Expression of L-Lactate Dehydrogenase Isoenzymes during Root Development in *Crocus sativus* L. Corm

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Abstract
        Flavocytochrome b_2 activity was detected in *Crocus sativus* L. corm extracts prepared before and at 10 and 30 days after rooting. The activity was measured spectrophotometrically by following the reduction of cytochrome *c* at 550 nm or that of potassium ferricyanide at 420 nm. pH activity profiles revealed optima at pH 5.5, 7.5 and 9.5 before rooting, at pH 5.5 and 9.0 after 10 days rooting, and at pH 5.5, 7.5 and 9.5 after 30 days rooting, using potassium ferricyanide as substrate. Results were similar when cytochrome *c* was used as substrate, with optima at slightly higher pHs (7.0, 8.0, 9.0-10.0). Kinetic parameters (K_m, V_{max}) were measured for each extract at pH optima. Results showed that the catalytic efficiency at a given pH would change as rooting was taking place. Using potassium ferricyanide as substrate, at pH 5.5 the catalytic efficiency (expressed per mg protein in the extract) was 2.1 ± 0.3 min^{-1} before rooting, 8.1 ± 0.8 min^{-1} after 10 days rooting, and 2.5 ± 0.4 min^{-1} after 30 days rooting. At pH 7.5, it was 5.7 ± 0.4 min^{-1} before rooting, and 9.2 ± 0.8 min^{-1} after 30 days rooting. At pH 9.5, it was 0.4 ± 0.04 min^{-1} before rooting, 2.1 ± 0.3 min^{-1} (pH 9.0) after 10 days rooting, and 6.0 ± 0.8 min^{-1} after 30 days rooting. When the extracts were submitted to non-denaturing gel electrophoresis, activity staining for lactate dehydrogenase revealed three distinct bands before rooting, two bands after 10 days rooting, and three bands after 30 days rooting. Data suggested the presence of isoenzymes of lactate dehydrogenase selectively expressed before and during rooting in *C. sativus* corms. This may be relevant to the metabolic control of the cell respiration under these conditions.

INTRODUCTION

        Cellular respiration, one of the key energy production processes in most living systems, requires a number of enzymes involved in electron transfer. Among them cytochrome *c*, a ubiquitous redox protein within eukaryotes, is able to interact with several redox partners. Flavocytochrome b_2 (L-(+)-lactate ferricytochrome *c* oxidoreductase, EC 1.1.2.3) is one of them that transfers electrons resulting from the oxidation of L-lactate into pyruvate directly to cytochrome *c*. Studies in yeast have shown that the enzyme contains both FMN and protoheme capable of taking part in the catalytic reaction that transfers electrons from lactate to cytochrome *c* (or artificial electron acceptor such as potassium ferricyanide) (Pajot and Claisse, 1974; Capeillere-Blandin et al., 1975). As a respiratory enzyme, the production of flavocytochrome b_2 is induced by the presence of oxygen and more specifically by L-lactate. In addition to producing pyruvate from lactate, flavocytochrome b_2 participates in the following respiratory chain:
L-lactate → cytochrome b_2 → cytochrome *c* → cytochrome *c* oxidase → oxygen.
        Presumably four cytochromes *c* interact with a tetramer of flavocytochrome b_2 (Tegoni et al., 1993).
        The electron transfer steps, summarized by Capeillere-Blandin (1982) are as follows. The first step is a two-electron transfer from L-lactate to flavin mononucleotide within the flavodehydrogenase and the second a reversible intramolecular one-electron transfer between lactate-reduced flavin and heme b_2 taking place within an active site assembling these two related groups. The molecular structure of the enzyme, a tetramer of

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molecular weight 230,000 has been characterized (Xia and Mathews, 1990). Each subunit comprises two domains, one binding a heme and the other an FMN prosthetic group. The heme domain (residues 1 to 99) is folded in a fashion similar to the homologous soluble fragment of cytochrome b_5; the FMN domain (residues 100 to 486) is mostly sequestered from solvent. A cysteine cluster is critical for flavin binding (Pompon and Lederer, 1983). Mutants determining the relationship between structure and function have also been investigated (Tegoni et al., 1995; Cunane et al., 2002). Two molecular forms of flavocytochrome b_2 have been reported, one of them resulting from a proteolytic cleavage of the other and being thus smaller (Jacq and Lederer, 1972).

