthemum crystallinum* (Forsthoefel et al. 1995). In the mature castor oil seed, the rapid decline in enolase activity did not parallel the slow decline of enolase protein levels whereas in ice plant leaves, the significant induction of enolase activity by salt stress was not accompanied by a corresponding increase in the levels of enolase protein. In both studies, it was suggested that the discrepancy between enolase protein levels and activities could be due to posttranslational modification via reversible phosphorylation. It has been noted that most enzymes involved in the degradative pathways are active when phosphorylated and inactive when dephosphorylated (for a discussion see Ranjeva and Boudet 1987, Randall and Blevins 1990, Bennett 1991, Creighton 1993).

Enolases have been purified to apparent homogeneity from several plant sources such as potato tubers (Boser 1959) and spinach leaves (Sinha and Brewer 1984), and partially purified from maize (Lal et al. 1994), castor oil seeds (Miernyk and Dennis 1984) and pea seeds (Miller 1958). However, in none of these cases was the phosphorylation of the enzyme reported. Enolase phosphorylation has been demonstrated in Rous sarcoma virus-infected chicken embryo cells in which a single tyrosine is phosphorylated (Eigenbrodt et al. 1983, Cooper et al. 1984) and also from rabbit muscle (Reiss et al. 1986, Nettelblad and Engstrom 1987) and *Escherichia coli* (Dannelly et al. 1989).

To examine the regulation and eventually the role of enolase in anaerobic tolerance in *Echinochloa* species, we developed a purification scheme for enolases from *E. phyllopogon* and *E. crus-pavonis*. The catalytic and physical properties of the purified enzymes were compared for the tolerant and intolerant species which exhibit differences in induction of activity under stress. Furthermore, using the purified enzyme we examined whether posttranslational modification via phosphorylation might be involved in regulating enolase activity under anoxia.

Materials and Methods

Plant materials—Seeds of *Echinochloa phyllopogon* (Stev.) Koss and *E. crus-pavonis* (H.B.K.) Schult were surface sterilized with 50% Clorox (2.5% NaOCl), rinsed three times with sterile deionized water and vacuum infiltrated with sterile water for 10 min. The seeds were germinated aerobically in the dark in an incubator at 28°C for 5 days in Petri dishes lined with one layer of germination paper (Anchor Paper Co., St. Paul, MN, U.S.A.) wetted with 10 ml of sterile deionized water. The seedlings were transferred to an anaerobic chamber at 28°C (Forma Scientific, Inc., U.S.A.) which was flushed continuously with a 90% nitrogen–10% (v/v) hydrogen gas mixture. After 15 h of anaerobic treatment the seedlings were harvested, frozen with liquid nitrogen, and the shoots were separated from seeds and roots. All tissues were stored at −80°C until needed.

Enzyme extraction and purification—Initial steps of purification were done at 0 to 4°C. Subsequent chromatographic separations were conducted at 25°C unless indicated otherwise. Each experiment was repeated at least twice and all enzyme assays were run in duplicate.

Tissue homogenization—Shoot tissue (10 g fr wt) was ground to a fine powder in a mortar containing liquid nitrogen. When the temperature reached approximately 0°C, 10 ml of Buffer I [50 mM Tris (pH 7.6), 100 mM KCl, 10 mM $MgSO_4$, 0.5 mM EDTA, 0.05% 2-mercaptoethanol, 1 mM 2-PGA, 1 mM phenylmethylsulfonyl flouride, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone and 0.1 mM leupeptin] was added. The tissue was homogenized for 5 min and the homogenate clarified by centrifugation at 27,000 × *g* for 20 min at 4°C. The supernatant was collected and the pellet resuspended in 10 ml of Buffer I and recentrifuged as described. The supernates after the first and second centrifugations were combined and referred to as clarified homogenate.

Ethanol and ammonium sulfate fractionations—Cold 100% ethanol (stored at −20°C) was added slowly with gentle mixing to the clarified homogenate (kept in ice water) to bring the solution to 20% (v/v) ethanol. The solution was incubated for 30 min in ice water and centrifuged at 12,000 × *g* for 10 min at 4°C. The inactive precipitate was discarded and the supernatant fraction was adjusted to 60% (v/v) ethanol and allowed to stand for 30 min. After centrifugation, the pellet was redissolved in 6 ml of Buffer I and brought to 40% $(NH_4)_2SO_4$ saturation by adding saturated $(NH_4)_2SO_4$ (4.05 M in Buffer I without protease inhibitors). The solution was incubated for 30 min and centrifuged as described. The inactive precipitate was discarded and the supernatant fraction was brought to 80% saturation and allowed to stand for 15 h at 0°C. After centrifugation, the pellet was redissolved with 1.5 ml of Q-Sepharose Fast Flow Buffer II [50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.05% 2-mercaptoethanol and 1 mM 2-PGA]. The enzyme solution was dialyzed twice at 4°C for 2.5 h against 200 ml of Buffer II. The dialyzed sample was clarified by centrifugation at 12,000 × *g* for 10 min and the inactive precipitate was discarded.

Q-Sepharose fast flow ion exchange chromatography—The $(NH_4)_2SO_4$ fractionated extract was applied to a column (1.5 cm i.d. × 20 cm) of Q-Sepharose equilibrated with 5 bed volumes of Buffer II. After sample application, the column was washed with 2 bed volumes of Buffer II followed by a linear 200 ml NaCl gradient (0–0.25 M) in

Buffer II. Fractions of 5 ml were collected at a flow rate of 25 ml h^{-1}.

Sephadex G-150 gel filtration—The peak fractions of enolase activity from Q-Sepharose chromatography were pooled and concentrated at 4°C to a final volume of 0.5 ml by spinning (5,000 × *g*) in a Microsep Centrifugal Microconcentrator (MW cutoff = 10 kDa; Filtron Technology Corp., Northborough, MA, U.S.A.). The concentrated enzyme solution was applied to a column (1.5 cm i.d. × 100 cm) of Sephadex G-150 equilibrated with Buffer II. Five ml fractions were collected at a flow rate of 15 ml h^{-1}. Mol wt markers from a MW-GF-200 kit (Sigma, U.S.A.) were used to generate the calibration curve.

