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Oxygen consumption and lipoxygenase activity in isolated tomato fruit chromoplasts

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Abstract: This Study using purified tomato fruit chromoplasts has shown significant rates of oxygen consumption even in the absence of external precursors. Oxygen consumption rates increased up to 7-fold when chromoplast samples were incubated for 24 h at room temperature. This increase in oxygen consumption is most likely due to the activation of lipoxygenase in the chromoplasts.

Keywords: Chromoplasts, Lipoxygenase, Tomato, Oxygen Consumption

1. Introduction

Chromoplasts are nonphotosynthetic plastids that synthesize and accumulate carotenoids in plant organs such as fruit, flowers, roots, and tubers. They derived from chloroplasts or amyloplasts [1], and show great morphological diversity [2]. Important structural changes occur during the chloroplast to chromoplast transition, thylakoid disintegration being the most significant. The biogenesis of these novel membranous systems has been related with the biosynthesis and/or the storage of the newly formed carotenoids.

In heterotrophic cells, most of the ATP is synthesized in the mitochondria, transported to the cytosol and, from there, to the different cell organelles. In the mitochondria, ATP production is linked to respiratory processes. As a climacteric fruit, tomato shows the typical burst of respiration at the beginning of ripening concomitant to ethylene production [3, 4]. However, in spite that tomato fruit has served as a model system to study many aspects of fruit ripening (e.g. ethylene synthesis and perception) very little is known about the climacteric respiration process and their role in fruit ripening [3].

Chromorespiration is a respiratory process known to operate in the chromoplasts [5]. The existence of a respiratory process in the plastids appears to be well established in higher plants. In the chloroplast, chlororespiration involves a plastid encoded Ndh complex and a terminal oxidase (PTOX) located in the thylakoid membranes [5]. In chloroplasts and chromoplasts PTOX is also known to be involved in the desaturation of phytoene required for carotenoid biosynthesis [6], a process in which O_2 acts as an acceptors of electrons.

The results presented by [7] show that isolated tomato fruit chromoplasts have the capacity to synthesize ATP through the operation of an ATP synthase complex, driven by a membrane proton gradient generated by an electron transport process in which NADPH acts as an electron donor.

Chromorespiration seems to play an essential role not only in carotenoid biosynthesis but also in the synthesis of ATP in chromoplasts.

We have reported, that isolated tomato fruit chromoplasts supplied by labelled precursor are able to actively synthesize carotenoids after 24h. Taken together, it is possible that chromorespiration could be active for several hours [8].

2. Materials and Methods

2.1. Isolation and Purification of Tomato Chromoplasts

Chromoplasts were isolated using the method described by [8]. Fruits (about 300 g) were washed with 2.5% NaCl in

distilled water for 15 min. About 200 g of pericarp tissue, after the removal of the skin, seeds and the gelatinous material of the locular cavities, was cut into small pieces with a razor blade and mixed with 2 volumes of buffer A (100 mM Tris-HCl pH 8.2, 0.33 M sorbitol, 2 mM $MgCl_2$, 10 mM KCl, 8 mM EDTA, 10 mM ascorbic acid, 5 mM L-cysteine, 1 mM PMSF, 1% PVPP and 1 mM DTT). After homogenization with a Waring blender (three pulses at low speed) the homogenate was first filtered through 8 layers of gauze and then through 2 layers of Miracloth (Calbiochem). The debris retained in the gauze layers were recovered, mixed with one volume of buffer A and homogenized again. After filtration through gauze and Miracloth layers as described above, the homogenates were mixed and centrifuged for 2 min at 200xg. The supernatant was recovered and centrifuged for 10 min at 5,000xg. The obtained pellet was resuspended in 50 mL of buffer B (buffer A without PVPP) and centrifuged for 10 min at 5,000xg. The pellet was resuspended in 4 mL of buffer B and chromoplasts were fractionated by ultracentrifugation on a discontinuous sucrose gradient (15%, 30%, 40% and 50% in Tris– HCl pH 7.4 supplemented with 1 mM DTT) for 1 h at 100,000xg (Beckman SW 28 rotor). Chromoplast fractions banding at the 15-30%, 30-40% and 40-50% interfaces were recovered by gentle aspiration with a Pasteur pipette. The collected fractions were washed with one volume of buffer B and chromoplasts were recovered by centrifugation (10 min, 5,000 xg).

Chromoplasts were incubated at 23 ºC in buffer A, containing 100 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM KH_2PO_4, 1 mM NADPH, 1 mM NADP, 20 mM FAD and 330 mM sorbitol.

2.2. Respiration/Oxygen Consumption Measurements

Oxygen consumption was measured using a liquid-phase Clarktype oxygen electrode of Qubit systems' dissolved O_2 Package under the control of the Logger Pro 3.4.6 software. Activity values of oxygen consumption correspond to the slopes of the recorded layouts and are expressed in nmol O_2 consumed by minute and by mg of protein.

