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Growth Temperature Control of the Linoleic Acid Content in Safflower (*Carthamus tinctorius*) Seed Oil

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The temperature and oxygen regulation of the microsomal oleate desaturase (FAD2) from safflower (*Carthamus tinctorius* L.) seeds was investigated. Heat-resistance profiles obtained in vivo and in vitro showed that the FAD2 enzyme maintained its maximal activity until 30 °C. A temperature increase from 10 to 40 °C caused a decrease of the FAD2 activity. However, when the temperature was decreased from 40 to 10 °C, no increase in the activity level was detected. The removal of hulls from safflower seeds followed by incubation in air did not change the FAD2 activity level, whereas incubation under nitrogen caused a strong decrease. Air replacement brought about the recovery of the initials levels. Oxygen concentrations less than 3% produced the inactivation of the enzyme. These data indicate that the higher thermal stability and the lower dependence on oxygen availability of the safflower FAD2 enzyme, compared with that of sunflower, could be the main factors to explain why the linoleate content of safflower seeds is more independent of growth temperature than that of sunflower seeds.

KEYWORDS: ***Carthamus tinctorius*; safflower; microsomal oleate desaturase; oilseed; oxygen regulation; temperature regulation**

INTRODUCTION

The nutritional characteristics of vegetable oils are highly dependent on their fatty acid composition, with oleic and linoleic acids being the major fatty acids in storage triacylglycerols (TAG) of oilfruits and oilseeds. Therefore, the linoleic content is an important parameter that determines nutritional properties of edible oils (*1*).

The enzyme responsible for the synthesis of linoleic acid from oleic acid, which is the main product of the plastidial fatty acid biosynthesis, is the microsomal oleoyl phosphatidylcholine desaturase (FAD2; EC 1.3.1.35) (*2*). This is a membrane-bound enzyme that converts oleic acid, preferentially esterified in the *sn*-2 position of phosphatidylcholine, to linoleic acid. The reaction involves the concomitant reduction of molecular oxygen to water and requires the presence of NADH, NADH-cyt b_5 reductase, and cyt b_5 as electron donor system (*3*).

Environmental temperature during oilseed development modifies the proportion of linoleic acid depending on the geographical area and year, resulting in an unwanted and uncontrolled variation of the oleic/linoleic acid ratio in the final fatty acid composition of the oil (*4*). Low temperatures increase the linoleic acid content of oilseed oils. Nevertheless, the mechanism by which temperature affects the percentage of linoleate in oilseeds is still not completely elucidated.

Currently, the market demands oils with a constant degree of unsaturation, and therefore, it is a goal to obtain oilseed varieties with fatty acid composition independent of the environmental temperature. To achieve that, it is necessary to understand how temperature controls the linoleate content in oilseeds.

Our group has studied this phenomenon in sunflower seeds, in which the percentage of linoleate is highly dependent on growth temperature (*5*, *6*). These studies have shown that temperature affects the de novo oleate biosynthesis (*7*) and its mobilization from preformed TAG (*8*, *9*), thus modifying the available amount of oleate for desaturation. In addition, temperature regulates the FAD2 enzyme. Two separate mechanisms were proposed to be involved in the temperature regulation of the FAD2 activity: a direct effect mainly related to the low thermal stability of the enzyme (*10*), and an indirect effect by which temperature determines the availability of oxygen, which, in turn, regulates the FAD2 activity (*11*).

To further investigate this hypothesis, we have studied the changes in the level of FAD2 activity under different temperature conditions and oxygen availability in safflower seeds, where the linoleate content is much less temperature dependent than that in sunflower (*12*). The results explain the differences in the temperature effect on the seed linoleate content between the two species.

MATERIALS AND METHODS

Plant Material. High linoleate safflower (*Carthamus tinctorius* L. cv. Rancho) seeds (*13*) were provided by Dr. J. M. Fernández-Martínez, IAS, CSIC, Córdoba, Spain. Plants were cultivated in a growth chamber

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with a 16 h photoperiod, photon flux density of 300 μmol m^{-2} s^{-1} (fluorescent lamps; Cool white and Gro-lux; Sylvania, Dansvers, MA) at 25/15 °C (day/night). Although the fruit of safflower is referred to as a cypsela or achene, for the purposes of this study, the more familiar term seed will be used. Seeds of 14−18 days after flowering (DAF), which corresponds to the period of active triacylglycerol biosynthesis, were collected for the experiments.