We previously identified the presence of flavoprotein b_2 in *Crocus sativus* L. dormant corms (Keyhani and Sattarahmady, 2002). Our results suggested the presence of up to three isoenzymes. The purpose of this research was to investigate the characteristics and the kinetic properties of ferricytochrome b_2 in *C. sativus* corms that had rooted for 10 and 30 days in distilled water and to compare them to the properties of the isoenzymes active in dormant corms.

MATERIALS AND METHODS

Extract Preparation

Dormant and 10-days- or 30-days-rooting *C. sativus* (hereafter named "saffron") corms were used throughout these studies. Dormant corms were without roots and shoots; 10-days-rooting corms exhibited 25 mm roots (average length) and 22 mm shoots (average length); 30-days-rooting corms exhibited 30 mm roots (average length) and 83 mm shoots (average length). For rooting, unearthed dormant corms were depleted from their sheathing leaves, cleaned from any dirt particle and placed in distilled water at room temperature. Corms were collected 10 and 30 days later. Extracts were prepared from corms weighing each between 3 and 6 g (weight before rooting), by homogenization in phosphate buffer 0.1 M, pH 7.0, and containing 0.02% phenylmethanesulfonyl fluoride as protease inhibitor. For 10-days- and 30-days-rooting corms, roots and shoots were carefully removed before preparation of the extracts. After centrifugation at 3,000 g for 10 min. then at 35,000 g for 30 min, a clear, transparent supernatant termed "crude extract" was obtained and used for our studies. Protein concentrations were determined by the Lowry method.

Enzymatic Activity Assays

L-Lactate dehydrogenase activity was determined by following the reduction of potassium ferricyanide at 420 nm with extinction coefficient 1040 $M^{-1}.cm^{-1}$. The activity was expressed in units (u) per mg extract protein; one unit was defined as the amount of enzyme that would reduce 1 μmole ferricyanide per minute. Assays were carried out at room temperature (~ 22-25°C), in 0.1 M phosphate buffer, pH 8.4, in the presence of 1 mM EDTA, 50 mM L-lithium lactate and 0.25 mM potassium ferricyanide, using Aminco DW2 and Milton Roy spectrophotometers. Results were average of 3 different experiments.

The pH activity curves were determined using a citrate-phosphate-borate buffer system (range 3-12) at a concentration of 0.1 M.

Activity Staining after Non-Denaturing Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was prepared according to Laemli (1970), except that sodium dodecyl sulfate and mercaptoethanol were omitted from all solutions. The gels prepared were 7% in acrylamide. Activity staining for L-lactate dehydrogenase was done according to Jacq and Lederer (1972). Briefly, after the electrophoresis was over, the gel was stained in 0.2 M phosphate buffer, pH 8.0, 20 mM DL-lactate, 0.2% tetrazolium blue. Bands were revealed after 10 to 30 minutes.

RESULTS AND DISCUSSION

Effect of pH and Substrate Concentration on Enzymatic Activity

Figure 1 shows the pH activity profiles for saffron corm extracts prepared before and after 10 and 30 days of rooting. The activity, expressed as units of enzyme per mg extract protein, was measured in the presence of 50 mM L-lithium lactate at different pHs, ranging from 4.0 to 10.0, and using ferricyanide as the electron acceptor. Clearly, the three peaks, respectively at pH 5.5, 7.5 and 9.5, found for dormant corms were reduced to two peaks, respectively at pH 5.5 and 9.0 for 10-days-rooting corms, while they were restored for 30-days-rooting corms. Results were similar when cytochrome c was used as substrate, with optima at slightly higher pHs (7.0, 8.0, 9.0-10.0).

