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Respiratory Metabolism of Pear Fruit and Cultured Cells Exposed to Hypoxic Atmospheres: Associated Change in Activities of Key Enzymes

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Abstract. **'Bartlett' pears (*Pyrus communis* L.) at two physiological stages, climacteric minimum or approaching the climacteric peak as achieved via storage for 2 or 8 weeks in air at 0C, respectively, were either ripened at 20C in air immediately or after exposure to 0.25% 0_2 for 4 days at 20C. Fruit stored for 2 weeks had relatively stable phosphofructokinase (PFK), pyrophosphate : fru-6-P phosphotransferase (PFP), and pyruvate kinase (PK) activities but decreasing succinate dehydrogenase (SDH) activities during ripening in air. Similar fruit treated with 0.25% O_2 had slightly increased PFK, PFP, and SDH activities and decreased PK activity. Fruit stored for 8 weeks exhibited higher levels of PFK and PFP activity upon transfer to 20C, in accordance with their more advanced physiological state. In general, the enzymic changes in these fruit upon exposure to 0.25% O_2 and subsequent ripening in air were similar to those observed in the less-mature counterparts, most notable being an increase in mitochondrial SDH. Exposure of suspension-cultured pear fruit cells to hypoxia resulted in an accentuated rise in phosphoenolpyruvate carboxykinase activity and a dramatic rise in SDH activity upon transfer to air. Taken in concert, the enzymic analysis supports the hypothesis that the rise in succinate levels observed in hypoxic fruit tissues is the result of a partial reductive tricarboxylic acid cycle. Cytochrome oxidase activity did not change during hypoxia whereas soluble peroxidase decreased somewhat, perhaps a reflection of their Michaelis constants for O_2.**

Hypoxia, or very limited O_2 availability, is being tested as a quarantine treatment and for possible long-term storage of fresh fruit and vegetables (Ke and Kader, 1992). Little information is available regarding the effects of hypoxia on the metabolism of horticultural commodities. Ethanol is a known major end product of anoxic metabolism (fermentation), and it and acetaldehyde have been shown to accumulate in hypoxic pears (Nanos et al., 1992). Other probable anoxic end products include lactate, alanine, malate, α-aminobutyrate, and succinate (Davies, 1980).

The accumulation of succinate in anoxic fruit (Davies, 1980; Vanlerberghe et al., 1989) is commonly attributed to the suppression of succinate dehydrogenase (SDH) (Butler, 1989). However, an alternative explanation for succinate accumulation suggests that phosphoenolpyruvate (PEP) may be carboxylated via PEP carboxylase (PEP-C) and possibly PEP carboxykinase (PEP-CK) to produce oxaloacetate that, via partial reversal of the tricarboxylic acid (TCA) cycle, is in turn reduced to malate, fumarate, and finally succinate (Davies, 1980; Vanlerberghe et al., 1990). Intermediates and some key enzymes in this sequence are shown in Fig. 1. In the search for underlying metabolic events, we investigated the changes in the activity of these several regulatory enzymes in ripening pears and fruit cells during and after their exposure to hypoxia.

Since Nanos et al. (1992) found that preclimacteric pears seem less stressed and to have greater potential for posthypoxia recovery than pears of a more-advanced physiological age, maturity was incorporated as a variable. Cytochrome oxidase (CytOx) and peroxidase (PO) activities were also examined, as oxidase activity may be especially compromised during anoxia. Finally, suspension-cultured fruit cells were subjected to hypoxic stress with subsequent enzymic analyses to assess if the cells could be used to advantage in these types of studies.

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Materials and Methods

Plant material and treatments. Mature-green 'Bartlett' pears were obtained on the day of harvest from Sacramento and Lake counties, Calif. Fruit were stored at 0C until separate experiments were conducted on the pears from each area after 2 and 8 weeks of storage. Fruit were selected for uniformity of size and freedom from defects. Individual fruit were put in 450-ml jars and ventilated with air or 0.25% O_2 (balance N_2) using a continuous flow-through system at 30 $ml \cdot min^{-1}$. After 4 days under 0.25% O_2, the fruit were ventilated with air for 6 additional days. All atmospheric treatments were conducted at 20C. Initially, and for every subsequent sampling period, three individual fruit replicates per treatment were evaluated. The sampling periods were 1, 2, 4, 6, and 8 days during normal air treatment, 2 and 4 days for fruit under 0.25% O_2, and 1, 2, 4, and 6 days after their transfer to air. Changes in physiological state were assessed by measuring flesh firmness, color change, and C_2H_4 and CO_2 production. Enzyme assays included those for phosphofructokinase (PFK), pyrophosphate : fru-6-P phosphotransferase (PFP), pyruvate kinase (PK), and soluble PO. Mitochondria were isolated and SDH and CytOx activities were measured.

Suspension-cultured 'Passe Crassane' pear fruit cells were grown as described by Pech and Romani (1979). At the end of their log growth phase, the cells were transferred to a medium lacking 2,4-dichlorophenoxy acetic acid (2,4-D) for 4 days and then to aging medium, whereupon the atmospheric treatments were initiated. Aging medium contained one-fourth the concentration of nutrients found in growth medium and no 2,4-D, but was supplemented with 0.4 M mannitol and 0.015 M sucrose. Two identical experiments were conducted at 25C. The cell suspensions (three flask replicates per treatment) were treated atmospherically and periodically sampled exactly as the pears. Associated changes in culture growth, cell vitality, and C_2H_4 and CO_2 production have been reported (Nanos et al., 1992).