Chemicals. Organic salts, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), sorbitol, NADH, 1-palmitoyl-lysophosphatidylcholine, and butylhydroxytoluene were purchased from Sigma (St. Louis, MO). Inorganic salts, acids, chloroform, methanol, ethyl ether, heptane, toluene, acetonitrile, and thin-layer chromatography Silicagel 60 plates were from Merck (Darmstadt, Germany). [1-^{14}C] Oleoyl-CoA was supplied by American Radiolabeled Chemicals (St. Louis, MO).

Seeds Incubations. Lots of twenty peeled safflower seeds (achenes without hull and seed membrane), corresponding to approximately 0.5 g, were collected from different capitula and plants to ensure homogeneity. The seeds lots were incubated at the indicated temperatures and times in a stream of water-saturated gas, either air or nitrogen with different oxygen concentrations. For details, see figure captions.

Subcellular Fractionation. The incubated seeds lots were ground in a pre-cooled mortar with 10 mL of 50 mM HEPES buffer (pH 7.2) containing 0.6 M sorbitol, 40 mM Na-ascorbate, 1 mM Na_2EDTA, and 1 mM $MgSO_4$ (grinding buffer). All manipulations were done at 4 °C. The homogenate was centrifuged at 10 000*g* for 5 min. The fat layer was discarded, and the supernatant was centrifuged for 1 h at 100000*g*. The pellet containing the microsomal fraction was resuspended in 1 mL of grinding buffer and stored at −80 °C.

Lipid Extraction and Analysis. Aliquots of the homogenate (0.5 mL) were mixed with 1.25 mL of chloroform/methanol/glacial acetic acid (50:50:1, by vol.) and shaken. Total lipids were recovered from the lower phase, evaporated to dryness with nitrogen, and converted into the corresponding fatty acid methyl esters by heating at 80 °C for 1 h in a 2-mL solution of methanol/toluene/96% H_2SO_4 (80:20:2, by vol.) (*14*). After cooling, the methyl esters were extracted with 2 mL of heptane and analyzed by gas−liquid chromatography (*7*).

In Vitro Assay of FAD2 Activity. The in vitro assay of microsomal oleate desaturase activity was carried out as described by García-Díaz et al. (*7*), using 5 μL of microsomal suspension (corresponding to approximately 2.5 mg of fresh seed tissue and 30 μg of microsomal protein) and 30 min as incubation time. Incubation temperature was 25 °C, except when indicated.

Determination of the Michaelis−Menten Constant (K_m) for Oxygen. Safflower seeds microsomal fraction was assayed for FAD2 activity using standard conditions, except that oxygen concentration was varied. The reaction mixture was bubbled with nitrogen containing different oxygen percentages (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 20) during the time of assay. Oxygen concentration in the assay mixture was calculated from the solubility data according to Wilhelm et al. (*15*) and corrected for the percentage of oxygen in the gas mixture. Hanes−Woolf plot was used to obtain the K_m value.

Statistics and Data Analysis. Results are mean of two independent experiments, with duplicate determinations of fatty acid composition and FAD2 activity. In all cases, the SD was <3% of mean value.

RESULTS

To study the direct effect of temperature on the linoleic acid content and the FAD2 activity separately from the temperature effect on oxygen availability, peeled safflower seeds were used. After 6 h of incubation at temperatures between 15 and 50 °C, the microsomal fraction was isolated and the FAD2 activity determined. **Figure 1** shows that the activity of the enzyme remained intact until 30 °C and then decreased as temperature increased, reaching a very low level at 45 °C. On the other hand, the fatty acid composition remained unaffected; the linoleic acid content being 77% at all studied temperatures.

The thermal properties of the safflower FAD2 enzyme were also characterized in vitro using microsomal membranes isolated from developing seeds. Optimal temperature under standard assay conditions was 25 °C (**Figure 2A**). When the heat-resistance profile (**Figure 2B**) was obtained, preincubating the microsomes at different temperatures for 30 min and then assaying the activity under standard conditions, the enzyme showed maximal activity until 30 °C. The temperature at which 50% of the enzyme activity was recovered was 34 °C, whereas no activity was detected at 45 °C.