SDS-PAGE and immunostaining—The purity and identity of enolase were monitored using 12.5% SDS-PAGE as described by Laemmli (1970) and by immunostaining, respectively. After electrophoresis, the gels were stained with Coomassie brilliant blue G250 (Neuhoff et al. 1988), or transferred to Immobilon P membrane (Millipore Corp., Bedford, MA, U.S.A.) by electroblotting for 15 h at 15°C and 30 V using the methods of Towbin et al. (1979). The Immobilon P membrane was immunostained using the alkaline phosphatase system with bromo-chloro-indoyl-phosphate and nitroblue tetrazolium as substrates for color development as described by the manufacturer (Promega Technical Manual, Madison, WI, U.S.A.). Partially purified polyclonal enolase antiserum was prepared in rabbits as previously described (Fox et al. 1995).

Enolase assay—Enolase activity was monitored during purification in the direction of PEP formation by coupling the assay with pyruvate kinase and LDH reactions and monitoring NADH oxidation at 340 nm (Bergmeyer 1974). The reaction mixture contained 50 mM MOPS-NaOH (pH 7.0), 10 mM $MgSO_4$, 1 mM 2-PGA, 50 mM KCl, 15 mM NADH, 25 mM ADP, 6 units of LDH and 2 units of pyruvate kinase. The reaction was started by adding 10 µl of enzyme extract to a final volume of 1.0 ml.

To characterize the purified enzyme, enolase was assayed directly by measuring the increase in absorbance at 240 nm due to the formation of PEP (Wold 1971). The reaction mixture contained 1 mM 2-PGA, 10 mM $MgCl_2$, 50 mM MOPS-NaOH (pH 7.0) and 10 µl purified enolase in a final volume of 1.0 ml. Enolase activity was corrected for the variation of extinction coefficient of PEP at 240 nm as a function of pH (Wold 1971). One unit of enolase activity converted 1 µmol of 2-PGA to PEP min^{-1} at pH 7.0 and 30°C.

Protein determination—Protein in fractions from all column chromatography steps was monitored by absorbance at 280 nm. At all other steps of purification, protein was determined according to Bradford (1976) using the Bio-Rad (Hercules, CA, U.S.A.) protein stain with BSA as the standard.

In vitro phosphorylation using [γ-^{32}P]ATP or [γ-^{32}P]GTP—Crude homogenate from anaerobically-treated shoots of *E. phyllopogon* was incubated with 40 µCi of either [γ-^{32}P]ATP (3,000 Ci $mmol^{-1}$) or [γ-^{32}P]GTP (6,000 Ci $mmol^{-1}$) in 100 µl total reaction mixture consisting of 200 mM buffer (sodium acetate, pH 5.0 or Tris-HCl, pH 7.2 and 9.0), 50 mM KCl, 5 mM ATP or GTP, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, for 15 min with shaking at 30°C. Total proteins were precipitated in 12% trichloroacetic acid, washed with 100 mM ammonium acetate in methanol, subjected to SDS-PAGE and Western blotted to Immobilon P membrane for immunostaining and autoradiography.

Purified enolase (5 µg of pooled active fractions from Sephadex G-150) was phosphorylated in vitro at pH 9.0 in the presence or absence of crude extract (5.0 µg total protein; kinase source). The mixture was subjected directly to SDS-PAGE and processed as described above.

Determination of enolase activity after in vitro phosphorylation at different pHs—Crude slurry from anaerobically treated shoots of *E. phyllopogon* and *E. crus-pavonis* was prepared by powdering fresh tissues in liquid nitrogen and further homogenizing with extraction buffer (1 : 1 [v/v]) containing 100 mM sodium acetate (pH 4.0 and 5.0) or 50 mM MES-NaOH (pH 6.0) or 100 mM Tris-HCl (pH 7.2 and 8.0), 5 mM $MgCl_2$, 0.05% 2-mercaptoethanol and 10% glycerol. The slurry was incubated in a 100 µl total reaction mixture consisting of 200 mM of the appropriate buffer at pH 4.0, 5.0, 6.0, 7.2 and 8.0, 50 mM KCl, 5 mM ATP or GTP, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, for 15 min with shaking at 30°C. The phosphorylation reaction was stopped by immersing the tubes in ice water and centrifuging at 4°C for 10 min. Enolase activity was assayed from the supernatant using the coupled enzyme assay as described above.

Treatment of purified enolase with alkaline phosphatase—Concentrated purified enolase was incubated with one unit of alkaline phosphatase-beaded agarose (Sigma, U.S.A.) previously washed with water and resuspended in 50 µl reaction mixture containing 50 mM Glygly buffer (pH 8.5), 5 mM $MgCl_2$, 0.5 mM $ZnCl_2$ and 0.05% 2-mercaptoethanol essentially as described by Zhang et al. (1993). The reaction mixtures were incubated for various times at 30°C. The reaction was stopped by centrifugation to remove the phosphatase beads. A mixture with no alkaline phosphatase served as control. The supernatant fractions were analyzed for enolase activity using the coupled enzyme assay as described above.

Results and Discussion

Purification of enolase—A typical purification scheme for anaerobically induced enolase from *E. phyllopogon* is presented in Table 1. A similar scheme was used in purifying *E. crus-pavonis* enolase (data not shown). Inclusion of 2-PGA and $MgCl_2$ in the buffer stabilized enolase activity

Table 1 Purification of anaerobically induced enolase from shoots of *E. phyllopogon*

Purification step	Units (μmol min^{-1})	Protein (mg)	Specific activity (Units mg^{-1})	Purification factor	Yield (%)
Crude homogenate	33.2	40.26	0.82	1.0	100
20–60% EtOH	18.7	11.70	1.59	1.9	56
40–80% $(NH_4)_2SO_4$	19.4	3.40	5.69	6.9	58
Q-Sepharose	9.5	0.47	20.21	24.6	29
Sephadex G-150	7.2	0.07	102.80	125.4	22

during extraction and purification. In the absence of the substrate, 2-PGA, enzyme activity decreased by 50% within two days after extraction regardless of storage temperature (−80°C, −20°C, 4°C or 25°C).

