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Lactate Dehydrogenase in *Oryza sativa* L. Seedlings and Roots

Identification and Partial Characterization

Jean Rivoal[1], Bérénice Ricard[2], and Alain Pradet*

Institut National de la Recherche Agronomique, Station de physiologie Végétale, BP 131, 33140 Pont-de-la-Maye, France

ABSTRACT

A lactate dehydrogenase activity is present in rice (*Oryza sativa* L.) seedlings and roots. Under aerobic conditions, lactate dehydrogenase activity is barely detectable in rice seedlings and is very low in rice roots. In 30 day old roots, the activity is increased two to three times by an anoxic or hypoxic treatment and can be detected on immunoblots by an antiserum raised against barley lactate dehydrogenase. The activity present in aerobic seedlings was partially purified. The native enzyme has a molecular mass of 160 kilodaltons, and is a tetramer of 2 subunit (38 and 39 kilodaltons) randomly associated. Studies of substrate specificity, native gel electrophoresis, and immunoblot analysis indicate that the partially purified enzyme is a typical lactate dehydrogenase. However, no increase of lactate dehydrogenase activity or protein was observed in seedlings transferred to anoxia.

Anaerobic stress in plants results in a rapid change in the pattern of gene expression (21). Under such conditions, the induction of fermentative metabolism (5) can be regarded as an adaptative phenomenon to maintain the capacity for ATP synthesis in the absence of aerobic respiration. Unlike the situation in animals, the main glycolytic end product in plants is ethanol (5). The two enzymes of the ethanol biosynthetic pathway, ADH[3] (EC 1.1.1.1) and PDC (EC 4.1.1.1), are induced by anoxic stress (6, 13, 15). However, fermentative pathways other than that leading to ethanol may operate in anaerobic seeds and seedling tissues. Lactate glycolysis has been reported to take place in anaerobic roots (22) and in germinating seeds (1, 12), but this may be a transient phenomenon (14). Indeed, Davies *et al.* (4) have hypothesized that in the first minutes of hypoxia, lactic acid produced by constitutive LDH (EC 1.1.1.27), acidifies the cytoplasm, thus inducing ethanol production after a lag phase. Failure to regulate this pathway leads to acidosis and subsequent death (19, 20). On the other hand, Hanson *et al.* (7, 10) showed that LDH activity was hypoxically inducible in barley roots and aleurone tissue and that lactate was secreted in the medium. They suggested that inducible LDH may have an unexplained role. However, in rice embryos germinated for 48 h and submitted to anoxia, ethanol fermentation occurs without a lag phase and in the absence of transient lactic fermentation (17). None of these phenomena are predicted by the Davies hypothesis. To further investigate these discrepancies, work was initiated to study the presence and role of LDH in rice seedlings and roots.

This paper presents results indicating that an LDH activity is present at low levels in 48 h rice seedlings; this level remains low upon transfer to anoxia. The aerobic enzyme was partially purified and characterized as an authentic L-lactate dehydrogenase. In rice roots, LDH activity and protein levels are hypoxically and anoxically inducible.

MATERIALS AND METHODS

Reagents and Buffers

All the chemicals were of reagent grade from Sigma or Merck. Purified potato LDH was from Sigma. Columns and gel matrices were obtained from Pharmacia, ^{35}S-labeled protein A and nitrocellulose (HYBOND C EXTRA) from Amersham, and products for electrophoresis from Serva.

Plant Material and Growth Conditions

Rice (*Oryza sativa* var Cigalon) seeds were decontaminated and germinated for 48 h under aerobic conditions, then incubated under anaerobic conditions as previously described (17). The treatment was terminated by quick-freezing in liquid nitrogen.