Extraction and analysis of PFK and PFP. These two enzymes were extracted and assayed via a modification of the method of

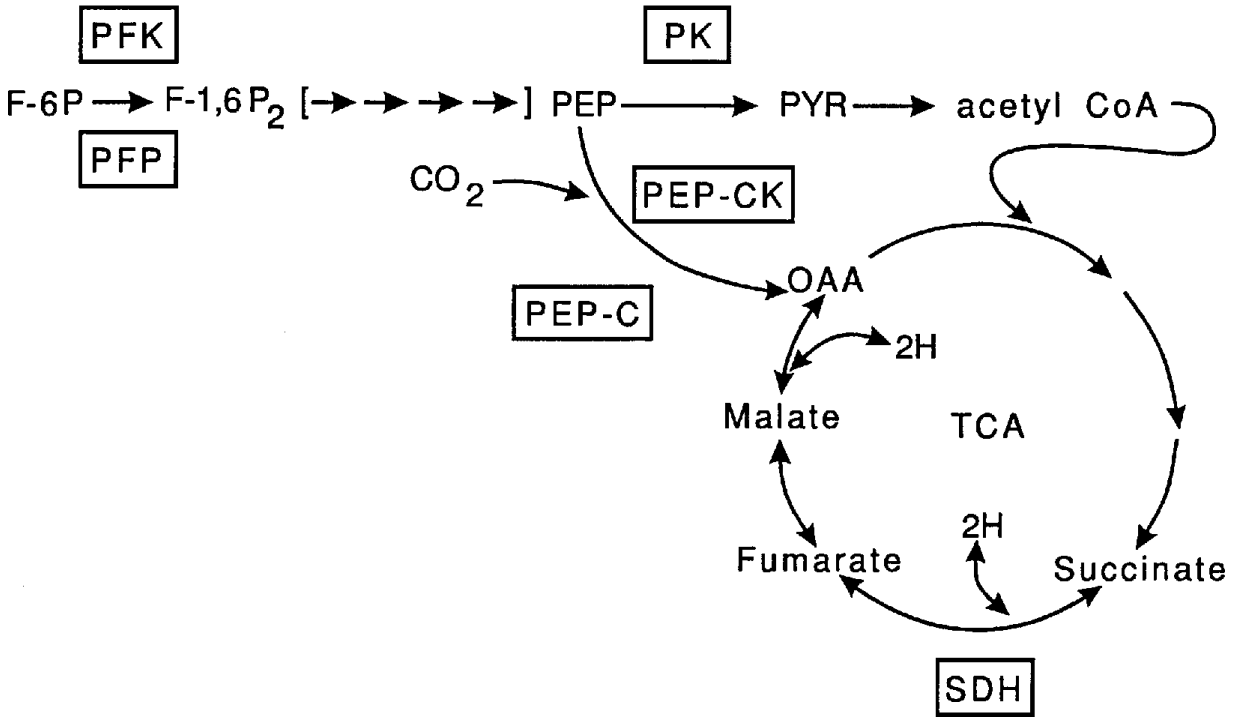

Fig. 1. Metabolic steps that may operate under hypoxia. Potential regulatory enzymes assayed in this study are shown.

Smyth et al. (1984). Tissue from the equatorial area of the fruit, including the skin, was ground to a fine powder under liquid N. Four grams of powder was extracted in 8 ml medium containing 100 mM tris-HCl (pH 8.0), 2 mM disodium-EDTA, 1 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 200 mg insoluble (40,000 molecular weight) polyvinylpyrollidone (PVP). The brei was centrifuged at 20,000× *g* for 25 min to remove debris, and the resulting supernatant (crude homogenate) was used for enzyme assay. The reaction medium for PFK and PFP (total volume 2 ml) contained 90 mM hepes-NaOH buffer (pH 8.0), 2.5 mM $MgCl_2$, 10 mM fru-6-Pm, 0.16 mM NADH, 1.2 units aldolase, 1.8 units glycerol-1-P dehydrogenase, 14 units triosephosphate isomerase, and 200 ml supernatant. The reaction was initiated by adding 1 mM sodium-ATP for PFK activity or 1 mM sodium-pyrophosphate and 2 mM fructose-2,6 P_2 for PFP activity. The absorbance at 340 nm was followed using a spectrophotometer at 25C (model PM2-DL; Zeiss, New York) and the rate was linear for at least 6 min. The activity is expressed in mmoles NADH oxidized/mg protein per min for PFK and change in absorbance/mg protein per min for PFP. The optimum extraction and assay pHs for these pear enzymes had been determined (Kerbel, 1987).