Enolase activity was detected in the 20 to 60% ethanol fraction of the crude extract. From this fraction, enolase precipitated in the 40 to 80% $(NH_4)_2SO_4$ saturation range. Chromatography of the dialyzed 40 to 80% $(NH_4)_2SO_4$ fraction on Q-Sepharose at pH 7.6 gave a single peak of enolase activity. This peak eluted at 150 mM NaCl (Fig. 1A). Only 29% of the original activity remained after this step, but about 99% of the total protein was removed (Table 1). The active fractions (11–14) were pooled, concentrated by Microsep centrifugal concentrators and further purified by Sephadex G-150 gel filtration. A single peak of enolase activity was obtained after gel filtration (Fig. 1B). Enolase from *E. phyllopogon* was purified 125-fold to near homogeneity with a specific activity of 103 units (mg protein)$^{-1}$ (Table 1) whereas *E. crus-pavonis* enolase was purified 129-fold with a specific activity of 110 units (mg protein)$^{-1}$ (data not shown).

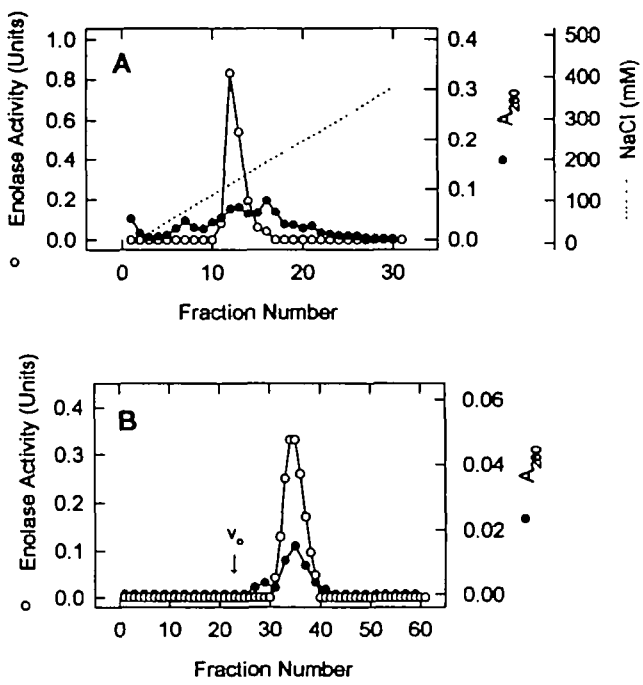

Fig. 1 Elution profiles of enolase from *E. phyllopogon* after Q-Sepharose ion exchange chromatography (A) and Sephadex G-150 gel filtration (B). One unit of enolase activity converted 1 μmol of 2-PGA min^{-1} at pH 7.0 and 30°C. V_o=void volume using blue dextran=57.5 ml.

SDS-PAGE and immunoblotting of the peak fraction from Sephadex G-150 resolved a predominant polypeptide with a molecular mass of about 52.5 kDa (Fig. 2A, B), similar to the values reported for spinach (Sinha and Brewer 1984) and yeast (Wold 1971) enolases which were 50 kDa and 47.6 kDa, respectively. The apparent native molecular mass of enolase from *E. phyllopogon* was estimated to be 100 kDa from gel filtration indicating that the native enzyme is a homodimer. However, enolase from *E. crus-pavonis* gave a much smaller native molecular mass of about 85 kDa (Fig. 3). This difference in mass may be due to the shape or three-dimensional structure of enolase in its native state, since the enzymes from both sources have similar subunit molecular mass of 52.5 kDa (Fig. 2). It is possible that posttranslational modifications such as phosphorylation may differentially modify the structure of enolase and hence its apparent molecular mass, as has been reported for other proteins (Martin 1993).

Attempts to further purify enolase by hydrophobic interaction chromatography using phenyl Sepharose CL 4B resulted in the complete loss of enolase activity. Likewise, enolase voided on a Con A agarose column suggesting the enzyme is probably not glycosylated.

During the course of our enzyme purification, we saw a single major peak of enolase activity after chromatography on Sephadex G-150 and Q-Sepharose (regardless of NaCl gradient used, i.e., 0–0.5 M, 0–0.25 M and 0–0.175 M). To investigate the possible presence of isozymes, we omitted the ethanol and $(NH_4)_2SO_4$ fractionation steps prior to chromatography and ran the crude homogenates directly on a Q-Sepharose column. A similar procedure was successfully employed to isolate enolase isozymes from developing castor oil seeds (Miernyk and Dennis 1984). In the aerobically grown and anaerobically treated *E. phyllo-*