For the hypoxic treatment of roots, rice seeds were germinated aerobically for 48 h as described above and then transplanted to 20 L plastic tanks and grown hydroponically in a sterilized nutrient solution (1.33 mM K^+, 0.62 mM Mg^{2+}, 1.3 mM Ca^{2+}, 1.49 mM NH_4^+, 0.2 mM Na^+, 4.8 mM NO_3^-, 0.62 mM SO_4^{2-}, 0.56 mM PO_4^{3-}, 0.2 mM Cl^-, 0.36 mM Fe^{3+}, 50 μM BO_3^{3-}, 20 μM Mn^{2+}, 6.9 μM Zn^{2+}, 2 μM Cu^{2+}, 0.08 μM $Mo_7O_{24}^{6-}$) (pH 5.7) in a phytotron (24°C, light 14 h, 250 μmol photons $\cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density) for 15

[1] Present address: MSU-DOE Plant Research Laboratory, East Lansing, MI 48824–1312.

[2] Present address: ILRAD, P. O. Box 30709, Nairobi, Kenya, Africa.

[3] Abbreviations: ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; pO_2, oxygen partial pressure.

or 30 d. The nutrient solution was continuously sparged with air (5 l/h). For the anaerobic treatment, the solution was sparged (5 l/h) with oxygen/nitrogen gas mixtures prepared and monitored to contain an oxygen partial pressure varying from 0.05 to 3 kPa. At various times, roots were removed, rinsed with sterile distilled water, blotted, quick-frozen in liquid nitrogen, and stored at −20°C until use. Under these conditions, microbial contamination was less than 10^6 germs per g of fresh plant tissue.

For the purification of LDH, rice seeds were grown for 7 d in the dark on vermiculite. Entire seedlings including roots were harvested, quick-frozen in liquid nitrogen, and stored at −20°C until use.

Extraction of LDH

Extractions were carried out at 4°C. Seedlings were homogenized in a small potter and roots in a mortar with a pestle in 20 mM Tris-HCl (pH 8), 5 mM Na ascorbate, 0.1 mM NAD, 1 mM EDTA, 5 mM DTT, 10% glycerol (v/v) (200 μL for 5 seedlings or 2 mL/g fresh weight for root tissue). The brei was centrifuged for 15 min at 12000*g*. Aliquots of the supernatant were analysed immediately for LDH activity or native gel electrophoresis.

Enzyme Asays and Protein Measurement

In the standard assay, LDH activity was measured spectrophotometrically in the pyruvate/lactate direction by recording the pyruvate-dependent NADH oxidation at 340 nm in the presence of pyrazole to inhibit ADH and KCN to lower the high background rate of NADH oxidation. The assay mixture contained 100 mM Tris-HCl (pH 8), 0.12 mM NADH, 15 mM Na pyruvate, 100 mM pyrazole, and 5 mM KCN. When activity was measured in the lactate/pyruvate direction, the assay mixture contained 100 mM glycine (pH 9), 10 mM Li lactate, 2 mM NAD, 100 mM pyrazole (17).

Protein was determined by the method of Bradford (3) using bovine γ-globulins as a standard.

Partial Purification of Rice LDH

The procedure was adapted from Jervis *et al.* (11) and optimized for rice LDH: 300 g of 7 d old dark grown rice seedlings were homogenized in 1 L of extraction buffer in a Waring blendor. After centrifugation for 15 min at 12,000*g* to remove cellular debris, the supernatant was fractionated with $(NH_4)_2SO_4$. The 30 to 80% pellet was resuspended in and dialyzed against 50 mM Tris-HCl (pH 8), containing 1 mM EDTA and 5 mM DTT. The dialyzed material was centrifuged for 15 min at 12,000*g* to remove the undissolved material and applied to a Blue Sepharose column (1.6 × 10 cm) at a flow rate of 60 mL/h. The column was washed with five bed volumes of dialysis buffer and LDH eluted with a linear gradient of 0 to 0.5 mM NADH in five bed volumes of the same buffer. The eluted proteins were concentrated and dialyzed over an Amicon YM 10 membrane. The enzyme solution, freed from NADH, was applied to a column of ATP agarose (2 mL bed volume). The column was washed with 20 mL of dialysis buffer. LDH was eluted with a linear gradient of 0 to 0.5 mM NADH in 10 mL of dialysis buffer adjusted to pH 7.9. The enzyme solution was concentrated, freed from NADH over Centriflo CF 25 membrane (AMICON), and kept at 4°C until further use.

