Lactate Dehydrogenase in Fresh-cut Carrot Shreds Stored under Low Oxygen Atmosphere

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ABSTRACT. Carrot (*Daucus carota* L.) root shreds were stored under a continuous flow of 0.5% and 2% O₂ (balance N₂) or air at 5 °C to investigate the effect of low O₂ atmosphere on respiratory metabolism, particularly on lactate dehydrogenase (LDH) activity and its isozyme composition. Low O₂ atmospheres caused a decrease in CO₂ production and an increase in lactate concentration and LDH activity compared to air. By day 2, CO₂ production rate decreased 0.4- and 0.5-fold, lactate increased 3.5- and 2.2-fold, and LDH activity increased 2.3- and 1.7-fold in carrot shreds stored in 0.5% and 2% O₂, respectively, compared to samples in air. Based on nondenaturing electrophoresis, LDH isozyme composition analysis revealed five bands consisting of a tetrameric enzyme with subunits encoded by two different *Ldh* genes. Changes in staining intensity of the isozymes indicated that the increase in LDH activity in carrots under low O₂ atmospheres resulted from increased enzyme synthesis and that there was preferential induction of one *Ldh* gene. These results suggest that lactic acid fermentation may be accelerated more under 0.5% than 2% O₂ atmospheres due to greater expression of the *Ldh* genes.

Hypoxic and anoxic conditions in plants result in rapid changes in respiratory metabolism and in the pattern of related gene expression (Millar et al., 1994; Sachs and Ho, 1986). Fermentative metabolism is induced under such conditions, which is regarded as an adaptive phenomenon to maintain the capacity for ATP synthesis (Davies, 1980; Kennedy et al., 1992). Lactate dehydrogenase (LDH) is the main terminal enzyme of fermentative metabolism in animals and microorganisms (Everse and Kaplan, 1973). In contrast, although LDH is not an uncommon enzyme in plants, the main terminal enzyme of fermentative metabolism in plants is alcohol dehydrogenase (ADH) (Good and Muench, 1993; Kennedy et al., 1992). The role of LDH in plants, therefore, may differ from those in animals and microorganisms. Information on the properties and metabolic function of plant LDH is scarce compared to that on animal and bacterial LDH (Davies, 1980; Rivoal and Hanson, 1994).

Much research on the effects of controlled and modified atmospheres has been directed toward optimizing storage conditions for horticultural commodities (e.g., Hardenburg et al., 1990; Weichmann, 1987) including carrot roots (e.g., Abdel-Rahman and Isenberg, 1974; Hansen and Rumpf, 1974; Liew and Prange, 1994). However, research data on the physiological and biochemical responses of carrot roots to controlled and modified atmospheres is limited. A better understanding of basic biochemical and physiological reaction to controlled atmospheres is needed to effectively evaluate storage conditions (Kader, 1986).

The objective of this research was to investigate the effect of low O₂ atmosphere on respiratory metabolism, particularly the reactions associated with LDH. Thus, respiration rate, lactate concentration, and LDH activity were determined in fresh-cut carrots stored under low O₂. Also, LDH isozymes were characterized to help elucidate the function of LDH under low O₂ atmosphere.

Materials and Methods

PLANT MATERIALS AND TREATMENTS. Carrot roots (*Daucus carota*) were purchased from a local wholesaler and washed with water.

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The roots were sterilized in a 0.2% (w/v) sodium hypochlorite for 15 min, rinsed thoroughly with distilled water, and shredded (≈50 mm long, 5 mm wide, and 4 mm thick). Shreds of the carrots were stored in 4-L glass jars (100 g fresh mass per jar) at 5 °C as described previously (Kato-Noguchi and Watada, 1996). Each jar was sealed with a rubber cap that had inlet and outlet glass tubes. A stream of 0.5% or 2% O₂ (balance N₂) or air was metered through the jar at a rate of 10 mL·min⁻¹. Distilled water (100 mL) was placed at the bottom of the jar to maintain a high relative humidity, and the carrots were placed above the water. A 10-mL gas sample was collected at the inlet and the outlet of each jar and injected into a gas chromatograph (GC-12A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector to analyze O₂ and CO₂ concentration. Oxygen was analyzed using a molecular sieve 13×30/60 column (3 mm × 4 m; GL Science, Tokyo, Japan) at 40 °C. Carbon dioxide was analyzed using a Unibeads C80/100 column (molecular sieve, 3 mm × 3 m; GL Science). The oven temperature was initially at 40 °C and increased at 20 °C·min⁻¹ to 220 °C. Carrier gas flow rate was 30 mL·min⁻¹ of helium in both cases.