As suggested by Fullbrook (1996) the presence of various pH optima indicates the presence of distinct isoenzymes. Thus under our experimental conditions, while three distinct isoenzymes of L-lactate dehydrogenase were detectable in dormant saffron corms, only two of them were detectable in 10-days-rooting corms, but all three were detectable again in 30-days-rooting corms.

The effect of ferricyanide concentration on its reduction rate by extracts from dormant, 10-days-rooting and 30-days-rooting saffron corm, at pH 5.5, is shown in Figure 2. Maximum activity was observed at about the same substrate concentration for dormant and 10-days-rooting corm extract (225 μM and 235 μM respectively), with substrate inhibition at higher concentration. In contrast, for 30-days-rooting corm extract, maximum rate was observed within a substrate concentration range of 220-380 μM; at higher concentrations, only mild substrate inhibition was observed, so that for 550 μM ferricyanide, the activity was reduced by only 20%. For dormant corms, the activity was reduced by 90% at 700 μM ferricyanide and for 10-days-rooting corms it was reduced already by 30% at 275 μM ferricyanide.

Figure 3 shows the effect of substrate concentration on the rate of reduction of ferricyanide by dormant and 30-days-rooting corm extracts, at pH 7.5. Maximum activity was observed at a substrate concentration of 70 μM for dormant corm and within a substrate concentration range of 70-210 μM for 30-days-rooting corms; thereafter, substrate inhibition reduced the activity by 87% for 350 μM ferricyanide in dormant corms while the activity was reduced by 55% only for 550 μM ferricyanide in 30-days-rooting corms.

As shown in Figure 4, at pH 9.5, the effect of ferricyanide concentration on its reduction rate was different for each extract. For dormant corms, maximum activity was observed for 570 μM substrate; thereafter, substrate inhibition was observed and the activity was reduced by 40% for a substrate concentration of 750 μM. For 10-days-rooting corms, at pH 9.0, maximum activity was observed for 280 μM substrate; thereafter, substrate inhibition was observed and the activity was reduced by 55% for a substrate concentration of 700 μM. For 30-days-rooting corms, at pH 9.5, maximum activity was observed over a substrate concentration range of 200-700 μM substrate; no substrate inhibition was observed with up to 700 μM ferricyanide.

Over all, substrate inhibition was much milder in 30-days-rooting corms than in dormant or 10-days-rooting corms at all pHs. Maximum activity was observed for approximately the same substrate concentration for dormant and 10-days-rooting corms at pH 5.5 and at approximately the same substrate concentration for dormant and 30-days-rooting corms at pH 7.5. At pH 9.0, maximum rate was observed for 10-days-rooting corms at about half the substrate concentration when compared to dormant corms. Interestingly, for 30-days-rooting corms, maximum activity was observed at a ferricyanide concentration less than half that required for dormant corms, but comparable to that observed for 10-days-rooting corms. However, contrary to dormant and 10-days-rooting corms, no substrate inhibition was observed with up to 700 μM ferricyanide.

Models have been proposed for L-lactate dehydrogenases substrate inhibition. By obtention of mutation in human heart L-lactate dehydrogenase, it was suggested that substrate inhibition was caused by the formation of a covalent adduct between pyruvate

and the oxidized form of the cofactor (Hewitt et al., 1999). Another model to explain substrate inhibition has also been presented by Kühl (1994). However, despite some progress, the mechanism of substrate inhibition is still unknown (Lin et al., 2001).

Kinetic Parameters

Table 1 shows the Michaelis-Menten constant (K_m), V_{max}, catalytic efficiency (calculated per mg extract protein) and the pseudo-first order rate constant for the L-lactate dehydrogenase activity found in dormant saffron corms at pHs 5.5, 7.5 and 9.5. The K_m, catalytic efficiency and pseudo-first order constant were different for all three pHs examined while the V_{max} was almost the same for pH 5.5 and 7.5, but diminished for pH 9.5.

