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Low-oxygen Atmosphere Increases Fructose 2,6-bisphosphate in Fresh-cut Carrots

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Abstract. **This study was undertaken to determine the effect of low-O_2 atmosphere on the concentration of fructose 2,6-bisphosphate (Fru-2,6-P_2), which can activate the enzyme pyrophosphate-dependent:phosphofructokinase (PPi-PFK) to catalyze the reaction from fructose 6-phosphate to fructose 1,6-bisphosphate (Fru-1,6-P_2). Fru-2,6-P_2 remained unchanged in carrot (*Daucus carota* L.) root shreds stored under air, but it increased 3.0- and 5.3-fold at 2% and 0.5% O_2 atmosphere, respectively, at 5C, and the increases were almost twice as great at 15C. The concentration of PPi ranged from 17 to 33 nmol·g^{-1} fresh weight, which is more than sufficient for the PPi-PFK to proceed. Thus, low-O_2 atmosphere appeared to hasten glycolysis of carrot shreds by increasing Fru-2,6-P_2, which activated PPi-PFK toward glycolysis.**

At least three pathways are involved in the interconversion of fructose 6-phosphate (Fru-6-P) and fructose 1,6-bisphosphate (Fru-1,6-P_2), and this interconversion has been recognized as an important regulatory site for the flow of carbon between carbohydrate storage products (sugar and starch) and carbohydrate use (Black et al., 1987; Solomos, 1988). The reactions are catalyzed essentially by two irreversible enzymes: ATP-dependent : phosphofructokinase (conversion of Fru-6-P to Fru-1,6-P_2) and fructose 1,6-bisphosphatase (FBPase, conversion of Fru-1,6-P_2 to Fru-6-P). A third enzyme, pyrophosphate-dependent : phosphofructokinase (PPi-PFK), readily reversible in vitro, catalyzes the interconversion of Fru-6-P and pyrophosphate (PPi) to Fru-1,6-P_2 and inorganic phosphate (Pi) (Black et al., 1987; Nielsen, 1994).

Fructose 2,6-bisphosphate (Fru-2,6-P_2) is a regulatory metabolite that makes an important contribution to the regulation of carbohydrate metabolism in different types of eukaryotic organism (Hers et al., 1982; Ueda et al., 1982). In plants, Fru-2,6-P_2 promotes glycolysis and restricts gluconeogenesis by activating PPi-PFK and inhibiting FBPase, respectively (ap Rees and Dancer, 1987; Hatzfeld et al., 1989). Fru-2,6-P_2 is present in plant tissues in significant amounts, and its concentration fluctuates depending on physiological or environmental conditions such as flooding, wounding, and photosynthetic light (ap Rees and Dancer, 1987; Paz et al., 1985; Van Schaftingen and Hers, 1983). The glycolytic form of PPi-PFK, that is induced by Fru-2,6-P_2, requires PPi as a substrate for the reaction (Smyth and Black, 1984).

Although much research on controlled and modified atmosphere has been directed toward determining optimum conditions for various fruits and vegetables (Hardenburg et al., 1990), little is known about the mode of action of O_2 and CO_2 on respiratory metabolism of these commodities (Kader, 1986). The contribution of Fru-2,6-P_2 to respiratory metabolism in harvested plant organs under controlled or modified atmosphere is not clear.

We previously observed that activity of the reversible enzyme PPi-PFK increased with the accumulation of Fru-1,6-P_2 in carrot shreds held under low-O_2 atmosphere (Kato-Noguchi and Watada, 1996). In this report, we show that low O_2 increased the level of Fru-2,6-P_2, which in the presence of adequate supply of PPi, PPi-PFK was activated toward glycolysis.

Materials and Methods

Plant materials and treatments. Carrot roots were purchased from a local wholesale distributor (Jessup, Md.). The carrots were shredded (about 50 mm long, 5 mm wide, and 4 mm thick) and stored in 3.8-liter glass jars (100 g fresh weight per jar, at least six jars for each sampling date) at 5 and 15C as described previously (Kato-Noguchi and Watada, 1996). A stream of 0.5% or 2% O_2 (balance N_2), or air was metered through the jar at a rate of 10 ml·min^{-1} at 5C and 15 ml·min^{-1} at 15C, which was sufficient to keep CO_2 accumulation below 0.3% and to measure respiration (Kato-Noguchi and Watada, 1996).

To quantify Fru-2,6-P_2, PPi, Pi, and ATP, carrot shreds for initial sample (day 0) were frozen in liquid N_2 within 5 sec after shredding and stored at –80C until analysis. One jar for each treatment was removed from storage on day 1, 3, 6, and 9, and carrot shreds were frozen as same manner as initial sample.