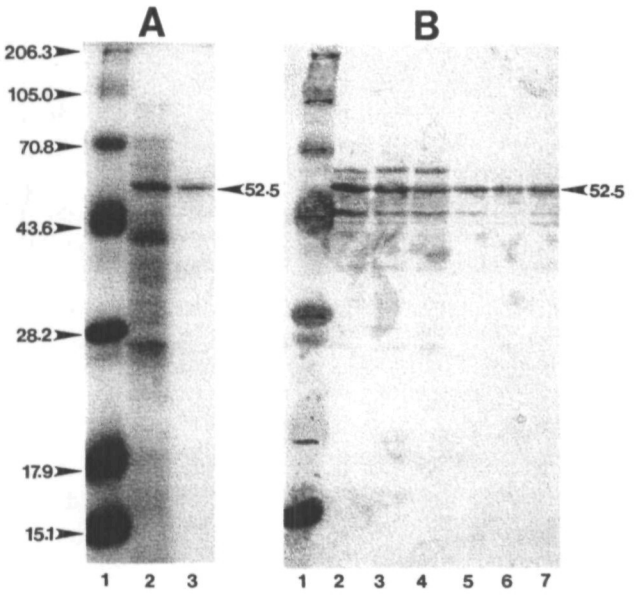

Fig. 2 Coomassie brilliant blue-stained gel after SDS-PAGE of purified *E. phyllopogon* enolase (A) and immunoblot of enolase at different stages of purification (B). (A) Lane 1, pre-stained SDS-PAGE mol wt markers (GIBCO BRL, U.S.A.) used: myosin heavy chain (206.3 kDa), phosphorylase B (105.0 kDa), bovine serum albumin (70.8 kDa), ovalbumin (43.6 kDa), carbonic anhydrase (28.25 kDa), β-lactoglobulin (17.9 kDa) and lysozyme (15.1 kDa). Lane 2, crude extract. Lane 3, peak fraction of purified enolase after Sephadex G-150 gel filtration. (B) Lane 1, pre-stained SDS-PAGE mol wt markers. Lane 2, crude extract. Lane 3, 20–60% ethanol pellet. Lane 4, 40–80% $(NH_4)_2SO_4$ pellet. Lane 5, peak fraction after Q-Sepharose chromatography. Lanes 6 and 7, peak fractions after Sephadex G-150 gel filtration of enolase from *E. phyllopogon* and *E. crus-pavonis*, respectively.

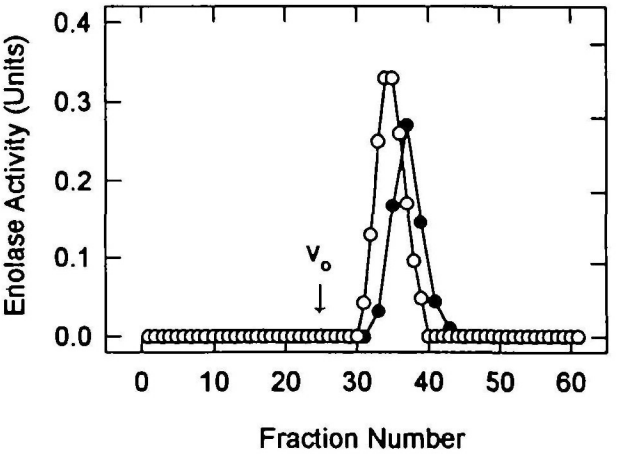

Fig. 3 Elution profiles of purified enolase from anaerobically grown shoots of *E. phyllopogon* (○) and *E. crus-pavonis* (●) after Sephadex G-150 gel filtration.

pogon shoots, a minor peak of activity preceded the elution of the major peak (data not shown). However, this minor peak disappeared when 2-PGA was included in the extraction and elution buffers, suggesting that this peak may be a product of limited enolase proteolysis in the absence of 2-PGA. The minor peak was not detected from shoots of anaerobically treated seedlings of *E. crus-pavonis* or from the green shoots of either species.

Overall, the above results do not indicate the presence of multiple molecular forms of enolase in *Echinochloa*. This conclusion is supported by Southern blot analysis of genomic DNA from *E. phyllopogon* and *E. crus-pavonis* which indicated the presence of only a single copy of the gene (Fox et al. 1995). Similarly, only one gene was reported for *Arabidopsis thaliana* (Van Der Straeten et al. 1991) and maize (Lal et al. 1991). In spinach (Sinha and Brewer 1984) and potato (Boser 1959, Sinha and Brewer 1984) only one enzyme was purified and characterized. However in spinach, three electrophoretic forms were detected in some pure preparations. The authors suggested that the two additional isoforms could be artifacts of the purification process (Sinha and Brewer 1984). In contrast, the existence of at least three gene copies has been demonstrated for rice, tomato and tobacco based on Southern blot analysis (Van Der Straeten et al. 1991). Furthermore, plastidic and cytosolic isozymes were partially purified and characterized from developing castor oil seeds (Miernyk and Dennis 1984).

pH optima and stability—Purified enolases from *E. phyllopogon* and *E. crus-pavonis* exhibited optimal activity at pH 7.0 (Fig. 4). The buffers used (MES, MOPS, HEPES and Glygly) had no significant effect on enolase ac-

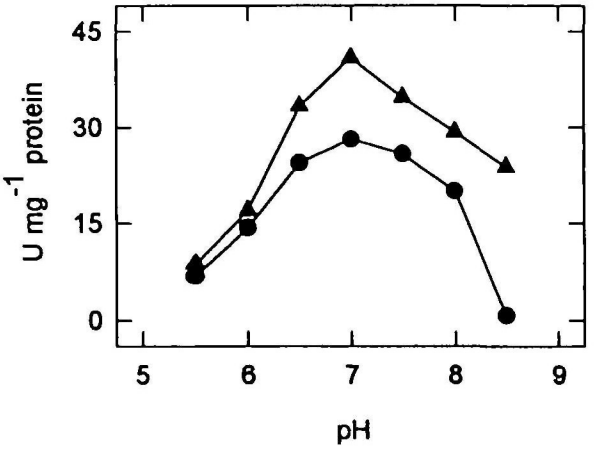

Fig. 4 Effect of pH on enolase activity. The following buffers were used: MES-NaOH (pH 5.5–6.5), MOPS-NaOH (pH 6.5–7.5), HEPES-NaOH (pH 7.5–8.0) and Glygly-NaOH (pH 8.0–8.5). ▲ (*E. phyllopogon*) and ● (*E. crus-pavonis*).

tivity. The optimum pH for activity is similar to that of castor oil seed enolase (pH 7.5) (Miernyk and Dennis 1984) and is one pH unit lower than those from spinach (pH 7.8–8.0) (Sinha and Brewer 1984) and pea seed (pH 8.0) (Miller 1958). *E. phyllopogon* enolase was stable when incubated for 1.5 h at 25°C from pH 7.0 to 8.5. However, when incubated at pH 5.5, only 25% of the original activity remained (data not shown).