The direct effect of temperature on the FAD2 activity was further studied using peeled safflower seeds subjected to temperature transitions. When peeled seeds were incubated at 10 °C for 14 h (**Figure 3A**), the enzyme activity remained practically constant. After temperature shifted to 40 °C, the

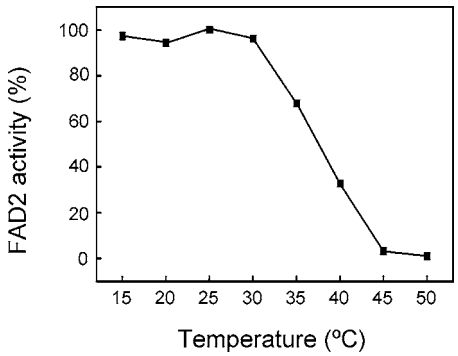

Figure 1. Effect of temperature on the FAD2 activity in peeled developing safflower seeds. Peeled seeds were incubated at the indicated temperatures in a stream of water-saturated air for 6 h, homogeneized, and stored at −20 °C. The homogenate was used to isolate the microsomal fraction, and the FAD2 activity was measured as described in Materials and Methods (100% = 38 nmol 18:2 (g FW)$^{-1}$ h^{-1}).

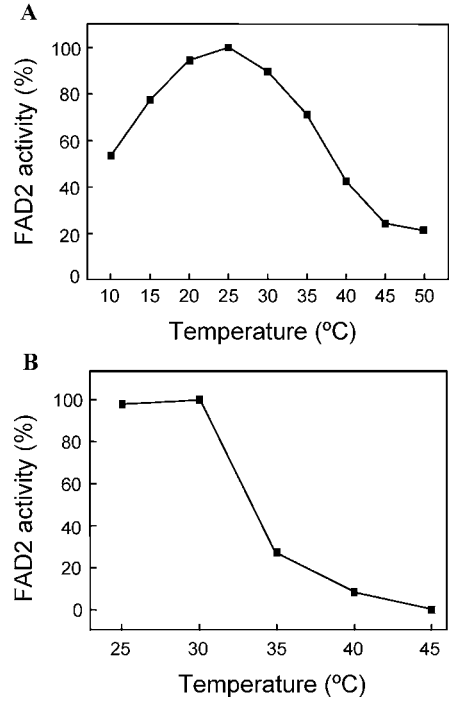

Figure 2. Optimal temperature (**A**) and heat-resistance profile (**B**) of FAD2 enzyme from developing safflower seeds. Microsomal samples isolated from developing seeds homogeneized immediately after dehulling were either assayed for FAD2 activity using the standard conditions, as described in Materials and Methods, except the indicated temperatures (**A**), or incubated at the indicated temperatures, and after 30 min, cooled rapidly on ice and assayed for FAD2 activity using the standard conditions (**B**) (100% = 45 nmol 18:2 (g FW)$^{-1}$ h^{-1}).

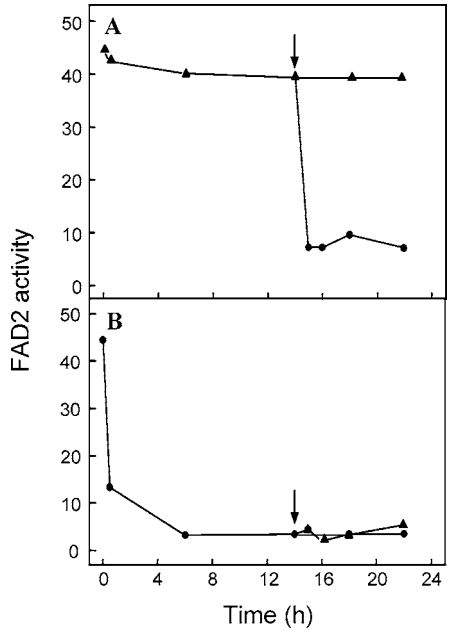

Figure 3. Effect of temperature changes from 10 to 40 °C (**A**), and from 40 to 10 °C (**B**), on the FAD2 activity in peeled developing safflower seeds. Peeled seeds were incubated at 10 °C (▲) or at 40 °C (●) in a stream of water-saturated air. Temperature was shifted or maintained after 14 h (denoted by an arrow). At the indicated times, the peeled seeds were homogeneized and stored at –20 °C. The homogenate was used to isolate the microsomal fraction, and the FAD2 activity was measured as described in Materials and Methods and expressed as nmol 18:2 (g FW)$^{-1}$ h^{-1}.