In Table 2, the K_m, V_{max}, catalytic efficiency (calculated per mg extract protein) and pseudo-first order rate constant for the L-lactate dehydrogenase activity found in 10-days rooting saffron corms are shown for pHs 5.5 and 9.0. All kinetic parameters were different at the two pHs examined. The K_m value found at pH 5.5 for 10-days rooting corms was the same as that found at pH 5.5 for dormant dorms; however, the K_m value found at pH 9.0 for 10-days rooting corms was half that found at pH 9.5 for dormant corms. Similarly, while the V_{max} values at pH 5.5 were close in dormant and 10-days rooting corms, the V_{max} found at pH 9.0 for 10-days rooting corms was approximately half the V_{max} found at pH 9.5 for dormant corms. Catalytic efficiencies were drastically different at both pH when dormant and rooting corms were compared, but pseudo-first order rate constants were similar for a given pH.

The K_m, V_{max}, catalytic efficiency (calculated per mg extract protein) and pseudo-first order rate constant for the L-lactate dehydrogenase activity found in 30-days rooting saffron corms at pHs 5.5, 7.5 and 9.5 are shown in Table 3. At pH 5.5, K_m was doubled in 30-days rooting corms compared to 10-days rooting and dormant corms, while at pH 7.5, K_m was similar to that in dormant corms. Interestingly, the value of K_m found at pH 9.5 was highest in dormant corm; it was halved in 10-days rooting corms and halved again in 30-days rooting corms. V_{max} was lowest in 30-days rooting corms at pHs 5.5 and 7.5, when compared to dormant and 10-days rooting corms; at pH 9.5, the V_{max} found in 30-days rooting corms was close to that found in 10-days rooting corms. Catalytic efficiency in 30-days rooting corms was close to that in dormant corms at pH 5.5; at pHs 7.5 and 9.5, it was highest in 30-days rooting corms. Pseudo-first order kinetic constant was 4 times lower in 30-days rooting corms than in dormant and 10-days rooting corms at pH 5.5; it was 3 times lower in 30-days-rooting corms than in dormant corms at pH 7.5, but at pH 9.5, it was 3 times higher in 30-days-rooting corms than in dormant and 10-days-rooting corms.

Non-Denaturing Polyacrylamide Gel Electrophoresis and Activity Staining

When extracts from dormant, 10-days-rooting and 30-days-rooting corms were submitted to non-denaturing polyacrylamide gel electrophoresis, staining for L-lactate dehydrogenase activity revealed three bands for dormant and 30-days-rooting corms and two bands for 10-days-rooting corms. This confirmed that at least three isozymes of L-lactate dehydrogenase were present in dormant and 30-days-rooting corms, and that at least two isozymes were present in 10-days-rooting corms. As shown in the electrophoretogram of Figure 5, two of the three bands present in lanes 1 and 3 and the two bands in lane 2 were thick and migrated the same distance. The two thinner bands present in lanes 1 and 3 also migrated the same distance, indicated similar apparent molecular weight. As shown in Tables 1-3, even at a single pH optimum, differences were found between the kinetic parameters of dormant corms and corms that rooted for various lengths of time. This suggested that more than one isozyme could be present that showed the same pH optimum, but the electrophoretic conditions were not suitable to separate them.