Measuring Fru-2,6-P_2. To measure content of Fru-2,6-P_2, the frozen carrot shreds (3 g fresh weight equivalent) were placed in a mortar containing liquid N_2 and ground to a fine powder using a pestle. Before the liquid N_2 had evaporated, 10 ml of ice-cold 50 mM NaOH was added to the sample and homogenized. The homogenate was kept for 30 min at 0C with occasionally shaking. The mixture was centrifuged at 10,000× *g* for 10 min at 0C. The supernatant was heated for 5 min at 0C, cooled in ice, and centrifuged again at 10,000× *g* for 5 min. The resulting supernatant was used to quantify Fru-2,6-P_2.

Quantification was carried out spectrophotometrically by monitoring the oxidation of NADH at 340 nm for 15 min at 30C in the following 1-ml reaction mixture according to Van Schaftingen and Hers (1983): 50 mM Tris-acetate (pH 8.0), 2 mM magnesium acetate, 5 mM Fru-6-P, 0.15 mM NADH, 0.5 mM PPi-tetrasodium, 1.2 units of aldolase, 1.8 units of glycerol-3-phosphate dehydrogenase, 7.5 units of triosephosphate isomerase, 0.5 units of PPi-PFK (Sigma Chemical Co., F-7006), and up to 0.1 ml of the extract. The assay was started by adding PPi-tetrasodium after 5 min

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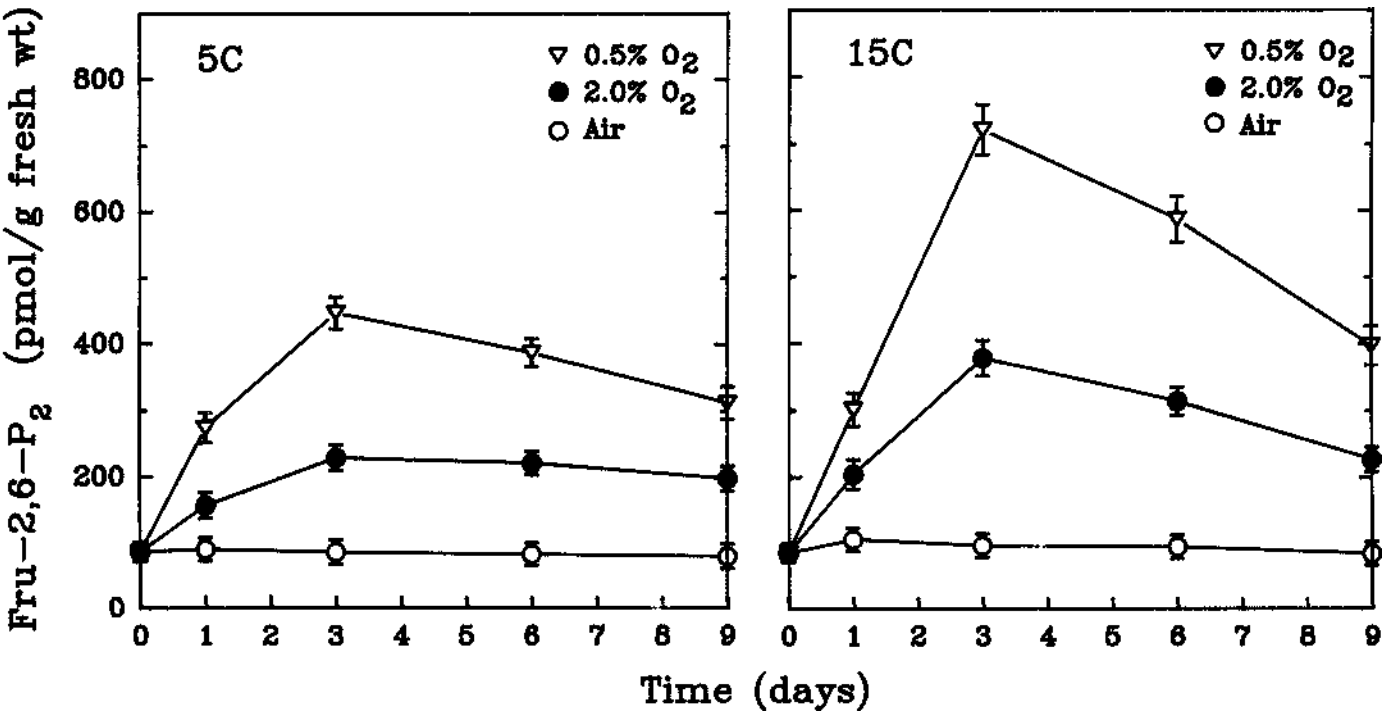

Fig. 1. Changes in the level of Fru-2,6-P_2 in carrot shreds held in 0.5% O_2, 2% O_2, and air atmosphere at 5 and 15C. The content of Fru-2,6-P_2 on day 0 was 87.2 pmol·g^{-1} fresh weight. Mean ± SE from three experiments with at three assays for each determination.

preincubation. The amount of Fru-2,6-P_2 in each sample was calculated by comparing the extent of activation with that induced by known amounts of standard Fru-2,6-P_2. The recovery of Fru-2,6-P_2 added to the extraction medium containing carrot shreds before homogenation exceeded 90%.