Kinetic constant—From the Lineweaver-Burk plot (Fig. 5A), the apparent K_m for 2-PGA of *E. phyllopogon* enolase at pH 7.0 was estimated to be 80 μM and V_{max} was 104 units (mg protein)$^{-1}$. For *E. crus-pavonis* enolase the apparent K_m for 2-PGA was 50 μM (Fig. 5B) and V_{max} was 83 units (mg protein)$^{-1}$. The K_m values for enolase from

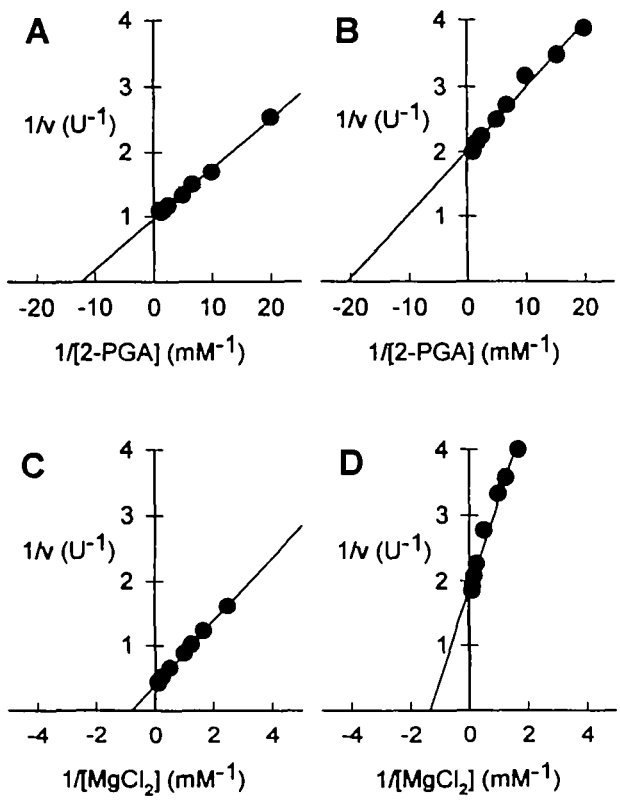

Fig. 5 Lineweaver-Burk plots of enolase activity from *E. phyllopogon* (A and C) and *E. crus-pavonis* (B and D) towards 2-PGA and $MgCl_2$. An apparent K_m of 80 μM and 50 μM for 2-PGA was calculated using the regression equations for enolase from *E. phyllopogon* ($y=0.9626+0.0774x$; $r^2=0.99$) and *E. crus-pavonis* ($y=2.008+0.0975x$; $r^2=0.99$), respectively. For $MgCl_2$, the apparent K_ms were 1.26 mM for *E. phyllopogon* ($y=0.3902+0.4919x$; $r^2=0.99$) and 0.73 mM for *E. crus-pavonis* ($y=1.863+1.365x$; $r^2=0.99$). V_{max} values for 2-PGA were estimated to be 104 units (mg protein)$^{-1}$ for *E. phyllopogon* and 83 units (mg protein)$^{-1}$ for *E. crus-pavonis* based on the concentration of the following purified enolase proteins: 0.010 mg ml^{-1} for *E. phyllopogon* and 0.006 mg ml^{-1} for *E. crus-pavonis*.

both *Echinochloa* species are similar to those from spinach (55 μM) (Sinha and Brewer 1984), castor oil seed (61 μM, cytosolic enolase) (Miernyk and Dennis 1984) and yeast (70 μM) (Wold 1971). In contrast, a higher K_m of 250 μM for 2-PGA was reported for pea seed enolase (Miller 1958).

The apparent K_m for Mg^{2+} for *E. phyllopogon* enolase was 1.26 mM (Fig. 5C) while that for *E. crus-pavonis* enolase was 0.73 mM (Fig. 5D). These values are similar to those obtained from castor oil seed enolase plastid isozyme (1.4 mM) and the cytosolic isozyme (1.2 mM) (Miernyk and Dennis 1984). In contrast, lower K_m values for Mg^{2+} were reported from spinach (60 μM) (Sinha and Brewer 1984), rabbit muscle (80 μM) and yeast (150 μM) enolase (Wold 1971).

Temperature coefficient and stability—The effect of temperature on activity at pH 7.0 was similar for both *E. phyllopogon* and *E. crus-pavonis* enolase. The temperature coefficient, Q_{10}, was 1.97 for *E. phyllopogon* enolase and 2.04 for *E. crus-pavonis* enolase between 25°C and 35°C. The Arrhenius activation energies (E_a) were 12.3 kcal mol^{-1} and 12.9 kcal mol^{-1} for *E. phyllopogon* and *E. crus-pavonis* enolase, respectively. The enzyme from both sources is thermostable. *E. phyllopogon* enolase remained fully active after 15 min of incubation at 50°C, 79% active at 60°C and was completely inactivated only after incubation for 15 min at 80°C (Fig. 6). *E. crus-pavonis* enolase exhibited a similar response to heat treatment except that it was only 50% active after incubation at 60°C (data not shown). SDS-PAGE of the heat-treated samples showed a decrease in the level of a 52.5 kDa protein in samples incubated at 70°C and 80°C (Fig. 6). Subsequent immunostaining identified this protein as enolase (data not shown).