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CONCLUSIONS

L-Lactate dehydrogenase has been shown to be in single (Betsche, 1981), or three (Rothe, 1974) isoforms in various organisms. Kinetics as well as non-denaturing gel electrophoresis results reported in this paper showed the presence of at least three isozymes of L-lactate dehydrogenase in dormant and 30-days-rooting saffron corms and at least two isozymes in 10-days-rooting corms. It is likely that at least one of the isozymes active in 10-days-rooting corms was also detectable in dormant corm (that with an optimum pH of 5.5) while the other (that with an optimum pH of 9.5/9.0) showed different kinetic parameters and was probably an isoform. Similarly, the isozyme with optimum pH of 7.5 detectable in dormant corms was also active in 30-days-rooting corms, while the other enzymes in 30-days-rooting corms showed different kinetic parameters and could be isozymes different from those present in dormant or 10-days-rooting corms. Thus isoenzymes of L-lactate dehydrogenase were expressed at different stages of corm development. The multiplicity of isoenzymes as well as their sequential expression with subtle differences in kinetic parameters reflected their important role in the metabolism taking place in the bulb, such as the switch from hypoxy in dormant corms to high oxidative activity in rooting corms. Furthermore, while various isoenzymes of NADH-dependent lactate dehydrogenase have been identified in plants, and particularly in seeds and tubers (Rothe, 1974; Jervis at al., 1981), here we show the important role played by the NADH-independent lactate dehydrogenase, flavocytochrome b_2.

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Tables

Table 1. Kinetic parameters at optima pHs for dormant corms

pH	K_m (μM)	V_{max} (μM.min^{-1})	V_{max}/K_m (min^{-1})[1]	k[2] (min^{-1})
5.5	70 ± 5	130 ± 10	2.1 ± 0.2	0.77 ± 0.05
7.5	30 ± 5	150 ± 10	5.7 ± 0.4	2.2 ± 0.1
9.5	270 ± 10	100 ± 10	0.4 ± 0.04	0.2 ± 0.02

[1]Calculated per mg extract protein; [2]pseudo-first order rate constant

Table 2. Kinetic parameters at optima pHs for 10-days-rooting corms

pH	K_m (μM)	V_{max} (μM.min^{-1})	V_{max}/K_m (min^{-1})[1]	k[2] (min^{-1})
5.5	70 ± 5	170 ± 10	12 ± 2	0.92 ± 0.08
9.0	126 ± 10	55 ± 5	02.1 ± 0.1	0.21 ± 0.02

[1]Calculated per mg extract protein; [2]pseudo-first order rate constant

Table 3. Kinetic parameters at optima pHs for 30-days rooting corms

pH	K_m (μM)	V_{max} (μM.min^{-1})	V_{max}/K_m (min^{-1})[1]	k[2] (min^{-1})
5.5	138 ± 5	66 ± 8	2.5 ± 0.4	0.22 ± 0.03
7.5	28 ± 4	43 ± 5	9.2 ± 0.8	0.8 ± 0.08
9.5	55 ± 5	70 ± 10	6.0 ± 0.8	0.6 ± 0.05

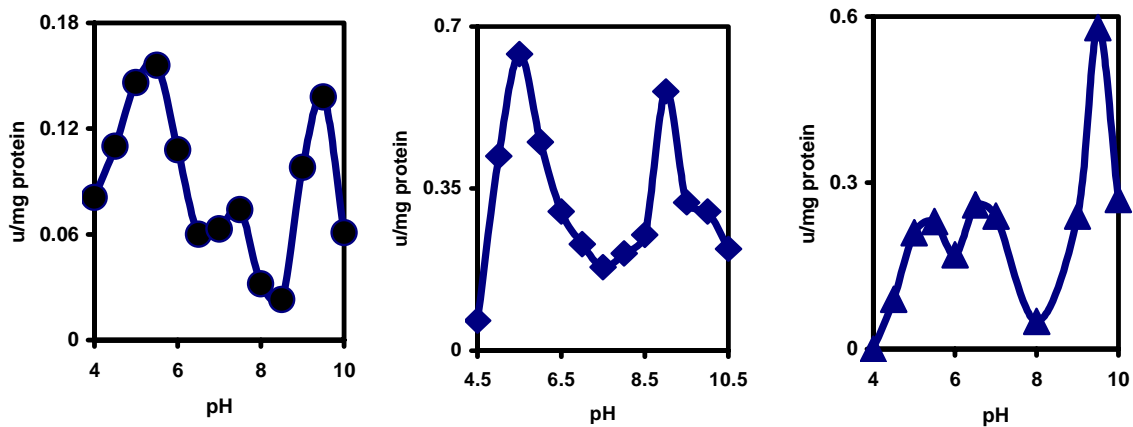[1]Calculated per mg extract protein; [2]pseudo-first order rate constant