Extraction and analysis of PK. Five grams of tissue from the equatorial area, including skin, was homogenized in 20 ml extraction buffer [100 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 6.5, 5 mM DTT, and 0.5% soluble PVP] for 20 sec using a homogenizer (Polytron; Brinkmann Instruments, New York). The homogenate was filtered through four layers of cheesecloth and centrifuged at 27,000× *g* for 10 min. The supernatant was immediately used for enzyme assay. The PK assay mixture contained 50 mM MES buffer (pH 6.0), 2.5 mM ADP, 1.5 mM PEP, 4 mM $MgCl_2$, 25 mM KCl, 0.1 mM NADH, and 3 units of lactate dehydrogenase. The reaction was initiated by adding 100 µl supernatant (total volume 1 ml), and the decrease in absorbance at 340 nm was followed at 25C. The rate was linear for at least 3 min, and the activity is reported as mmoles NADH oxidized/mg protein per min. Preliminary experiments were conducted to determine the optimum extraction and assay pH (data not shown).

Isolation of mitochondria and analysis of SDH and CytOx. Pear fruit mitochondria were isolated using the method of Romani et al. (1969). Fifty grams of peeled pear fruit tissue was macerated in 150 ml isolation medium. The isolation medium consisted of 0.25 M sucrose, 50 mM potassium phosphate (pH 7.2), 6 mM EDTA, 10 mM β-mercaptoethanol, 0.5% soluble PVP, and bovine serum albumin (BSA) at 1 $mg \cdot ml^{-1}$. The pH of the slurry was continuously adjusted to 7.2 with drop-wise additions of 5 N KOH. The homogenate was squeezed through two layers of cheesecloth and centrifuged at 2000× *g* for 10 min. The supernatant was filtered through four layers of cheesecloth and centrifuged at 10,000× *g* for 15 min, and the pellet was resuspended in 4 ml wash medium containing 0.25 M sucrose, 50 mM potassium phosphate (pH 7.2), and BSA at 1 $mg \cdot ml^{-1}$. The resultant suspension was then centrifuged at 2000× *g* for 5 min, the resultant supernatant was centrifuged at 8000× *g* for 10 min, and the final pellet was resuspended in 0.5 ml wash medium to provide once-washed mitochondria.

The method of Frenkel and Patterson (1973) with some modifications was used to assay for SDH. The reaction mixture contained 100 mM potassium phosphate (pH 7.2), 10 mM KCN, 6 mM phenazine methosulfate, 20 mM succinate, and 0.2 mM dichlorophenol indophenol. The assay was begun by adding 100 µl of the mitochondrial preparation, diluted 1:5 with water, to the reaction mixture. The decrease in absorbance at 600 nm was followed at 25C and was linear for at least 3 min. Activity is reported as the change in absorbance/mg protein per min.

CytOx activity was evaluated using the method by Hoekstra and van Roekel (1983). Cytochrome C [50 mM in 10 mM potassium phosphate (pH 7.0)] was first reduced by adding minimal solid dithionite. To start the assay, 100 µl of the mitochondrial preparation was diluted 10:1 with water and added to 0.9 ml of the reduced

cytochrome C. The decrease in absorbance at 550 nm was followed at 25C and was linear for at least 3 min. Activity is reported as mmoles of cytochrome C oxidized/mg mitochondrial protein per min.

Extraction and analysis of PK, SDH, PEP-C, and PEP-CK in suspension-cultured cells. During each sampling period, 200 ml of cell suspension was centrifuged at 3000× *g* for 3 min and the resulting pellet was ground in liquid N. For the PK assay, the cells were suspended in extraction buffer immediately following grinding and assayed as reported for the pears. SDH activity was measured in a cell extract consisting of 4 g of ground cells suspended in 10 ml of 100 mM potassium phosphate (pH 7.2) plus 1% soluble PVP. SDH was assayed in the same manner as reported above.

PEP-C and PEP-CK were assayed using the method by Blanke et al. (1986) with some modifications. Four grams of ground cells was suspended in 10 ml of extraction buffer: 50 mM 3-[*N*-morpholino] propanesulfonic acid (MOPS) (pH 8.0), 50 mM tricine, 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.25 mM EDTA, 2 mM DTT, and 2% insoluble PVP adjusted to a final pH of 8.0. The suspension was centrifuged at 27,000× *g* for 10 min and the supernatant was used for the enzyme assay. For PEP-C, the reaction mixture contained 25 mM MOPS (pH 8.0), 25 mM tricine, 5 mM $MgCl_2$, 0.25 mM EDTA, 2 mM DTT, 3 mM glycine, 5 mM $NaHCO_3$, 10 units malate dehydrogenase (MDH), 0.1 mM NADH, and 0.2 ml extract, and the reaction was initiated with 2 mM PEP (total volume 3 ml). The decrease in absorbance at 340 nm was followed at 25C and was linear for at least 3 min. The activity is reported as change in absorbance/mg protein per min. The same extract was also used to measure PEP-CK activity with the reaction mixture containing 50 mM trisma buffer (pH 7.0), 0.6 mM DTT, 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 units MDH, 2 mM ADP, 0.1 mM NADH, and 0.2 ml extract, and the reaction was initiated by adding 2 mM PEP (total volume 3 ml). The decrease in absorbance at 340 nm was followed at 25C and was linear for at least 3 min. The activity is reported as change in absorbance/mg protein per min. Preliminary experiments were conducted to determine the optimum extraction and assay pH for the last two enzymes (data not shown).