Acrylamide Gel Electrophoresis, Isozyme Staining, and Immunoblot Analysis

PAGE under nondenaturing conditions was done as described by Hoffman *et al.* (10). Gels were stained for LDH (10), ADH (15), malate dehydrogenase (MDH, EC 1.1.1.37) (17), hydroxypyruvate reductase (EC 1.1.1.81), and glycollate oxidase (EC 1.1.3.1) activity (2). For immunoblot analysis, separated peptides were electrophoretically transferred to nitrocellulose. Immunodetection of LDH was done by coupling a rabbit antiserum raised against barley LDH (9) with ^{35}S-labeled protein A. SDS-PAGE was done as described (15).

Analytical Gel Filtration

Analysis of the native molecular mass of LDH was performed on a SUPEROSE 12 FPLC column (Pharmacia) equilibrated with 50 mM KH_2PO_4, 150 mM NaCl (pH 7). An aliquot of 150 μg of different molecular mass markers (urease tetramer, 480 kD; urease dimer, 240 kD; bovine albumin dimer, 132 kD; bovine albumin monomer, 66 kD; chicken ovalbumin, 45 kD; carbonic anhydrase, 29 kD; α-lactalbumin, 14.2 kD) or 1 nkat of partially purified LDH diluted in 100 μL of equilibration buffer was loaded onto the column at a flow rate of 0.5 mL/min. The eluted peaks were detected at 280 nm with an ISCO recorder. For LDH, 250 μL fractions were collected and LDH activity tested in these fractions as described above.

RESULTS AND DISCUSSION

Detection of LDH Activity in Rice Tissues

Crude extracts of rice seedlings germinated for 2 d under aerobic conditions gave no detectable LDH activity unless protective agents (Na ascorbate, NAD, EDTA, DTT, glycerol) were added to the extraction buffer. Inclusion of Na borate and BSA, however, did not improve rice LDH extraction, contrary to what has been reported by Hoffman *et al.* (10) for barley root LDH. Using the modified extraction buffer described in "Materials and Methods," very low but above background LDH activity could be measured in seedlings as well as in roots from plants grown hydroponically for 15 d. In 30 d old roots, LDH activity was clearly detectable. Transfer to anoxic conditions for 2 d or to hypoxic conditions for up to 15 d resulted in still low but nevertheless increased levels of LDH activity in rice roots. In rice seedlings, LDH activity also appeared to increase but the figures were too low for definite conclusions to be drawn. These results, expressed in nkat/mg proteins are summarized in Table I. Similar results were obtained with desalted extracts. On a fresh weight basis, LDH activity is 0.5 nkat/g fresh weight in 2 d rice seedlings under aerobic conditions.

Because enzyme measurements were done in the presence of pyrazole, an ADH inhibitor, it was unlikely that the NADH-dependent reduction of pyruvate could be due to the

Table I. *Effect of pO_2 on LDH Activity in Different Organs of Rice*

Crude extracts were prepared and LDH activity was measured in the pyruvate to lactate direction as described in "Materials and Methods." Activity values are means ± SE for at least three independent experiments.

Organ	pO_2	Treatment Length	LDH Activity
	kPa	*d*	*nkat/mg proteins*
2 d Seedlings	21		0.05 ± 0.03
	0	2	0.1 ± 0.05
7 d Seedlings	21		0.08 ± 0.05
15 d Roots	21		0.1 ± 0.05
	3	4	0.12 ± 0.04
	1	4	0.07 ± 0.05
	0.1	4	0.08 ± 0.05
	0.05	4	0.12 ± 0.03
30 d Roots	21		0.53 ± 0.08
	0	2	1.55 ± 0.16
	0.1	4	0.85 ± 0.05
	0.1	6	0.97 ± 0.1
	0.1	15	1.7 ± 0.15