Fig. 1. pH activity profile of L-lactate dehydrogenase activity in dormant (●), 10-days-rooting (◆) and 30-days-rooting (▲) corms, using ferricyanide (280 μM) as electron acceptor.
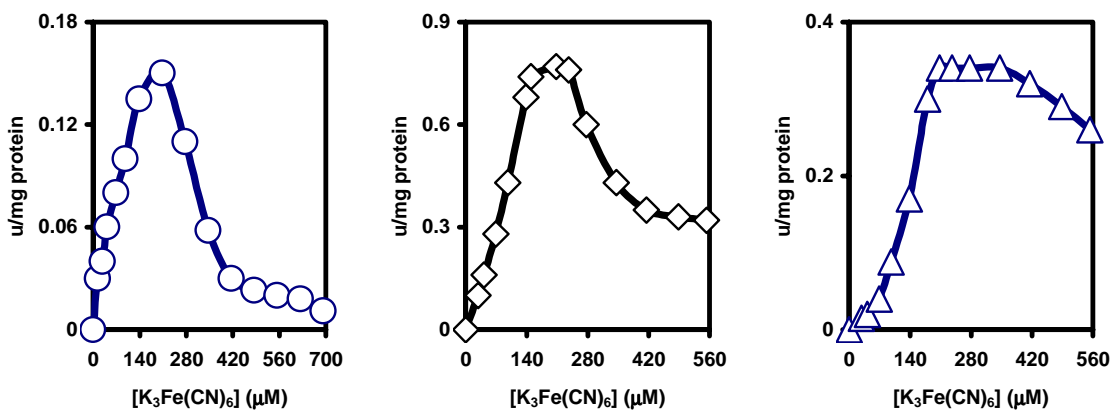

Fig. 2. Effect of ferricyanide concentration on L-lactate dehydrogenase activity in dormant (○), 10-days-rooting (◇) and 30-days-rooting (Δ) corms at pH 5.5. Note the strong substrate inhibition in dormant and, to some extent, in 10-days-rooting corms compared to 30-days-rooting corms.
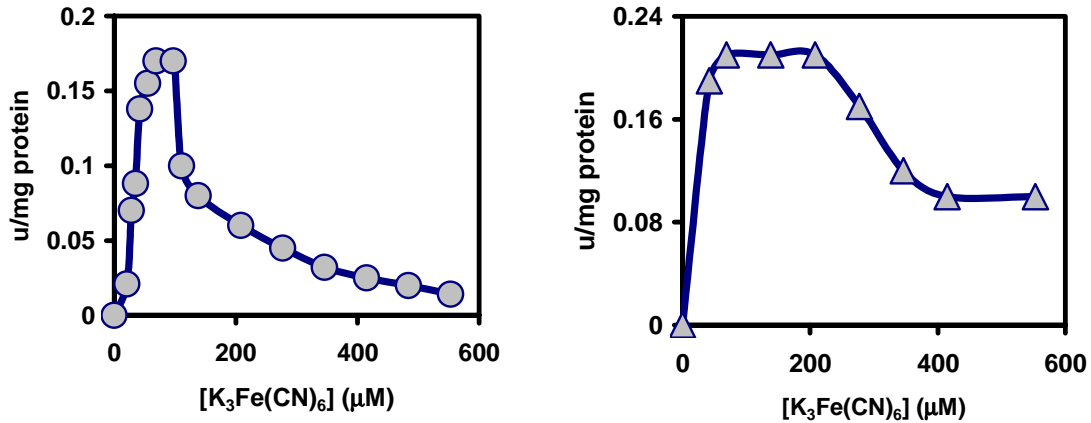
Fig. 3. Effect of ferricyanide concentration on L-lactate dehydrogenase activity in dormant (○) and 30-days-rooting (△) corms at pH 7.5. Note the marked substrate inhibition in dormant corms.
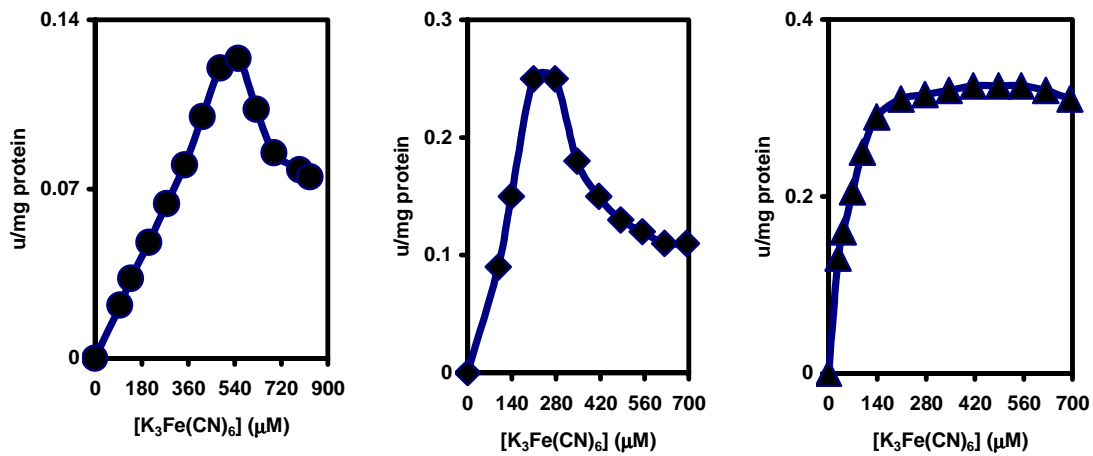