Extraction and analysis of PO. The enzyme was extracted and assayed using a modification of the method of Thomas et al. (1981). Three grams of pear tissue was homogenized in 10 ml extraction buffer consisting of 100 mM potassium phosphate (pH 8.0) and 1% insoluble PVP. The brei was filtered through four layers of cheesecloth and the debris was kept for isolating ionically bound PO. The filtrate was centrifuged at 27,000× *g* for 15 min and the supernatant was used for soluble PO determination. Debris from the above filtration was rinsed twice with 10 ml water then suspended in 10 ml of 1 M KC1 and stirred periodically for 1 h. The solution was then filtered and centrifuged at 27,000× *g* for 15 min and the supernatant was used to discern bound PO. The PO assay mixture contained 80 mM potassium phosphate (pH 4.6), 0.1% H_2O_2, and 200 ml extract. The reaction was initiated by adding 250 µl of 0.1 M guaiacol (final volume 3 ml). The increase in absorbance at 470 nm was followed at 25C and was linear for at least 6 min. The activity is reported as mg guaiacol oxidized/mg protein per min.

Protein content. The protein content of all extracts was estimated using the Bradford (1976) method with BSA standards.

Statistics. After 2 weeks of storage, two experiments were conducted with pears from each of two harvest areas for a total of four experiments. After 8 weeks of storage, one experiment was conducted with pears from each area. Two complete experiments were conducted with the suspension-cultured cells, with three replicates per sampling period during treatment. Two-way analysis of variance over treatment and time was calculated and the SE (n = 3) is shown, except for skin color, where the SE (n = 6) is reported.

Results and Discussion

Physiological state. All analyses reported below were conducted with fruit whose physiological state had been confirmed assessing their color, firmness, and rates of respiration and ethylene production (Nanos et al., 1992). After short-term (2-week) storage at 0C, the fruit were at their climacteric minimum and reached the peak ≈6 days after direct transfer to 20C. After long-term (8-week) storage at 0C, the fruit had advanced physiologically and exhibited a climacteric peak on the second day after transfer to 20C.

PFK and PFP activities. Pears stored at 0C for 2 weeks ripened normally upon transfer to air at 20C. There was little change in PFK (Fig. 2A) but some increase in PFP activity (Fig. 3A) coincident with the climacteric on day 6. When similarly stored fruit were placed under 0.25% O_2 at 20C, PFK and PFP activities increased slightly during the 4 days of hypoxia followed by a more marked increase upon transfer to air (Figs. 2A and 3A). Activities of PFK and PFP extracted from pears that had been stored at 0C for 8 weeks rose dramatically upon transfer to 20C, becoming 3- to 4-fold higher (Figs. 2B and 3B) than those observed after 2 weeks of storage. Maximum PFK and PFP activity was reached 2 days after transfer, again coincident with the climacteric respiratory peak.

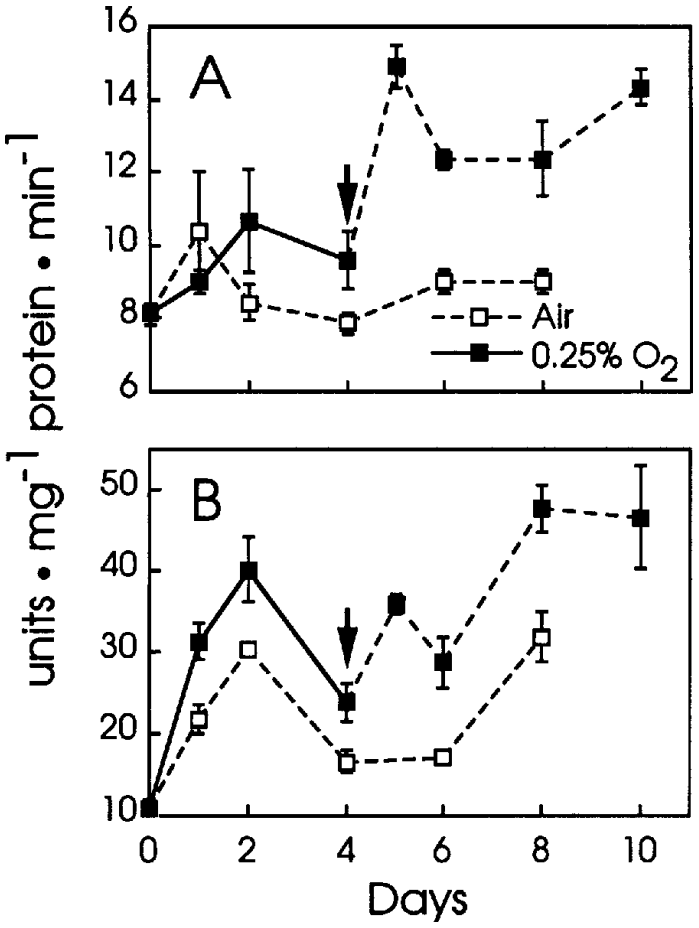

Fig. 2. Changes in the phosphofructokinase activity of 'Bartlett' pears first stored at 0C for 2 weeks (**A**) or 8 weeks (**B**) and then at 20C in air or in 0.25% O_2 and subsequently (↓) transferred to air. Units = mmoles NADH oxidized.