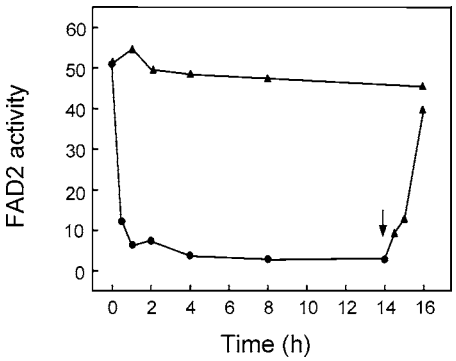

Figure 4. Effect of anoxia followed by oxygen replacement on FAD2 activity in peeled developing safflower seeds. Peeled seeds were incubated at 20 °C in a stream of water-saturated air (▲) or nitrogen (●). The seeds incubated under nitrogen were shifted to air after 14 h (denoted by an arrow). At the indicated times, the seeds were homogeneized and stored at –20 °C. The homogenate was used to isolate the microsomal fraction and the FAD2 activity was measured as described in Materials and Methods and expressed as nmol 18:2 (g FW)$^{-1}$ h^{-1}.

FAD2 activity decreased drastically during 30 min, remaining at very low levels afterward. In a similar experiment, but starting at 40 °C (**Figure 3B**), a rapid and strong decrease of the FAD2 activity was observed, and then remained unaffected. After changing to 10 °C, no change in the enzyme activity level was detected.

On the other hand, we have also investigated if different conditions of oxygen availability affect the FAD2 activity level of safflower seeds. First, the effect of anoxia followed by oxygen reposition on the safflower FAD2 activity was investigated (**Figure 4**). When peeled seeds were incubated at 20 °C in a stream of air, no significant change in the enzyme activity was

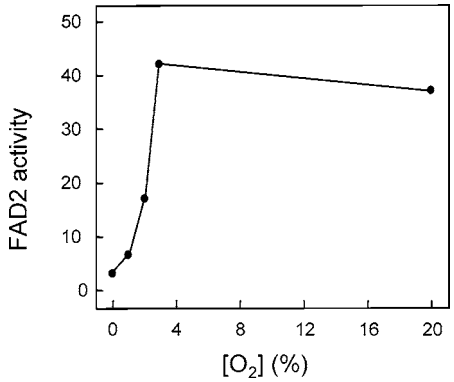

Figure 5. Effect of oxygen concentration on FAD2 activity in peeled developing safflower seeds. Peeled seeds were incubated at 20 °C in a stream of water-saturated nitrogen with different oxygen concentrations. After 30 min, the seeds were homogeneized and stored at –20 °C. The homogenate was used to isolate the microsomal fraction, and the FAD2 activity was measured as described in Materials and Methods and expressed as nmol 18:2 (g FW)$^{-1}$ h^{-1}.

detected after 16 h. In contrast, anoxia brought about a rapid and strong decrease of the activity during the first hour, remaining at a very low level afterward. Air replacement after 14 h of anoxia produced the recovery of the initial activity level in 2 h.

To determine the oxygen level that caused a decrease in the FAD2 activity, peeled seeds were incubated for 30 min at different concentrations of the gas (**Figure 5**). The enzyme activity was similar in seeds incubated either in the air (20% oxygen) or under nitrogen containing 3% oxygen, whereas when the oxygen level was reduced to less than 3%, a strong decrease of the enzyme activity was observed.

In addition, to measure the affinity of the safflower FAD2 enzyme for oxygen, we determined the Michaelis−Menten constant (K_m) for this substrate, obtaining a value of 12.7 μM.

DISCUSSION

Previous studies have shown that in sunflower seeds, where the linoleate content is highly dependent on growth temperature, the FAD2 enzyme is regulated by temperature by at least two different and independent mechanisms: a direct effect and an indirect effect modifying oxygen availability (*7*). To further characterize both mechanisms, we have investigated and compared the effect of temperature and oxygen availability on the FAD2 activity of safflower seeds, characterized by a high linoleate content, which is almost unaffected by environmental temperature.

To study the direct temperature effect with no interference of oxygen availability, we have used peeled safflower seeds to be sure that oxygen concentration is not limiting for oleate desaturation. The in vivo heat-resistance profile (**Figure 1**) showed that the safflower FAD2 maintained its maximal activity level until 30 °C, revealing a higher thermal resistance in vivo than the sunflower enzyme (*10*). In the same experiment, the linoleate content in these peeled seeds remained high and constant at all studied temperatures, in contrast to the situation in sunflower where a maximal in vivo oleate desaturation was observed at 20 °C (*10*). A mechanism controlling the maximal linoleate level of the safflower seeds should act in vivo, because the percentage of linoleate in seeds from plants grown in the field at different temperatures never exceeds 82% (*12*). In addition, the in vitro thermal properties of the safflower FAD2 enzyme have been characterized. The optimal temperature was

25 °C (**Figure 2A**), similar to that reported in sunflower (*10*), but lower than that described for Δ12-desaturases from nonplant organisms such as fungi (*16*) and cyanobacteria (*17*), with values of 40 and 35 °C, respectively. The heat-resistance profile was also obtained in vitro, by preincubating the microsomes at different temperatures (**Figure 2B**), and corroborated the results obtained in vivo (**Figure 1**), confirming that the safflower FAD2 is more thermally resistant than the sunflower enzyme (*10*) but less than that of *Lipomyces staskeyi* (*16*).