coupled action of PDC and ADH. Furthermore, in rice roots, LDH was also assayed in the lactate/pyruvate direction. Although activities were even lower, they were generally sufficiently above background to be considered positive. In order to verify the low rates shown by the enzyme assays, activity gels of seedling and root extracts were run. In spite of the fact that activity gels are difficult to quantitate, they offered several advantages. Larger amounts of protein (200 μg instead of 20 to 100 μg in the spectrophotometric assay) could be analyzed; LDH activity could be distinguished from a number of nonspecific activities and the effect of inhibiting factors in crude extracts could be minimized. Identical amounts of proteins from rice seedlings or roots were analyzed on nondenaturing polyacrylamide gels which were then stained for LDH activity using the nitroblue tetrazolium method with L-lactate as substrate in the presence of pyrazole. No activity could be detected in extracts from rice seedlings, in agreement with the very low activities measured in enzyme assays. However, Figure 1 shows that LDH activity was revealed in rice roots of 30 d old plants. The activity in aerobic rice roots was barely detectable but clearly increased after 2 d of anoxic treatment. LDH activity was also induced by hypoxia and increased with the length of treatment. These results confirmed those obtained in Table I and provided the following additional controls (not shown): (a) There was no staining in the absence of lactate. (b) This native gel was also stained to reveal other enzymes which could have given nonspecific activity in the LDH assay (2). In the case of ADH and MDH, different activity profiles from LDH were obtained. In the case of glycollate oxydase, no activity could be detectable. (c) As expected for a LDH activity, pyruvate and hydroxypyruvate gave identical patterns on the native gel (2).

Immunoblot Analysis

To determine whether the increase in LDH activity in induced rice roots could be at least partially explained by an increase in LDH-specific protein, immunoblot analyses were done. Identical amounts of protein from hypoxically induced roots were separated on nondenaturing polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. Blots were probed with a 1:250 dilution of an antiserum to barley root LDH (a generous gift of A. D. Hanson). Visualization was with ^{35}S-labeled protein A. Figure 2 shows that the antiserum recognized rice LDH and that there was clearly an increased level of LDH-specific protein in rice roots after 2 d and 4 d of hypoxic treatment.

Partial Purification and Characterization of LDH from Aerobic Seedlings

Since activity gels and immunoblots did not allow us to substantiate the presence of LDH in aerobic seedlings, we partially purified the enzyme and characterized some of its properties. LDH activity in the crude extract was very low. The extract was concentrated by ammonium sulfate precipitation and LDH purified by sequential adsorption onto Blue Sepharose and ATP agarose affinity columns followed by specific elution with NADH, as summarized in Table II. Although the final enzyme preparation was purified about 1400-fold, the specific activity of 100 nkat/mg and SDS-PAGE analysis (not shown) demonstrated that it was still

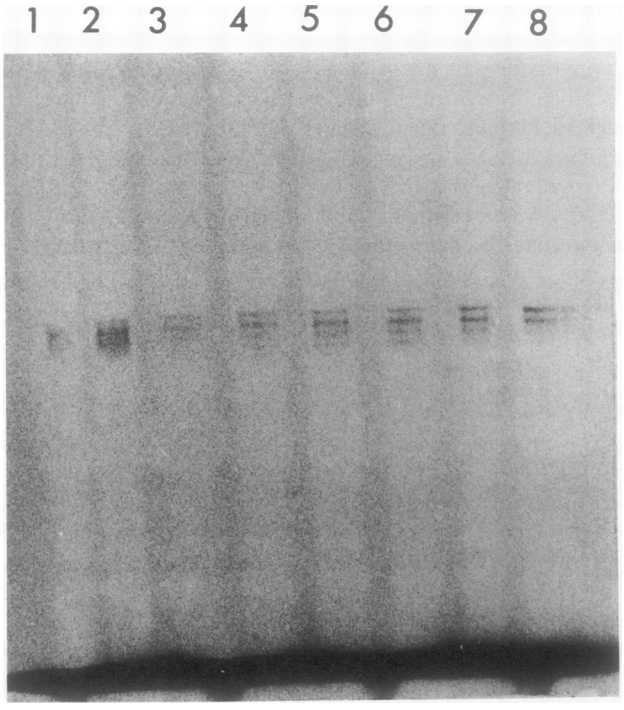

Figure 1. Analysis of 30 d old rice roots extracts by electrophoresis under native conditions. Proteins from crude extracts of rice roots grown during 30 d under aerobic conditions then transfered to hypoxia (0.1 kPa O_2) or anoxia for different times were separated on a native gel (200 μg on each lane) as described in "Materials and Methods." The gel was then stained for LDH activity by the nitro blue tetrazolium method. Lane 1, aerobic roots; lane 2, roots submitted for 2 d to anoxia; lanes 3 to 8, roots submitted to hypoxia for 2, 4, 6, 8, 10, and 15 d.