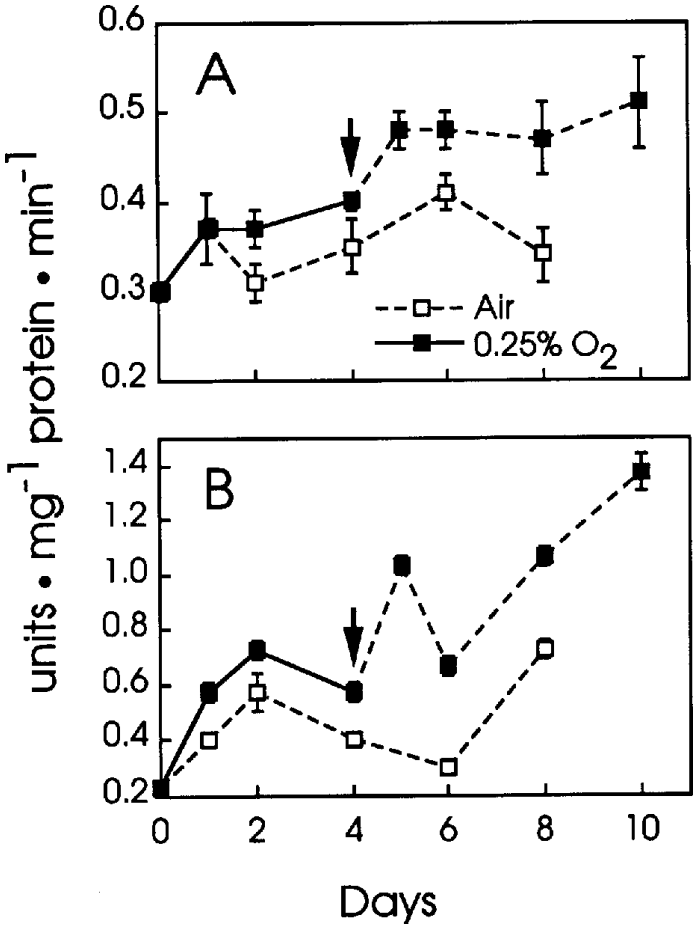

Fig. 3. Changes in the pyrophosphate : fructose-6-P phosphotransferase activity of 'Bartlett' pears stored at 0C for 2 weeks (**A**) or 8 weeks (**B**) and then at 20C in air or in 0.25% O_2 and subsequently (↓) transferred to air. Units = Δ absorbance at 340 nm.

Exposure of these fruit to hypoxia resulted in higher PFK and PFP activities that remained relatively high upon transfer to 20C (Figs. 2B and 3B). In general, the observations with air-treated fruit agree with previous results for banana (Beaudry et al., 1987; Salimen and Young, 1975) and avocado (Bennett et al., 1987), in that a rise in PFK and PFP is associated with the climacteric respiration increase. Storage at 0C and advancement in physiological state, while increasing the enzymic level appreciably, did not seem to affect the general trend. However, the prolonged and appreciably higher PFK and PFP activities during and especially after hypoxic treatment portend a disrupted metabolism not unlike that observed in anoxia-sensitive species; e.g., an increase in PFK activity due to anoxia was implicit in castor bean endosperm (Kobr and Beevers, 1971) and demonstrated in the alga *Selenastrum minutum* (Vanlerberghe et al., 1990); PFP activity increased in anoxic *Euglena gracilis* (Enomoto et al., 1990).

PK activity. Pears ripened after 2 weeks of 0C storage displayed relatively stable PK activity (Fig. 4). However, hypoxia resulted in a much lower PK activity that remained relatively low even after transfer to air. These results agree with the observation by Mertens et al. (1990) that PK activity in rice, an anoxia-tolerant species, remained relatively low during anoxia. In terms of PK and PFK activities, the short-term-stored fruit behaved as anoxia-tolerant tissues. This interpretation is supported by the lack of low-O_2 injury symptoms.

After long-term storage, the fruit had much lower PK activity overall (3.5 to 13.5 units/mg protein per min) and there were no

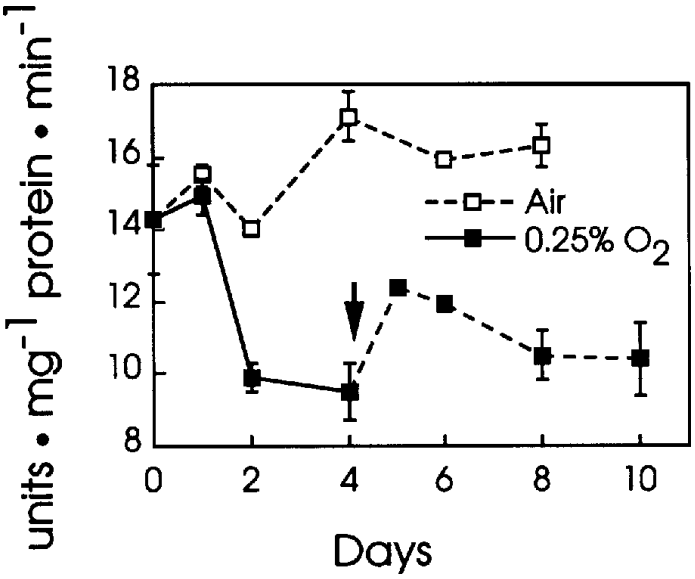

Fig. 4. Changes in the pyruvate kinase activity of 'Bartlett' pears first stored at 0C for 2 weeks and then at 20C in air or in 0.25% O_2 and subsequently (↓) transferred to air. Units = mmoles NADH oxidized.

significant differences between air controls and 0.25% O_2-treated fruit (data not shown).