Measuring PPi, Pi, and ATP. Tissue powder (5 g fresh weight equivalents) was prepared as described above and homogenized with 10 ml of ice-cooled 0.45 N $HClO_4$ at 0C for 30 min with occasional shaking. Insoluble materials in the homogenate were removed by centrifugation at 30,000× *g* for 15 min at 0C. The supernatant was neutralized to pH 7.0–7.5 with 5 N K_2CO_3. The precipitated potassium chlorate was removed by centrifugation (30,000×*g*, 5 min) and the supernatant was used for analyzing PPi, Pi, and ATP. PPi was determined immediately, whereas samples used for Pi and ATP were stored at –80C until analysis.

PPi, Pi, and ATP were quantified spectrophotometrically by monitoring the oxidation/reduction of NADH/NAD and NADP at 30C using 25 to 50 µl of the extract containing in the following 1-ml reaction mixture: 50 mM Tris-acetate (pH 8.0), 2 mM magnesium acetate, 2 µM Fru-2,6-P_2, 5 mM Fru-6-P, 0.15 mM NADH, 1.2 units of aldolase, 2 units of glycerol-3-phosphate dehydrogenase, 7.5 units of triosephosphate isomerase, 0.5 units of PPi-PFK for PPi (Van Schaftingen and Hers, 1983); 100 mM triethanolamine (pH 7.6), 1 mM EDTA, 2 mM $MgCl_2$, 1.3 mM NAD, 10 mM glucose, 1 mM ADP, 0.5 mM Fru-1,6-P_2, 2 units of aldolase, 9 units of glyceraldehyde-3-phosphate dehydrogenase, 3 units of hexokinase, and 16 units of phosphoglycerate kinase for Pi (Cornell et al., 1979); 100 mM triethanolamine (pH 7.6), 4 mM $MgCl_2$, 2 mM NADP, 2 mM glucose, 3.5 units of glucose-6-phosphate dehydrogenase, and 2.8 units of hexokinase for ATP (Mohanty et al., 1993).

The overall recovery of PPi, Pi, and ATP added to the extraction medium containing carrot shreds before homogenation was 88% ± 4%, 91% ± 2%, and 89% ± 3%, respectively, as calculated from five replications of the test run with pure PPi, Pi, and ATP.

Three entirely separate experiments were conducted, and determination was replicated at least twice in each experiment. All enzymes were purchased from Sigma Chemical Co.

Results and Discussion

The Fru-2,6-P_2 content was low on day 0 and did not change with samples stored in air (Fig. 1). The content increased under 0.5% and 2% O_2 atmosphere, and the increase was greater at the lower O_2 and higher storage temperature. By day 3, Fru-2,6-P_2 increased 3.0- and 5.3-fold with samples in 2% O_2 and 0.5% O_2 at 5C, and 4.0- and 7.8-fold with samples in 2% O_2 and 0.5% O_2 at 15C, respectively, compared with samples in air. After day 3, the content began to decrease under 0.5% O_2 at 5C and under 0.5% and 2% O_2 at 15C. The pattern of changes in the level of Fru-2,6-P_2 was similar to those of PPi-PFK activity and Fru-1,2-P_2 content (Kato-Noguchi and Watada, 1996). A similar correlation between content of Fru-2,6-P_2 and activity of PPi-PFK is found in rice seedlings after making tissue anoxia (Mertens et al., 1990).

The content of Fru-2,6-P_2 in samples under low O_2 ranged from 0.15 to 0.78 nmol·g^{-1} fresh weight. All available evidence strongly suggests that Fru-2,6-P_2 is confined to the cytosol in plants (ap Rees and Dancer, 1987; Paz et al., 1985). Assuming the plant cell cytoplasm is 10% of the cell volume (Black et al., 1987), then the concentration of Fru-2,6-P_2 is likely to be in the range 1.5 to 7.8 µM. This concentration is more than sufficient to activate PPi-PFK (Black et al., 1987; Kombrink et al., 1984; Stitt, 1989). The increase of Fru-2,6-P_2 in response to low O_2 atmosphere may be important in regulating glycolysis in carrot shreds.