Fig. 4. Effect of ferricyanide concentration on L-lactate dehydrogenase activity in dormant (■), 10-days-rooting (◆) and 30-days-rooting (▲) corms at pH 9.5. Note that no substrate inhibition was observed for 30-days-rooting corms with up to 700 μM ferricyanide.
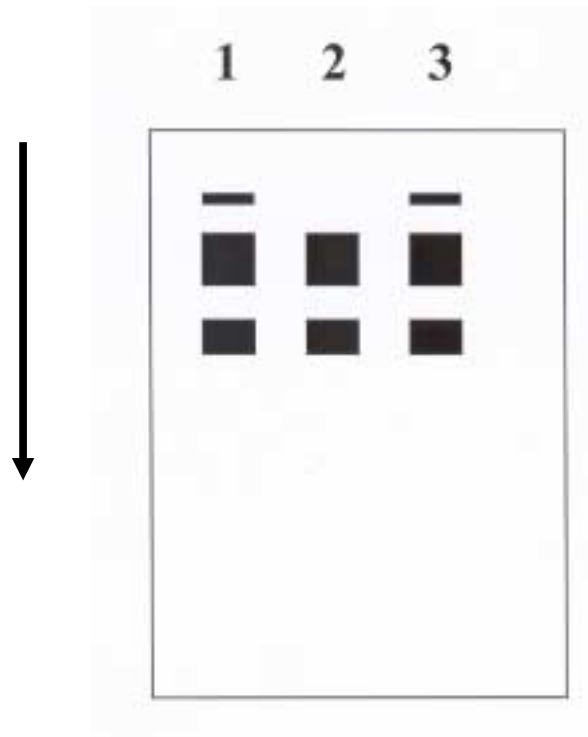
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Fig. 5. Polyacrylamide gel electrophoresis patterns of ʟ-lactate dehydrogenases. Lane 1 represents the isoenzymes pattern for dormant corm extract; lane 2 represents the isoenzymes pattern for 10-days-rooting corm extract; lane 3 represents the isoenzymes pattern for 30-days-rooting corm extract. Arrow indicates direction of electrophoresis which was performed in non-denaturing 7% polyacrylamide gel.