SDH activity. Mitochondrial SDH activity declined when fruit that had been stored for 2 weeks at 0C were ripened in air. However, similarly stored fruit exhibited an increased SDH activity when treated with 0.25% O_2 (Fig. 5A). After 8 weeks at 0C, the control, air-treated fruit retained low, stable mitochondrial SDH activity at 20C (Fig. 5B). However, mitochondrial SDH activity doubled during 0.25% O_2 treatment but, as with the fruit stored for 2 weeks, returned to normal within 2 days after the hypoxic treatment. The increase in SDH activity during low-O_2 treatment agrees with the hypothesis that succinate accumulation (often observed in anoxic tissues) can result from a partial reductive TCA cycle (Fig. 1), wherein PEP is carboxylated to produce oxaloacetic acid (OAA) and, via malate and fumarate, results in succinate accumulation (Davies, 1980; Vanlerberghe et al., 1990). The increase in PFK and PFP activities (Figs. 2 and 3) and decrease in PK activity (Fig. 4) also support the hypothesis.

Enzyme activities in cells. To further ascertain cellular potential for a partial reductive TCA cycle, suspension-cultured pear fruit cells were used to follow the activities of PK, SDH, and the two carboxylating enzymes at the PEP to oxaloacetate step. It has already been demonstrated that hypoxia-induced changes in C_2H_4 and CO_2 production and pyruvate decarboxylase and alcohol dehydrogenase activities were qualitatively the same but generally more pronounced in the suspension-cultured pear cells than for similarly treated pears (Nanos et al., 1992). Relative to corresponding controls, the PK and PEP-C activities of suspension-cultured cells were lowered (Fig. 6 A and B), whereas PEP-CK activity increased appreciably (Fig. 6C) and mitochondrial SDH activity increased somewhat (Fig. 6D) during exposure to the 0.25% O_2 atmosphere.

The changes in PK and PEP-CK activity persisted after transfer to air. PEP-C activity that had been diminished by hypoxia increased substantially to levels above the control fruit upon transfer to air. Most striking of the changes was the rise in mitochondrial SDH upon transfer of the fruit from hypoxia to air (Fig. 6D). It is conceivable that the increase in PEP-CK activity during hypoxia would lead to increased levels of OAA even though

carboxylation of PEP by PEP-C is partially inhibited. The increased PEP-CK activity in hypoxic cells is again in keeping with the hypothesis that succinate accumulates via a partial, reductive TCA cycle. The dramatic posthypoxia rise in SDH activity would be anticipated if succinate had accumulated during hypoxia as has generally been observed.

CytOx activity. There was little change in CytOx activity of short-term stored pear as they progressed through their climacteric whether or not they had been exposed to anoxia (data not shown). Long-term-stored pears ripened in air exhibited a rise and decline in CytOx activity (Fig. 7) coincident with the climacteric. Surprisingly, CytOx activity also increased under 0.25% O_2 and increased further by ≈50% upon transfer of the low O_2-treated fruit to air. This rise in CytOx is consistent with the elevated respiratory activity and signs of injury (skin browning) observed in these fruit (Nanos et al., 1992).

PO activity. The activity of PO (soluble fraction) in pears stored for 2 weeks subsequently ripened in air increased and reached a maximum on the second day at 20C (Fig. 8), well before the respiratory climacteric that occurred on day 6. This observation agrees with similar measurements on papaya (Silva et al., 1990). There are, however, conflicting reports in which soluble PO activity was found to increase continuously with ripening of pears and tomatoes (Frenkel, 1972) and bananas (Toraskar and Modi, 1984).