Thermostable enolases have been reported previously from other plant and microbial sources. In castor oil seeds, cytosolic enolase remained 50% active after 10 min incubation at 60°C (Miernyk and Dennis 1984). Likewise, an octameric enolase from the anaerobic bacterium *Clostridium difficile* remained fully active after incubation for 5 min at 55°C (Green et al. 1993). Other thermostable octameric enolases have been characterized from the thermophilic bacteria *Thermus aquaticus* (Stellwagen et al. 1973) and *Thermus* X-1 (Barnes and Stellwagen 1973). Enolase from *T. aquaticus* and *T.* X-1 exhibited optimum assay temperatures of 90°C and 70°C, respectively. The enhanced thermostability of enolase from these thermophilic microorganisms has been positively correlated with amino acid residues capable of forming side-chain hydrogen bonds and negatively correlated with the average hydrophobicity of the enzyme molecule (Barnes and Stellwagen 1973). Taken together, the thermal stability of enolase is consistent with a previous report that an isoform of this enzyme is a heat shock protein and thus confers thermal tolerance in the yeast *Saccharomyces cerevisiae* (Iida and Yahara 1985).

	Incubation Temperature				
	4°C	50°C	60°C	70°C	80°C
Unit Activity (μmol PEP/min)	1.38	1.55	1.09	0.02	0.00
Protein (mg)	0.21	0.12	0.05	0.01	0.005
Specific Activity (U/mg)	6.6	12.9	21.8	2.0	0.00
Residual Activity (%)	100.0	100.0	79.0	1.4	0.00

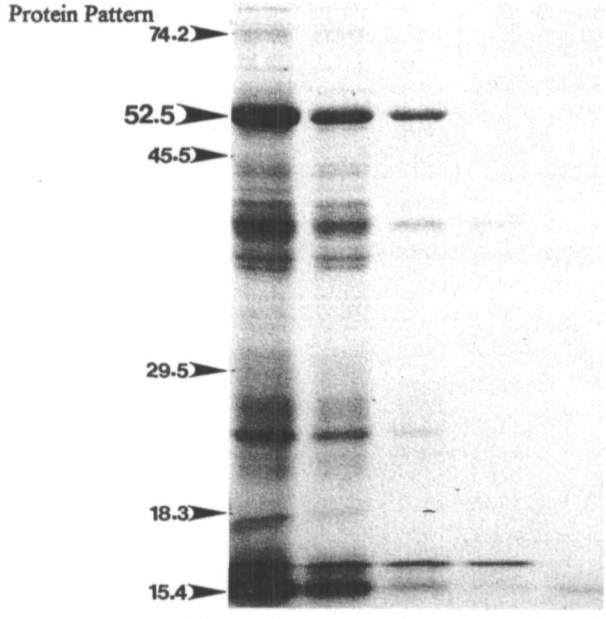

Fig. 6 Protein patterns and residual enolase activity from *E. phyllopogon* after heat treatment at various temperatures. Aliquots (100 μl containing 210 μg protein) of the partially purified enolase [40–80% $(NH_4)_2SO_4$ fraction dialyzed to pH 7.0] were incubated at various temperatures as specified above for 15 min, cooled rapidly in ice water for 5 min and spun at 4°C. Supernates were assayed for residual enolase activity at 30°C using the direct assay as described in "Materials and Methods". For SDS-PAGE, 25 μl of the supernatant were loaded in each gel lane. Protein patterns were visualized using Coomassie brilliant blue G-250. Numbers preceding arrows specify molecular masses in kDa of the indicated proteins. The 52.5 kDa protein is enolase based on immunoblotting (data not shown).

Divalent cation activation and inhibition—Enolase from *E. phyllopogon* and *E. crus-pavonis* required Mg^{2+} for maximal activity (Table 2). The enzyme was completely inactive when no metal ion was included in the reaction mixture. Saturating concentrations of Mn^{2+} or Co^{2+}, in the absence of Mg^{2+}, partially restored enolase activity from both sources. Zn^{2+} also activated *E. crus-pavonis* enolase (4%) but did not activate enolase from *E. phyllopogon*.

The requirement for Mg^{2+} in maintaining enolase activity has been well documented in all the characterization studies of the enzyme. It has been reported that under physiological conditions, each enolase subunit has a Mg^{2+} cation that is strongly bound in the conformational site and is necessary for catalysis (Brewer 1981). The partial activation of enolase by Mn^{2+}, Co^{2+} and Zn^{2+} in the absence of other metal ions has been reported previously for yeast and other plant enolases (Miernyk and Dennis 1984, Miller 1958, Sinha and Brewer 1984, Wold 1971).

Except for Li^+ and Na^+, all the other metal ions tested inhibited enolase activity from *E. phyllopogon* and *E. crus-pavonis* in the presence of 5 mM $MgCl_2$ in the reaction mixture (Table 3). Thus, in the presence of Mg^{2+}, the activating cations Mn^{2+}, Co^{2+} and Zn^{2+} inhibited enolase activity. In yeast, this inhibition was due to the binding of these metal ions to "inhibitory" sites whereas, inhibition by Ca^{2+} (a non-activating cation) was due to its binding to the catalytic site subsequently displacing Mg^{2+} (Elliott and Brewer 1980).

Enolase activity after in vitro phosphorylation and alkaline phosphatase treatment—During the course of our enzyme characterization, we were expecting to find differences in the catalytic properties of the two enzymes such as pH optimum, K_m or cation requirements in order to explain the differential regulation of activity under anoxia. However, since the physico-chemical and catalytic properties of enolase from *E. phyllopogon* and *E. crus-pavonis* were found to be the same, we explored the possibility that covalent modification such as phosphorylation might regulate its activity under anaerobic stress. To determine if enolase activity is regulated by phosphorylation, crude extracts of *E. phyllopogon* were incubated with either [γ-^{32}P]-ATP or [γ-^{32}P]GTP. We examined both ATP and GTP since different kinases have specific requirements for either nucleotide as substrate. The pattern of phosphorylated proteins at pH 5.0, 7.2 and 9.0 is shown in Fig. 7A. A 52.5 kDa protein which was labeled at pH 7.2 (lanes 2, 5 and 8) and more heavily labeled at pH 9.0 (lanes 3, 6 and 9) also immunostained with enolase antibody (Fig. 7B). EGTA did not inhibit phosphorylation (Fig. 6A, lanes 8 and 9) indicating that the kinase that phosphorylates enolase does not need Ca^{2+} for activity. Since the crude extract potentially contained other proteins of similar molecular mass to enolase which could also be phosphorylated, we repeated this labeling using the purified enolase from *E. phyllopogon* and *E. crus-pavonis* and a minimal amount of crude extract (5 μg protein) as the kinase source. A labeled immunoreactive band was detected with radiolabeled nucleotides and