Isolated chromoplasts were incubated in buffer A for 1/2 h, 1h, 2h, 4h, 8h, 24h and then 0.4ml aliquots were transferred into the electrode chamber containing 0.6 ml of buffer B (300 mM sorbitol, 10mM N-tris (hydroxymethyl) methyl-2 aminoethane sulfonic acid (TES), 2 mM $MgCl_2$, 5 mM KH_2PO_4, 0.33 mM EDTA, pH 7.4. Depletion of oxygen was linear in the stirred solution of the closed cuvette, except at low concentrations of O_2.

The reaction was carried out at 25°C under constant stirring. Linoleic acid (LA) 3mM, salicylhydroxamic acid (SHAM), octyl gallate (OG) (0.2, 0.4, 0.6, 0.8 1 mM) were added to the electrode chamber using a Hamilton syringe from a stock solution dissolved in distilled water and DMSO, respectively.

3. Results

As a first approach to measure oxidase activity in tomato fruit chromoplasts, purified chromoplast samples were used to measure respiration rates. In the assays, 400 µl of freshly purified chromoplasts were added to 600 µl of respiration buffer and oxygen consumption analyzed using and oxygen electrode. Although at relatively low rates, respiratory activity could be measured at 60 min (43 ± 4 nmol of $O_2.mg^{-1}$ protein.min^{-1}). Surprisingly, very high rates of oxygen consumption were observed when chromoplasts were incubated for 24 h at room temperature in isolation buffer (760 ± 19 nmol of $O_2.mg^{-1}$ protein.min^{-1}) (Table 1). However, large variations of respiration rates were usually observed between chromoplast samples. The obtained results indicated that the very high rate of oxygen consumption measured in chromoplasts (similar to those of reported in isolated mitochondria) could reflect an as yet uncharacterized respiratory process operating during tomato fruit ripening and that could be associated with the active anabolic activities found in this organelle.

Table1. *Respiration rates of isolated tomato fruit chromoplasts at different times (1/2h, 1h, 2h, 4h, 8h, 24h) after isolation. Chromoplasts were used in an O_2 cuvette at 25ºC. Values shown are means ± SE of 4 to 9 replicates.*

Time of incubation (h)	Oxygen consumption (nmol $O_2.mg^{-1}$ protein.min^{-1})
1/2	18 ± 5
1	43 ± 4
2	136 ± 7
4	210 ± 5
8	425 ± 12
24	760 ± 19

3.1. Analysis of the Sensitivity to Respiratory Inhibitors

To better characterize the respiratory process observed, inhibitors acting on different cell oxidases were used: octyl gallate (OG), a common inhibitor of PTOX and lipoxygenase, and salicylhydroxamic acid (SHAM), inhibitor of alternative oxidase. When these compounds were present in the respiration assays a clear reduction in the oxygen consumption rates was observed. In the case of OG, a 90% of inhibition was observed at concentrations higher than 0.6-0.8 mM (Figure 1).

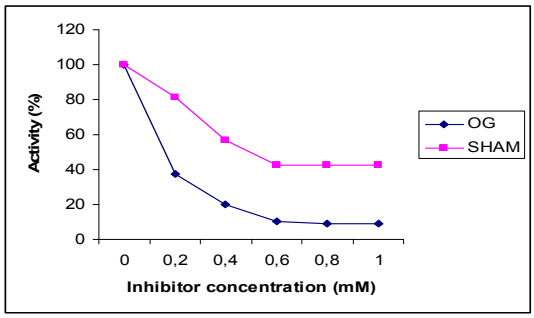

Figure 1. *Effect of OG and SHAM on the oxygen consumption rate of isolated tomato fruit chromoplasts. Inhibitors were added at different concentrations (0.2 -1 mM) to the electrode chamber.*

SHAM respiration was reduced to about 40% at concentrations higher than 0.6 mM.

SHAM and propyl gallate are reported to be inhibitors of AOX by preventing the binding of it substrate [9,10]. PTOX has a similar enzymatic function than AOX as a quinol oxidase. In our assays using isolated chromoplasts, SHAM is not a specific inhibitor. The isolated chromoplasts show a differential sensitivity to both inhibitors, with a relatively low sensitivity to SHAM.

3.2. Detection of Lipoxygenase Activity in Isolated Chromoplasts

The unexpected high rates of oxygen consumption observed in chromoplasts 24 h after isolation could be explained either by the activation of a respiratory mechanism(s) or the induction of an oxidase activity. Such oxidase activity could be provided by lipoxygenase (LOX). In tomato at least five lipoxygenases (TomLoxA, TomLoxB, TomLoxC, TomLoxD and TomLoxE) have been identified [11-13]. In addition, TomloxC is a plastidic isoform known to be induced during ripening, were it seems to be involved in the synthesis of fruit flavor volatiles [13].