To determine lactate concentration and LDH activity, carrot shreds were removed from three jars of each treatment, frozen immediately in liquid N₂, and stored at -80 °C until extraction.

EXTRACTION AND QUANTIFICATION OF LACTATE. Frozen carrots (3 g fresh mass equivalent) were ground to a fine powder with a mortar and pestle containing liquid N₂. The powder was homogenized with 15 mL of ice-cold 0.6 M HClO₄. The homogenate was kept in a ice bath for 30 min with occasional shaking and then centrifuged at 30,000 g_n for 15 min at 4 °C. The supernatant was neutralized with 3 M KOH and centrifuged again at 30,000 g_n for 5 min. The supernatant was used for analyses.

Lactate was quantified spectrophotometrically by monitoring the reduction of NAD at 340 nm for 15 min at 25 °C in a 3-mL reaction mixture of 116 mM glutamate buffer (pH 8.9), 0.93 mM NAD, 4 units of alanine aminotransferase, 12 units of LDH, and 0.2 mL of the sample as described by Bergmeyer (1983). Recovery of lactate added to the extraction medium containing the carrots before homogenization was 89% ± 7%, as calculated from five replications.

EXTRACTION AND ASSAY OF LDH. Carrot powder (3 g fresh mass equivalent), prepared as described above, was homogenized with 15 mL of ice-cold solution containing 100 mM Tris-HCl (pH 8.0), 10 mM Na-ascorbate, 10 mM DTT, 50 mg bovine serum albumin, and 15%

Fig. 1. Effect of low O₂ atmosphere on CO₂ production of carrot root shreds held in 0.5% O₂ (●), 2% O₂ (○), and air (□) at 5 °C. Means ± SE are from three experiments with four replications and four measurements per day for each determination.

(v/v) glycerol. The homogenate was centrifuged at 30,000 *g*_n for 20 min and the supernatant was assayed.

LDH was assayed at pH 8.0 by measuring the decrease in A₃₄₀ associated with NADH oxidation. The 1-mL assay mixture contained 100 mM Tris-HCl (pH 8.0), 0.24 mM NADH, 5 mM 4-methylpyrazole, 3 μM NaCN, 1.5 mM Na-pyruvate, and 0.2 mL of sample. 4-Methylpyrazole was used to inhibit interference from the coupled action of pyruvate decarboxylase (PDC) and ADH (Hoffman et al., 1986). A unit of enzyme activity was defined as the oxidation of 1 μmol NADH/min, which was equivalent to a decrease of 6.21 absorbance units/min. The overall recovery of enzyme activity through the quantification process was estimated to be 87% ± 8% based on five repeated assays with pure enzyme (Sigma Chemical Co., St. Louis) in the extract. Protein was determined by the method of Bradford (1976) using bovine gamma-globulin as a standard.