Table 2 Divalent cation requirements of *Echinochloa* enolase

	% Enolase activity in 10 mM metal chloride				
	None	Mg^{2+}	Mn^{2+}	Co^{2+}	Zn^{2+}
E. phyllopogon	0	100	24	16	0
E. crus-pavonis	0	100	16	9	4

Metal ions were removed by passing samples through a small column of chelating resin (iminodiacetic acid) (Sigma, U.S.A.). The reaction mixture contained 10 mM of each cation and was preincubated for 2 min prior to the addition of 2-PGA. Enolase activity was determined at 240 nm as described in "Materials and Methods". The following chloride or sulfate salts did not activate the reaction: Ca^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Li^{+} and Na^{+}.

purified *E. phyllopogon* enolase at pH 9.0 (Fig. 8, lanes 2 and 4). Both ATP and GTP served as phosphate donors. Enolase was not phosphorylated when crude homogenate was omitted from the reaction mixtures (Fig. 8, lanes 3 and 5), suggesting that *E. phyllopogon* enolase cannot autophosphorylate and that a protein kinase is likely required for phosphorylation. In contrast, purified enolase from *E. crus-pavonis* was not phosphorylated with either ATP or GTP at pH 9.0 in the presence or absence of crude extracts from either *E. crus-pavonis* or *E. phyllopogon* (data not shown). In a control experiment in which phosphorylation was carried out with only the crude lysate from *E. phyllopogon* or *E. crus-pavonis* (5 μg protein) in the absence of purified enolase, no labeling of the band corresponding to a 52.5 kDa protein was detected (data not shown). This result ruled out the possibility that the label

Table 3 Response of *Echinochloa* enolase to varying concentrations of metal ions supplied as chloride salts

Metal ion added	Final concentration (μM)	% Enolase activity	
		E. phyllopogon	*E. crus-pavonis*
None		100	100
Ca^{2+}	10	108	87
	50	100	73
	500	53	63
Co^{2+}	10	100	84
	50	72	68
	500	44	49
Cu^{2+}	10	80	71
	50	61	61
	500	44	12
Fe^{3+}	10	100	85
	50	98	71
	500	93	73
K^{+}	10	104	87
	50	104	76
	500	99	87
Mn^{2+}	10	91	96
	50	65	72
	500	51	59
Zn^{2+}	10	56	80
	50	34	57
	500	28	44

Enolase activity was determined at 240 mm in the presence of 5 mM $MgCl_2$ as described in "Materials and Methods".

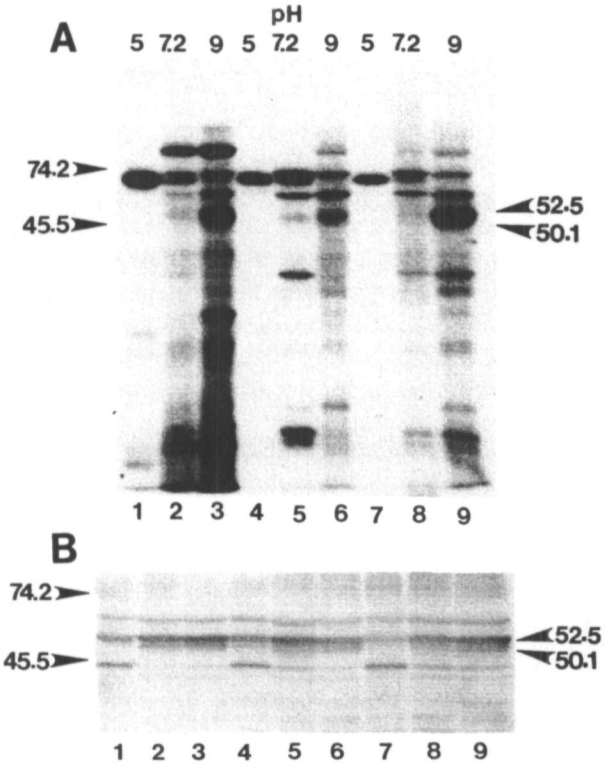

Fig. 7 Effect of pH on in vitro phosphorylation of crude homogenate from *E. phyllopogon* with [γ-^{32}P]ATP or [γ-^{32}P]GTP (A) and enolase immunoblot (B). Crude homogenates (200 μg protein) were phosphorylated in vitro, subjected to SDS-PAGE, and Western blotted to Immobilon P membrane. The membrane was autoradiographed and later immunostained for enolase. A and B, lanes 1–3, samples labeled with [γ-^{32}P]ATP; lanes 4–6, samples labeled with [γ-^{32}P]GTP; and lanes 7–9, samples labeled with [γ-^{32}P]-GTP in the presence of 5 mM EGTA. A 52.5 kDa protein was phosphorylated at pH 7.2 and 9.0. This same protein also immunostained with enolase antibody at pH 7.2 and 9.0.