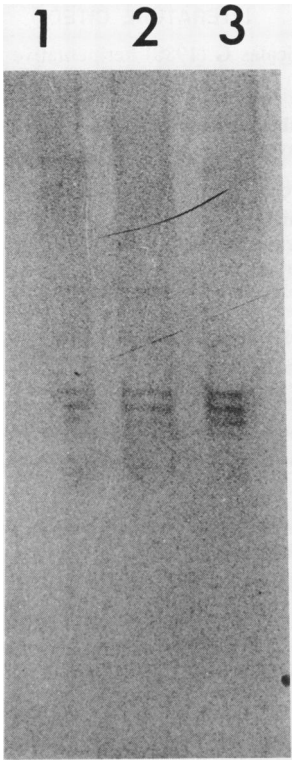

Figure 2. Immunodetection of LDH in extracts from rice roots submitted to hypoxia. Proteins from crude extracts of rice roots grown during 30 d under aerobic conditions then transferred to hypoxia were electrophoretically separated on a native gel (200 μg on each lane). Lane 1, aerobic roots; lane 2, 2 d hypoxic roots; lane 3, 4 d hypoxic roots. Immunodetection was carried out with an antiserum raised against barley LDH (1/250 dilution) and ^{35}S-protein A (10^5 cpm per lane).

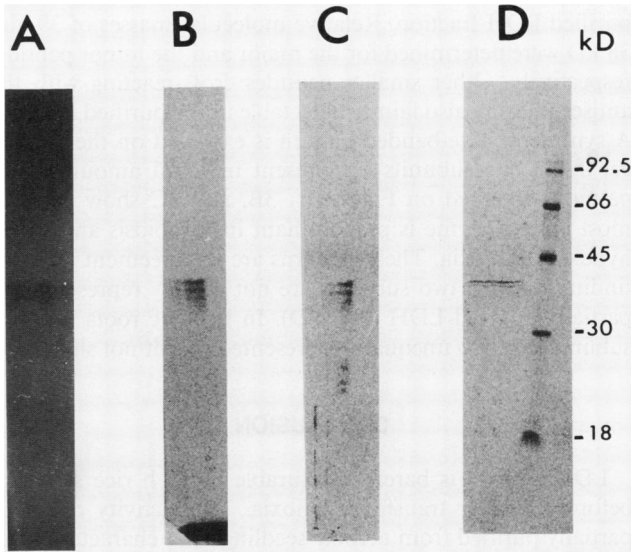

Figure 3. Electrophoretical and immunological analysis of partially purified LDH. Twenty micrograms of proteins of the partially purified LDH preparation were separated on a native gel (lanes A, B, C) or by SDS-PAGE (lane D). Lane A was stained for LDH activity in the pyruvate to lactate direction. Lane B was stained for LDH activity in the lactate to pyruvate direction. Lanes C and D were transferred to nitrocellulose and submitted to immunodetection with an antiserum raised against barley LDH as described in Figure 2.

contaminated with other proteins. The possible degradation or inactivation of LDH in the crude extract or during the first steps of the purification was ruled out because, when purified potato LDH was added to the crude extract, up to 70% of the activity was recovered in the dialysed $(NH_4)_2SO_4^{2-}$ pellet.

As expected for an L-lactate dehydrogenase, the enzyme catalyzed NAD^+-dependent L-lactate oxidation as well as NADH-dependent pyruvate reduction and showed no activity with D-lactate.