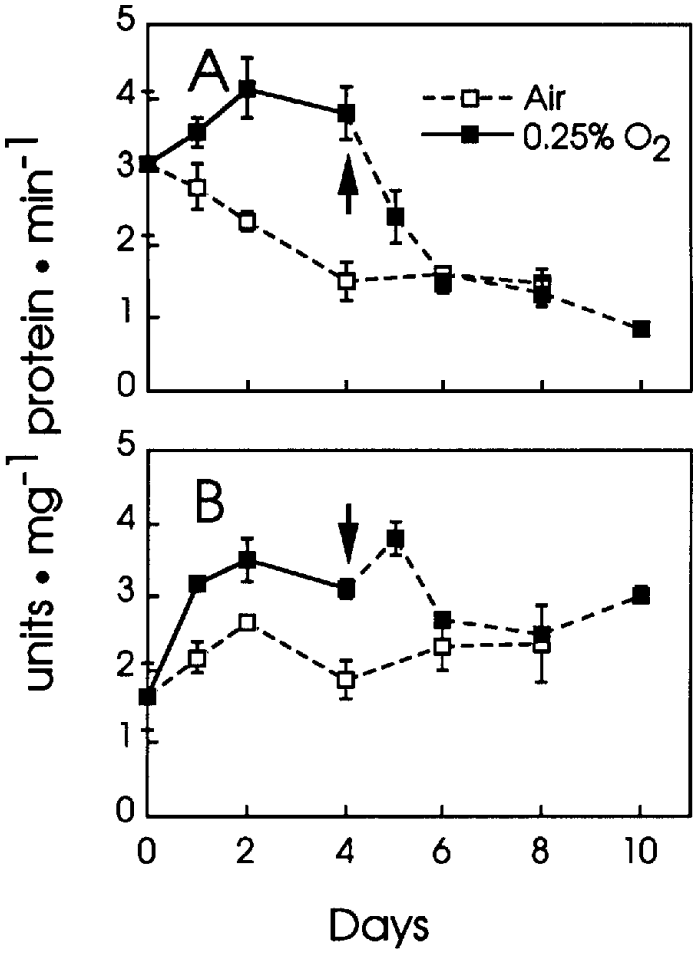

Fig. 5. Changes in the succinate dehydrogenase activity of 'Bartlett' pears first stored at 0C for 2 weeks (**A**) or 8 weeks (**B**) and then at 20C in air or 0.25% O_2 and subsequently (↓) transferred to air. Units = Δ absorbance at 600 nm.

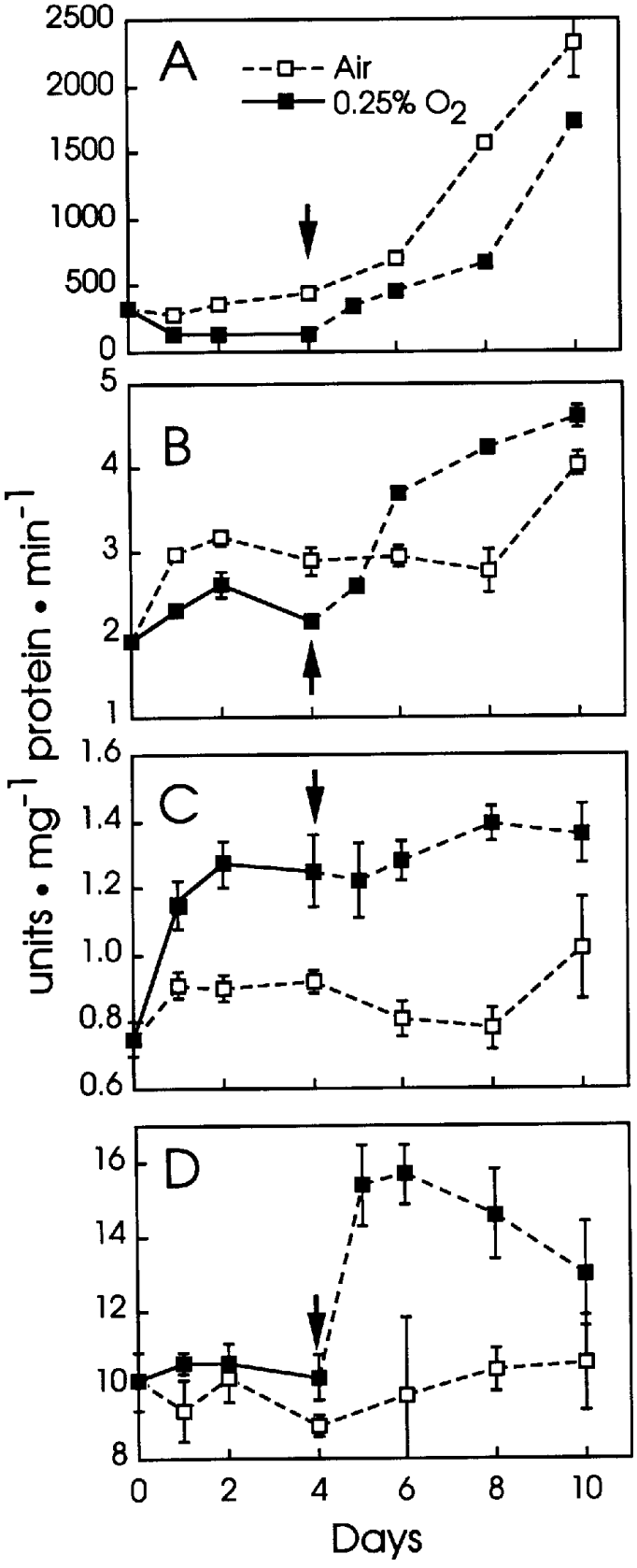

Fig. 6. Changes in the pyruvate kinase (**A**), PEP-carboxylase (**B**), PEP-carboxykinase (**C**), and succinate dehydrogenase (**D**) activities of suspension-cultured 'Passe Crassane' pear fruit cells kept at 25C in air or 0.25% O_2 with subsequent (↓) transfer to air. Units = (**A**) nmoles NADH oxidized; (**B** and **C**) absorbance at 340 nm; (**D**) Δ absorbance at 600 nm.