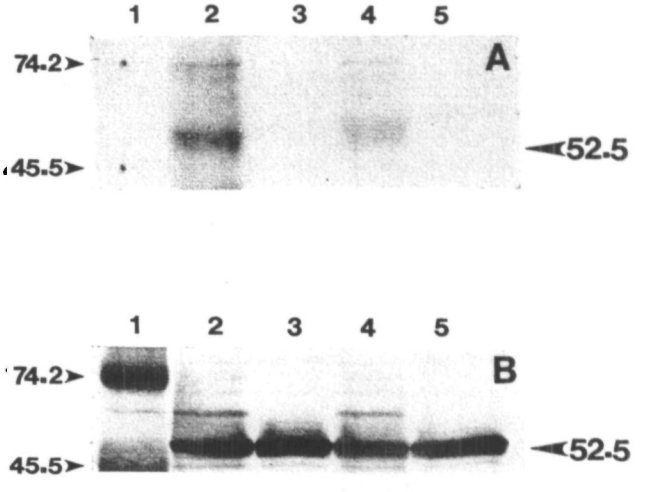

Fig. 8 In vitro phosphorylation of purified enolase from *E. phyllopogon* with [γ-^{32}P]ATP and [γ-^{32}P]GTP. Purified enolase (5 μg from concentrated peak fractions after Sephadex G-150) was phosphorylated in vitro at pH 9.0, in the presence or absence of crude extract (5 μg protein as kinase source), subjected to SDS-PAGE and Western blotted to Immobilon P membrane. The membrane was autoradiographed (A) and later immunostained for enolase (B). Lane 1, pre-stained SDS-PAGE mol wt markers; lanes 2–3, samples labeled with [γ-^{32}P]ATP in the presence and absence of crude homogenate, respectively; lanes 4–5, samples labeled with [γ-^{32}P]GTP in the presence and absence of crude homogenate, respectively.

in purified *E. phyllopogon* enolase was coming from the phosphorylation of a crude lysate protein.

Phosphorylation of enolase with either ATP or GTP did not appear to stimulate or inhibit enolase activity in the presence of $MgCl_2$ and $CaCl_2$ over a pH range of 4.0 to 8.0 (Table 4). Likewise, treatment with alkaline phosphatase at pH 8.5 for 0.5, 1 and 2 h at 30°C had no effect on enolase activity (data not shown). It is possible that if only a small

Table 4 Enolase activity after phosphorylation at different pH's

pH	Enolase activity (U (g fr wt)$^{-1}$)					
	E. phyllopogon			*E. crus-pavonis*		
	−ATP	+ATP	+GTP	−ATP	+ATP	+GTP
4.0	0.80	0.75	0.80	0.68	0.67	0.69
5.0	0.64	0.64	0.64	0.45	0.45	0.43
6.0	1.34	1.39	1.45	0.63	0.63	0.64
7.2	1.18	1.34	1.07	0.60	0.60	0.64
8.0	1.34	1.39	1.34	0.58	0.58	0.59

Crude slurry from anaerobically treated shoots of *Echinochloa* was incubated with either ATP or GTP and the supernatant assayed for enolase activity.

fraction of *Echinochloa* enolase is phosphorylated, as reported for Rous sarcoma virus enolase (Cooper et al. 1984), we may not be able to detect the effect of this modification on enzyme activity. The absence of any such effect may be due to masking by a larger non-phosphorylated active population of enolase. Thus, it is necessary to determine if different populations of enolase protein exist and whether they can be separated and have different functions. Finally, it should also be noted that in order to prove the physiological significance of this modification, enolase phosphorylation needs to be demonstrated in vivo.

Interest in whether there is a specific phosphorylated population of enolase arises because this protein may function other than as an enzyme, for example in translocation into the nucleus, processing or DNA-binding and transcription-activation. Such possibilities have been reported previously for phosphotyrosine-containing proteins in animals such as the STAT proteins and MAPK (Wang 1994, Shuai et al. 1993a, b). Tyrosine phosphorylation is essential for translocation into the nucleus, and also for the DNA-binding and transcription-activation function of the STAT protein. Similarly, phosphorylation of a threonine and a tyrosine residue in the kinase domain of MAPK by a variety of extracellular signals is required for entry of MAPK into the nucleus. It is interesting to note that yeast enolase has been shown to bind single-stranded DNA (Al-Giery and Brewer 1992). The authors noted that the interaction is stronger at more acid pH values and could be due to the formation of ion pairs between the positively charged side chains of histidine in enolase and the negatively charged phosphates of DNA.

Enolase, in addition to its glycolytic activity, has also been identified as a structural protein in several organisms whereby it protects the cellular contents against environmental insults (Wistow and Piatigorsky 1987, Wistow et al. 1988, Ingolia and Craig 1982). In yeast, for example, ENO1 is inducible by heat shock where it is proposed to act as a thermal protectant by stabilizing DNA during stress (Iida and Yahara 1985). The lens structural protein τ-crystallin has been identified as α-enolase in different vertebrates (Wistow et al. 1988). Several of these lens crystallin proteins have similar amino acid sequences to nuclear heat shock proteins in *Drosophila* (Ingolia and Craig 1982). Moreover, $\gamma\gamma$ enolase was reported to be present at the centrosome of HeLa cells throughout the cell cycle, where it was suggested to play a structural role in the organization of the interphase cytoskeleton and the mitotic spindle (Johnstone et al. 1992). There is no comparable information relevant to higher plants. However, it is interesting to note that in the ice plant, *M. crystallinum*, enolase transcript levels increased significantly in the leaves in response to a variety of environmental stresses which include salt, low temperature, drought and anoxia (Forsthoefel et al. 1995). The transcript levels also increased in the roots in response to heat stress and all of the above stresses except drought.

It is legitimate to speculate that in anaerobic tolerant *Echinochloa*, the immediate induction of enolase protein following the imposition of anoxia (Zhang et al. 1994) is an essential survival response to prevent certain vital cellular structures from being destroyed. Since the appearance of enolase protein precedes the detection of its catalytic activity by about 12 h after the onset of anaerobic stress (Fox et al. 1995), the initial function of this protein may be more structural than enzymatic. The role of phosphorylation during this period remains to be determined. Ultrastructural studies coupled with immunolocalization, particularly during the early period of anaerobic stress, will be very useful in determining the subcellular distribution of enolase and hence, its possible structural role during anoxia.

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