Determination of the molecular mass of the native enzyme by FPLC SUPEROSE 12 gel filtration (Pharmacia) gave a value of 160 kD. A similar molecular mass was obtained when purified potato LDH was analysed under the same conditions. This value also agrees with the molecular mass of LDH purified from barley roots (8). The partially purified rice enzyme preparation was also analyzed on a native polyacrylamide gel and stained for LDH activity as described above. An identical gel was submitted to immunoblot analysis. A single difuse band was revealed when pyruvate was the substrate (Fig. 3A) and four or five bands specific for LDH activity were observed in the presence but not in the absence of lactate (Fig. 3B). Attempts to measure other possible contaminating activities (ADH, glycollate oxidase, MDH) and to reveal their presence by activity staining after native gel electrophoresis gave negative results. We conclude that the activity detected on gel is a lactate dehydrogenase. The immunoblot shown in Figure 3C supported this conclusion: the activity bands corresponded to LDH-specific protein. The other immunoreactive bands were probably degradation products of LDH. Multiple bands of LDH activity have been observed for other plant LDHs and are interpreted as due to the presence of isozymes that are tetramers of two different peptides (8). Figure 3D shows the Western blot of the partially

Table II. *Partial Purification of Rice LDH from Aerobic Seedlings*

Step	Volume	Protein	Total Activity	Specific Activity	Yield	Purification Factor
	mL	*mg*	*nkat*	*nkat/mg*	%	*-fold*
Crude extract	1050	1428	103	0.07	100	1
$(NH_4)_2SO_4$ pellet	40	650	52	0.08	50	1.14
Blue sepharose	5.5	1.32	37	28	35	400
ATP agarose	0.22	0.14	16.6	118	16	1473

purified LDH fraction. Relative molecular masses of 39 and 38 kD were determined for the major and the minor peptide, respectively. Other smaller peptides cross-reacting with the antiserum were also faintly detectable in the purified fraction. A symmetric five-banded pattern is expected on the activity gel if the two subunits are present in equal amounts. The patterns observed on Figures 1, 3B, and 3C show that the most basic isozyme is predominant in aerobiosis and under hypoxia or anoxia. These patterns are in agreement with the finding that the two subunits are not equally represented in partially purified LDH (Fig. 3D). In hypoxic roots, the two subunits are also unequally represented (result not shown).

CONCLUSION

LDH activity is barely measurable in 48 h rice seedlings before and after transfer to anoxia. This activity could be partially purified from aerobic seedlings and characterized as an authentic L-lactate dehydrogenase. Like other plant LDHs (8), rice LDH is tetrameric, with subunit molecular masses of 39 and 38 kD. It is specific for L-lactate and NAD(H). There is no evidence that low activities are due to inactivation or inhibition in crude extracts. Indeed, the negative results of immunoblots suggested that low activity is due to low levels of LDH protein. Under aerobic conditions, LDH activity is 3.8 nkat/g fresh weight in maize roots (10). On a fresh weight basis, LDH activity is thus 7 to 8 times lower in rice seedlings than in maize roots. This is consistent with the fact that, contrary to the situation found in maize root tips (18), lactic fermentation cannot be detected when 2 d rice seedlings are transferred to anoxia (17). We therefore conclude that LDH cannot play a preponderant role in the initiation of ethanolic fermentation in rice seedlings. Roberts *et al.* (19, 20) showed that cytoplasmic acidosis was a determinant of flooding intolerance in plants. As uncontrolled lactic fermentation can lead to cytoplasmic acidosis (18), the very low level of LDH activity in rice embryo may be the result of an adaptative strategy for life under prolonged anoxia.

In rice roots of 30 d old plants, the low LDH activity was increased by hypoxia as well as by anoxia. There was a parallel increase in LDH-specific protein. The simplest explanation is that increased enzyme synthesis is responsible for the induction of LDH activity by low oxygen levels in rice roots. Because very few proteins are synthesized in root tissue under anoxia (16, 21), it would be logical to think that LDH synthesis serves a metabolic function. However, it should be pointed out that induction of LDH in rice roots is 10 to 20 times less than that observed in barley roots under similar conditions (10). What function could be served by such small amounts of LDH remains to be studied.

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