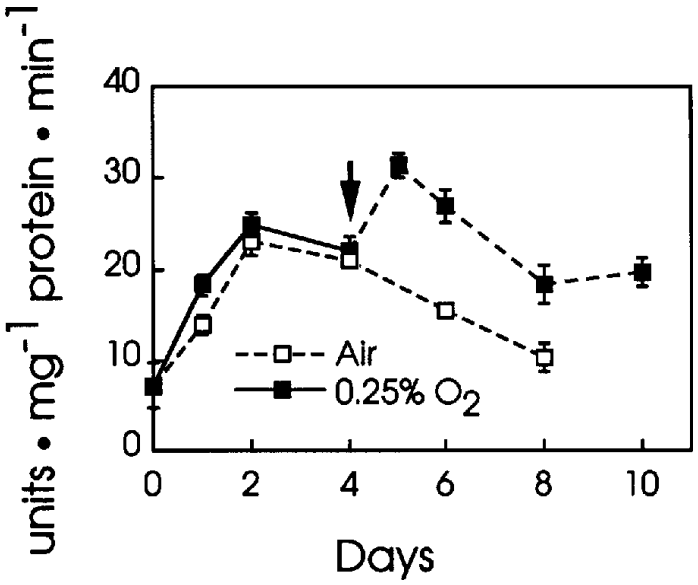

Fig. 7. Changes in the cytochrome oxidase activity of 'Bartlett' pears first stored at 0C for 8 weeks and then at 20C in air or in 0.25% O_2 with subsequent (↓) transfer to air. Units = nmoles cytochrome C oxidized.

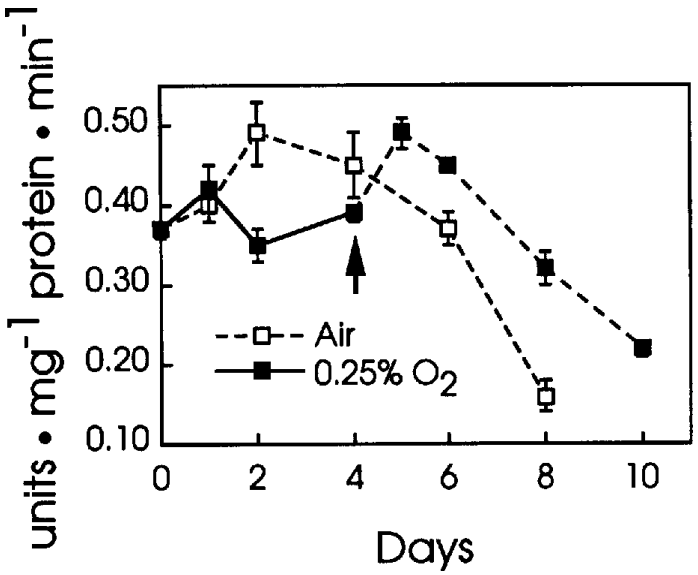

Fig. 8. Changes in the peroxidase (soluble fraction) activity of 'Bartlett' pears kept at 20C in air or in 0.25% O_2 and subsequently (↓) transferred to air. Units = mg guaiacol oxidized.

Exposure of similarly stored fruit to 0.25% O_2 resulted in some lowering of soluble PO activity. However, upon transfer to air, PO activity increased on the first day and then declined roughly coincident with the delayed climacteric (Fig. 8). The coincidence between PO activity and the course of fruit ripening irrespective of prior exposure to 0.25% O_2 is in keeping with the lack of cellular injury from the exposure of these preclimacteric fruit to 0.25% O_2.

Conclusions

Although of a survey nature, this study permits some informative observations. First, during normal ripening at 20C after 2 weeks of 0C storage, the activities of some enzymes (PFK, PFP, PK) remained relatively constant while those of others (SDH, CytOx, and soluble PO) declined. During hypoxia, PFK, PFP, and SDH activities increased while PK activity decreased. These latter transitions are consistent with the operation of a partial reductive TCA cycle leading to the accumulation of succinate an as end product.

It should be noted that these same hypoxic pears and fruit cells also exhibited alcoholic fermentation with increases in pyruvate decarboxylase and alcohol dehydrogenase (Nanos et al., 1992). Perhaps it is not surprising that two alternative means of generating albeit small amounts of energy should exist in cells whose normal electron transport pathway is blocked by the absence or near absence of O_2.

CytOx activity showed little change, while PO activity decreased during 0.25% O_2 treatment. The results support the hypothesis that hypoxia suppresses oxidases with high Kms for O_2 while not altering CytOx.

The more mature pears stored for 8 weeks were injured by subsequent exposure to 0.25% O_2, and their CO_2 and C_2H_4 production rates were irreparably affected (Nanos, et al., 1992). Nonetheless, except for PK and SDH, the general nature of the posthypoxia changes in the various enzymes tested was somewhat the same in the short-term- and long-term-stored fruit. It seems questionable, therefore, that the low-O_2 injury (uneven ripening and skin browning) of the latter was due to a significant difference in their metabolic response during hypoxia, but rather that their subsequent repair response was too short-lived to be effective, having been precluded by the climacteric peak that occurred 1 to 2 days after transfer to normal O_2 supply. If so, the observation is consonant with the hypothesis (Romani, 1987) that repair or homeostasis begins to decline after the inflection point of the climacteric rise and virtually ceases at the climacteric peak.

Enzymic activities that could serve as indexes to repairable or nonrepairable levels of hypoxia-induced fruit injury would be very useful in predicting the tolerance of various fruit to hypoxia as a quarantine treatment. SDH, PK, and PEP-CK seem to be good prospects. As such, they merit further study with the possibility of using the tractable cultured cells as a model system that, with respect to some key enzymes, mimics the hypoxic response of the intact fruit.

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