POLYACRYLAMIDE GEL ELECTROPHORESIS. Frozen carrot shreds were prepared as described in the previous section and applied to native slab polyacrylamide gel electrophoresis. Each gel track contained 85 μL of sample plus 15 μL of 90% glycerol containing 0.01% bromophenol blue. The running gel containing 8.6% (w/v) acrylamide, 0.11% bisacrylamide, 0.43 M Tris-HCl (pH 8.5), and 12% (w/v) glycerol was polymerized with 0.9 μL·mL⁻¹ TEMED and 0.4 mg·mL⁻¹ ammonium persulfate. The stacking gel containing 7.5% acrylamide, 0.1% bisacrylamide, 0.07 M Tris-HCl (pH 6.7), and 12% glycerol was polymerized with 0.6 μL·mL⁻¹ TEMED and 0.3 mg·mL⁻¹ ammonium persulfate for ≈6 h. Gels were run with Tris-glycine buffer (pH 8.8, 10 mM Tris, 77 mM glycine) in both electrode tanks at 15 mamps at 4 °C. When the dye front migrated 15 cm through the running gel, the gel was stained in a solution containing 50 mL of 0.1 M Tris-HCl (pH 8.0), 1.0 g lithium-lactate, 100 mg NAD, 20 mg MgCl₂, 10 mg nitroblue tetrazolium, 3 mg phenazine methosulfate, and 150 μmol of 4-methylpyrazole (Hoffman et al., 1986). Staining intensity of gel bands was measured using a densitometer (GS-700; Bio-Rad, New York). No bands were stained when lactate was omitted.

Results and Discussion

The rate of CO₂ production by carrot root shreds held in low O₂ atmospheres decreased up to day 2 and remained at this level during the experiment but did not change with samples held in air (Fig. 1).

The CO₂ production rate was lower at 0.5% than at 2% O₂ atmosphere, but differences were minimal. By day 2, CO₂ production of carrots held in 0.5% and 2% O₂ was 40% and 49% of that in air, respectively. The decrease in CO₂ production may suggest an induction of alternate respiratory metabolism (Kennedy et al., 1992).

Lactate concentration and LDH activity did not change in carrot root shreds held in air but increased in samples held in low O₂ atmospheres (Table 1). LDH has been isolated from several plants, however, little information exists on LDH induction under hypoxic conditions compared to that known about ADH induction (Davies, 1980). Lactate concentration and LDH activity were greater at 0.5% than 2% O₂ atmosphere and greater at day 2 than day 4 (Table 1). The decrease of LDH activity and lactate concentration at day 4 was consistent with the Davies-Roberts hypothesis described below (Davies et al., 1974; Roberts et al., 1984). By day 2, lactate concentration increased 3.5- and 2.2-fold in samples in 0.5% and 2% O₂, and LDH activity was 2.3- and 1.7-fold in samples in 0.5% and 2% O₂, respectively, compared with samples in air. The pattern of change in lactate concentration was similar to that of LDH activity of carrots held in low O₂ atmospheres. These results suggest that storage in low O₂ atmospheres resulted in a decrease in CO₂ production and increases in lactate concentration and LDH activity.

To investigate LDH isozyme composition and genetic control, carrot root shreds were stored under anoxic conditions (N₂ atmosphere) for 1 d, and the extracts then subjected to native slab polyacrylamide gel electrophoresis. Activity of LDH in the carrot extracts was resolved to five bands that migrate closely together (Fig. 2). This five-band pattern was typical of other LDH systems where the enzyme is a tetramer encoded by two *Ldh* genes (Hoffman and Hanson; 1986; Hoffman et al., 1986). In animals (Everse and Kaplan, 1973), potato (Asker and Davies, 1984), and four types of cereals (Hanson and Jacobsen, 1984), the LDH holoenzyme is a tetrameric compound that is encoded by two separate genes, and the products of these genes associate randomly to generate a set of five isozymes. If carrot LDH is also a two-gene system (gene *Ldh1* and *Ldh2*) in which the gene products associate randomly to form five tetramers (LDH1 tetramer, LDH1.LDH1.LDH1.LDH2, LDH1.LDH1.LDH2.LDH2, LDH1.LDH2.LDH2.LDH2, and LDH2 tetramer), the five isozymes would occur in the ratio 1:4:6:4:1 when both genes are expressed at the same level (Good and Crosby, 1989; Hoffman et al., 1986). This ratio is comparable to the relative staining intensities of the five LDH isozymes in carrots stored under anoxic conditions (Fig. 2). Thus, carrot LDH could be a tetramer of two subunits randomly associated and encoded by two different *Ldh* genes.

All LDH isozymes are not always found in plant tissues due to the limited expression of the specific isozymes (Sachs and Ho, 1986). Only three central LDH bands were clearly and consistently identified in extracts of carrots stored in air (Fig. 3, lanes 1, 2, and 5).