Although PPi-PFK is a reversible enzyme and can catalyze the reaction between Fru-6-P and Fru-1,6-P_2, activation by Fru-2,6-P_2 dramatically increases the V_{max} of PPi-PFK activity and increases the affinity of PPi-PFK for Fru-6-P (Black et al., 1987; Stitt, 1990). Accordingly, it has been proposed that Fru-2,6-P_2 can be viewed as a glycolytic signal (Stitt, 1990; Van Schaftingen, 1987). Of

Table 1. Content of PPi, Pi, and ATP in carrot shreds held in 0.5% O_2, 2% O_2, and air atmosphere at 5 and 15C.

Treatment		Content (nmol·g^{-1} fresh wt)[z] PPi	Pi	ATP
0 Days (initial)		33.4 ± 3.4	5410 ± 430	89.7 ± 8.4
5C	Air, 3 days	31.3 ± 2.1	7250 ± 820	84.5 ± 6.3
	Air, 6 days	29.2 ± 1.8	8320 ± 690	79.2 ± 7.3
	2%, 3 days	27.5 ± 2.1	6820 ± 870	69.6 ± 6.8
	2%, 6 days	21.6 ± 1.9	7620 ± 940	59.5 ± 5.9
	0.5%, 3 days	23.7 ± 2.4	6420 ± 690	58.7 ± 7.5
	0.5%, 6 days	18.2 ± 2.1	6980 ± 960	46.3 ± 6.9
15C	Air, 3 days	28.2 ± 2.4	7830 ± 860	75.6 ± 7.9
	Air, 6 days	24.8 ± 1.7	8670 ± 790	62.3 ± 6.6
	2%, 3 days	25.4 ± 1.8	7520 ± 850	55.1 ± 6.1
	2%, 6 days	18.3 ± 1.6	8120 ± 920	51.5 ± 6.8
	0.5%, 3 days	21.4 ± 2.5	7210 ± 860	52.4 ± 5.7
	0.5%, 6 days	17.5 ± 2.6	7880 ± 760	43.7 ± 4.5

[z]Mean ± SE from three experiments with at least two assays for each determination.

course, Fru-2,6-P_2 also is now known to influence the activity of several other enzymes in plant sugar metabolism, such as the cytoplasmic FBPase and gluconate dehydrogenase (ap Rees and Dancer, 1987). PPi-PFK is more sensitive to Fru-2,6-P_2 than other enzymes, hence it is a primary Fru-2,6-P_2 target site (ap Rees and Dancer, 1987; Stitt, 1990).

For PPi-PFK to function in the glycolytic direction, an adequate pool of PPi must be present as a substrate (Smyth and Black, 1984). We found the PPi pool, which ranged from 17.5 to 33.4 nmol·g^{-1} fresh weight, to be at least 35% to 40% of the size of the ATP pool (Table 1). The contents of PPi in other plant tissues were 5 to 39 nmol·g^{-1} fresh weight (Kesy and Kowalczyk, 1987; Smyth and Black, 1984). Contents of Pi in carrot shreds (Table 1) are comparable to those reported for other plants (ap Rees et al., 1985; Edwards et al., 1984). The PPi concentration in carrot shreds was estimated to be 17.5 to 33.4 μM with the assumption that 1 g fresh weight of tissue is equal to 1 ml. The K_m for PPi of partially purified plant PPi-PFK is 10 to 20 μM (Kombrink et al. 1984; Smyth and Black, 1984). Thus, the PPi concentration in carrot shreds exceeds the K_m values of PPi-PFK for PPi. These results suggest that PPi is present in carrot shreds as an appreciable pool and its presence provides the phosphate-donating substrate and the energy source for PPi-PFK.

These results indicate that low O_2 caused an increase in the level of Fru-2,6-P_2, which in the presence of adequate supply of PPi, PPi-PFK was activated toward glycolysis. Glycolysis has been shown to be accelerated in many other plants when O_2 is limiting and the glycolytic pathway was considered to replace the Krebs cycle as the main source of energy (Kennedy et al., 1992). The carrot shreds apparently increased glycolysis by activating the PPi-PFK pathway because ATP-PFK activity remained unchanged under low O_2 (Kato-Noguchi and Watada, 1996). The activity of ATP-PFK may have been restricted and could not be accelerated due to reduced supply of ATP, which had decreased by 30% under low O_2 (Table 1). Black et al. (1987) describes how the reaction catalyzed by PPi-PFK conserves energy compared to that catalyzed by ATP-PFK. Thus, when O_2 became limiting, carrot shreds perhaps increased glycolysis as a source of energy and activated a pathway that conserved energy.

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