To check for the presence of LOX activity in our chromoplast samples we used linoleic acid (LA) a substrate. When respiration assays were performed in the presence of LA a high increase in oxygen consumption was observed (Fig.2).

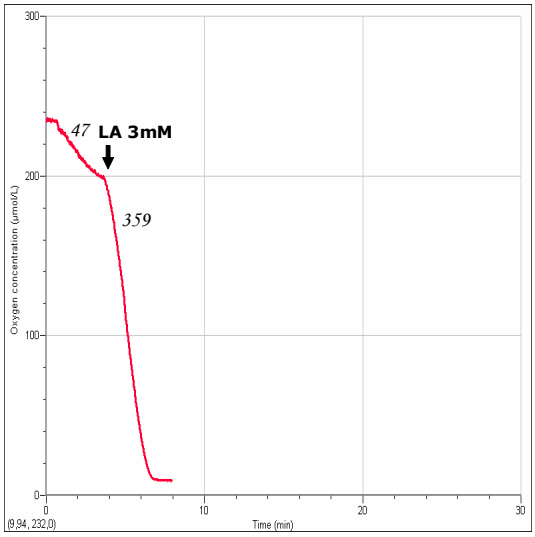

Figure 2. *Effect of linoleic acid (LA) on the respiration rate of tomato fruit chromoplasts after 1 h of isolation. The value indicates the rate of oxygen consumption expressed in nmol O_2.mg^{-1} protein.min^{-1}. The value was selected from at least three independent chromoplast preparations with similar results. LA were added into the electrode cuvette from stock solution.*

These results clearly show that LOX activity is present in tomato fruit chromoplasts. Since all known inhibitors of LOX also affect PTOX, the presence of this activity in tomato fruit chromoplasts represents a major drawback to study respiratory processes in this organelle.

4. Discussion

Although highly controversial in the past, the existence of a respiratory process in the plastids appears to be well established in higher plants. In the chloroplast, chlororespiration involves a plastid encoded Ndh deydrogenase complex and a terminal oxidase (PTOX) localized in the nonappressed thylakoid membranes [5]. PTOX is also known to be involved in the desaturation of phyotene required for carotenoid biosynthesis [6], a process in which O_2 acts as an acceptor of electrons. During chloroplast biogenesis and in nongreen plastids the amounts of chlororespiratory enzymes are higher than in mature green plastids. There are evidences suggesting that chlororespiration could contribute to the bioenergetic metabolism of nongreen plastids by supplying ATP or by reoxidizing metabolic compounds [5,14]

Our preliminary studies using purified tomato fruit chromoplasts have shown significant rates of oxygen consumption. To our knowledge, this is the first experimental description of a respiratory activity in intact chromoplasts with different incubation time . Previously, only respiratory assays using reconstituted chromoplast membrane vesicles from daffodil petals were reported [14]. In this daffodil system, oxygen consumption increased significantly in the presence of NADPH, indicating the existence of redox systems in daffodil chromoplasts linked to a possible respiratory process. This respiratory activity seemed to be linked to ATP production [14].

In the respiratory assays performed with intact tomato fruit chromoplasts we found that oxygen consumption rates increased up to 7-fold after incubation for 24 h at room temperature. This unexpected activation of oxygen consumption rates could be explained assuming an inactivation of PTOX during chromoplast preparation followed by a gradual recovery of activity afterwards. However, another possibility was the presence of lipoxygenase (LOX) which activity could be activated during the 24 h incubation period.

LOX (linoleate:oxygen oxidoreductase, EC 1.13.11.12) refers to a group of enzymes that catalyze the dioxygenation of fatty acids using molecular oxygen [15]. Although the functional role of LOX in plants is still largely unknown, metabolites of unsaturated fatty acids have been involved in growth and development, plant senescence and response to diseases and wounding. Furthermore, in fruits and other plant food products, LOX plays a role in the formation of volatile flavour compounds [15-17].

At least five LOX isoforms (TomloxA, TomloxB, TomloxC, TomloxD, and TomloxE) are present in tomato fruit [13]. Interestingly, it has been reported that TomloxC is expressed during fruit ripening and targeted to plastids, where it seems to be involved in the synthesis of fruit flavor volatiles [13]. Experiments using linolenic acid has revealed the presence of LOX in chromoplast extracts (fig.2). All together, it is likely that the increase of oxygen consumption observed in isolated chromoplasts could reflect an activation

of LOX. Since all known inhibitors of LOX also affect PTOX, the presence of this activity in chromoplasts represents a major drawback to study chromorespiration. Further experiments are needed to establish the relative contribution of these enzymes in the oxygen consumption observed in tomato fruit chromoplasts.

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