Table 1. Effects of low O₂ on LDH activity (protein basis) and lactate concentration in carrot root shreds at 5 °C. Means ± SE are from three experiments with at least three assays for each determination.

Treatment	LDH activity (nmol·mg ⁻¹ ·min ⁻¹)	Lactate concn (nmol·g ⁻¹)
Day 0 (initial)	11.7 ± 1.3	59 ± 5
Day 2		
Air	10.7 ± 1.2	60 ± 6
2% O ₂	18.5 ± 1.9	134 ± 12
0.5% O ₂	24.8 ± 2.3	211 ± 19
Day 4		
Air	10.3 ± 1.1	58 ± 5
2% O ₂	14.5 ± 1.6	104 ± 12
0.5% O ₂	14.3 ± 2.0	177 ± 17

Fig. 2. Isozyme composition of LDH in carrot roots stored in N_2 atmosphere for 1 d at 5 °C. Isozyme subunit composition was performed according to Hoffman et al. (1986). The cathode was at the top and the anode was at the bottom.

Staining intensity of isozyme 1 (most anodal) and isozyme 5 (cathodal) was too low to show discernible bands. Under low O_2 atmospheres, where total LDH activity was higher than that of air (Table 1), staining intensity of the three central isozymes was greater, and additional two bands of isozyme 1 (LDH1 tetramer) and 5 (LDH2 tetramer) appeared (Fig. 3, lanes 3, 4, 6, and 7). However, isozyme 5 band was stained more than isozyme 1 band. This indicates that there was more LDH2 subunit present in carrots under low O_2 atmospheres than in air. Thus, these results suggest that low O_2 atmosphere may increase LDH activity in carrots due to activation of LDH2 subunit translation encoded by the *Ldh2* gene.

A physiological role for LDH has been proposed by Davies et al. (1974) and Roberts et al. (1984). According to their hypothesis, under hypoxic conditions, the first enzyme of ethanolic fermentation—pyruvate decarboxylase (PDC)—is inactive because the cytosol of plant cells is slightly alkaline. Pyruvate is initially metabolized to lactate via the lactic acid fermentation pathway, resulting in lactate accumulation, which lowers the cytoplasmic pH. This acidification progressively inhibits LDH and activates PDC and ethanolic fermentation. Thus, Davies et al. (1974) and Roberts et al. (1984) concluded that lactic acid fermentation is a self-limiting process controlled by cytoplasmic pH, which can avoid cytoplasmic hyper-acidification leading to cell death. Carrot roots have the capacity for lactic acid (Table 1) and ethanolic fermentation (Kato-Noguchi and Watada, 1997). A balance of these two pyruvate-using pathways may be required for survival during oxygen deprivation without serious injury, in terms of pH regulation (Roberts et al., 1992) and maintenance of capacity for ATP synthesis (Davies, 1980; Kennedy et al., 1992).

In summary, 0.5% and 2% O_2 atmospheres decreased CO_2 production of carrots (Fig. 1) and increased LDH activity and lactate

Fig. 3. Changes in LDH isozymes in carrot roots held in 0.5% O_2 , 2% O_2 , and air atmosphere at 5 °C. Day 0 (lane 1); day 1, air (lane 2), 2% O_2 (lane 3), and 0.5% O_2 (lane 4); day 3, air (lane 5), 2% O_2 (lane 6), and 0.5% O_2 (lane 7). The cathode was at the top and the anode was at the bottom.

concentration (Table 1). The decrease was lower and the increases were greater at 0.5% than 2% O_2 atmosphere, respectively. These results suggest that CO_2 production rate was decreased and lactic acid fermentation was accelerated under low O_2 atmosphere. Based on electrophoretic analysis (Fig. 3), changes in staining intensity of the isozymes suggests that the increased LDH activity of carrots held in low O_2 atmospheres occurred due to increased synthesis of the enzymes. However, there could be preferential induction of the *Ldh2* gene under the low O_2 atmospheres.

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