The effect of temperature changes on the FAD2 activity level was also investigated in peeled safflower seeds. When the seeds were incubated at 10 °C (**Figure 3A**), no change in the FAD2 activity level was observed, although it decreased strongly and rapidly when the temperature shifted to 40 °C, possibly due to the heat denaturation of the enzyme. These results are in agreement with that shown in **Figure 1**, indicating that 10 °C preserved the safflower FAD2 activity, while 40 °C brought about a strong decrease in the activity level. In contrast, when the peeled seeds were first incubated at 40 °C, the corresponding decrease in the FAD2 activity was detected, but no recovery of the activity level was observed after shifting to 10 °C (**Figure 3B**), confirming the presence of an irreversible heat denaturation process and not a typical reversible activation-inactivation mechanism. These data also corroborate the higher thermal stability of the safflower FAD2 enzyme compared with that of sunflower, where a similar behavior with respect to temperature changes has been previously reported, but using 30 instead of 40 °C (*10*).

To study if the safflower FAD2 is regulated by an indirect temperature effect on the oxygen availability as reported previously for the sunflower enzyme (*7*), we have measured the FAD2 activity in safflower seeds under different oxygen concentrations. Unlike sunflower seeds, where the low initial FAD2 activity increased dramatically after hull removing and incubation in air due to the increased oxygen availability (*7*), safflower seeds did not change the activity level significantly, keeping constant the initial high level (**Figure 4**). On the contrary, when the safflower seeds were incubated under nitrogen, a rapid decrease of the FAD2 activity was seen, similar to that reported for the sunflower enzyme (*18*), indicating that the safflower FAD2 activity is also controlled by oxygen availability. However, the inactivation of the safflower FAD2 enzyme produced by anoxia was not followed by a long-term slow increase of the activity level, as it was previously described for sunflower (*11*), suggesting that an additional mechanism is present in sunflower, but not in safflower seeds.

On the other hand, the value obtained for the K_m for oxygen (12.7 μM) is the first reported for an FAD2 enzyme, being lower than that described for the safflower stearate desaturase, which was 56 μM (*19*). These data indicate that in safflower seeds, the microsomal oleate desaturase exhibits a higher affinity for oxygen than does the stearate desaturase.

In contrast to sunflower seeds, where the FAD2 activity was partially inactivated at oxygen concentration of 3% (*11*), the safflower enzyme kept the maximal activity level at this concentration (**Figure 5**), pointing out that is active at lower oxygen concentrations than the sunflower FAD2. The effect of low oxygen concentration on safflower FAD2 activity was not observed in a previous work (*20*), because the safflower seeds were incubated in the range of 16−29% oxygen before the enzyme activity was measured. However, the oxygen concentration inside the tissue should be lower than that at atmospheric equilibrium at the studied temperatures, as indicated in sunflower seeds (*7*).

The high FAD2 activity level found in intact safflower seeds, in comparison with the characteristic low level detected in sunflower (*7*), could be explained by the lower sensitivity to oxygen concentrations observed for the safflower enzyme, together with a possible higher oxygen concentration inside the safflower seed due to a higher permeability of the hull and/or a lower respiration rate that competes for the available oxygen. Thus, in plants cultivated under the same physiological conditions, safflower seeds show a higher FAD2 activity level than do sunflower seeds.

In conclusion, the higher thermal stability of the safflower FAD2 enzyme and the lower dependence on oxygen availability could explain why the linoleate content in the safflower seed is less affected by temperature and oxygen than in sunflower seeds, where the FAD2 enzyme is less thermally resistant and more sensitive to oxygen shortage. The results indicate that sunflower and safflower represents two models for the temperature and oxygen regulation of oleate desaturation in oilseeds.

ABBREVIATIONS USED

DAF, days after flowering; FAD2, microsomal oleate desaturase; FW, fresh weight; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; TAG, triacylglycerol

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