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ARTICLE

Antioxidant System and Protein Pattern in Peach Fruits at Two Maturation Stages

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Peach fruits were selected to study the protein pattern and antioxidant system as well as oxidative parameters such as superoxide radical and hydrogen peroxide accumulation, at two maturity stages, which were chosen for being suitable for the processing industry and fresh consumption. The proteins phosphoenolpyruvate carboxylase, sucrose synthase, and 1-aminocyclopropane-1-carboxylate oxidase, as well as the antioxidants glutathione synthetase and ascorbate peroxidase, appeared as new in the mature peach fruits. Activities of superoxide dismutase (SOD) and components of the ascorbate−glutathione cycle were also measured to explore their role in the two maturity stages studied. Changes in the SOD isoenzyme pattern and an increase in the activities of ascorbate peroxidase, monodehydroascorbate reductase, and glutathione reductase were observed in mature fruits, revealing an efficient system to cope with the oxidative process accompanying ripening.

KEYWORDS: Antioxidant; oxidized ascorbate (DHA); oxidized glutathione (GSSG); peach; reduced glutathione (GSH); reduced ascorbate (ASC); ripening; SOD

INTRODUCTION

Peach (*Prunus persica* L.) is a climacteric fruit, and ripening is controlled by the production of ethylene, although other hormones are also involved (*1*). This process is a dynamic event that involves biochemical and physiological changes, and the main research effort has focused on characterizing the molecular components involved, including ethylene biosynthesis, cell wall depolymerization, signal transduction, pigment accumulation, increased respiration, and enhanced reactive oxygen species (ROS) accumulation (*2*). Some of the changes in the chemical composition and physical characteristics of the fruit taking place during ripening lead to easily perceivable alterations in fruit texture, firmness, pigmentation, aroma, and sweetness (*3*). These aspects assume special consideration in important economic fruit crops in temperate regions, such as peaches, which are chosen by consumers for their flesh texture and rich aroma and sweetness. Other changes during fruit ripening and senescence include important increases in respiration and, as a consequence, an enhancement in the ROS accumulation as well as changes in the antioxidant activities which promote the oxidative stress that contributes to a general deterioration of the cell metabolism, mainly producing peroxidation of membrane lipids, protein denaturation, and DNA mutation (*4*−*6*).

ROS are maintained and controlled by a battery of molecules with antioxidant capacity, which are distributed in cell organelles that play an important role in both senescence and ripening processes. Among these, superoxide dismutase (SOD), which removes superoxide radical ($O_2^{\bullet -}$), catalase, and the ascorbate−glutathione (ASC−GSH) cycle involved in the removal of hydrogen peroxide (H_2O_2), together with the more recently described thiol-specific proteins including the thioredoxin/peroxiredoxin system involved in the scavenging of hydroperoxides and in redox regulation (*7*), have been described as components of the antioxidant system. Vitamin C is a powerful antioxidant in its reduced form and is involved in the scavenging of ROS, quenching of excess excitation energy and regeneration of other antioxidants such as α-tocopherol, as well as in growth regulation and cell division (*8*). Glutathione (GSH) is another antioxidant and redox buffer and is the main nonprotein thiol cofactor of certain detoxifying enzymes and is involved among others, including ascorbate, in the regulation of defense gene expression (*9*). To maintain these compounds in their reduced forms, the ASC−GSH cycle is composed of a series of coupled redox reactions involving four enzymes, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), and its presence has been described in different cell compartments, including chloroplasts, cytoplasm, mitochondria, and peroxisomes, its function being related not only with the detoxification of toxic oxygen species but also with metabolism (*8*, *10*). Changes in the antioxidant system have been described during fruit ripening and senescence, and it has recently been proposed that SOD enzyme and components of the ASC−GSH cycle may play a role during fruit ripening as modulators of plastid ASC and GSH redox states and also of the other signal molecules such as $O_2^{\bullet -}$ and H_2O_2 (*4*, *11*).

Extensive studies on the role of ROS and antioxidants in fruit development and ripening have been conducted on climacteric and nonclimacteric fruits such as pepper (*11*), tomato (*4*), saskatoon fruit (*12*), apple (*13*), and pear (*14*). Peach is one of the most popular fruits in the world because of its high nutrient content and pleasant flavor, and fresh fruits are an excellent source of sucrose, citric and malic acids, carotenoids, and lactones, as well as polyphenol and pectic substances, which determine the sensory

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quality and nutritive values of the fruits. Some of these compounds are important antioxidants, and their levels in fresh fruits and vegetables have relevant health implications. However, there is little information related to the changes in the antioxidant system controlling ROS, which are involved in the recycling of vitamin C in peach fruits during ripening, with research on this fruit focused mainly on the search of alternative treatments that would delay fruit ripening, due to the short postharvest shelf life of the fruits and their rapid senescence (*15*). In any case, information about antioxidant properties in different fruit ripening stages is very useful for nutritionists and consumers so that both the right variety and fruit stage, where antioxidants are highest, can be selected, providing not only better nutritional values but also protection for the fruit itself, which is going to suffer postharvest processing or storage. Thus, this study examines changes in the protein pattern and antioxidants such as SOD and components involved in the ASC−GSH cycle, together with the changes in the $O_2^{\bullet-}$ and H_2O_2 levels, to characterize the antioxidant system and the main proteins involved in the ripening of peach fruits, in the two maturity stages that are suitable for the processing industry and fresh consumption.

MATERIALS AND METHODS

Materials and Sample Preparation. Assays were conducted with peach fruits (*P. persica* L.) grown in a commercial orchard in Murcia, southeastern Spain. The experiment was repeated at least three times to prove the repeatability of the experiment.

Immediately after harvest, fruits were manually selected for uniformity in color, size, and firmness and divided into two groups: one group was selected as immature fruits with a green color and a firmness between 88 and 102 N, and the second group was selected as mature fruits with uniform yellow color and a firmness between 70 and 74 N. The fruit firmness was determined using Durofell equipment (Mod. Copa Technology). Both stages were chosen as the ones usually used in the processing industry and for fresh consumption. Blemished and diseased fruits were discarded. For analysis, the peeled fruit flesh of at least five fruits per sample was cut into pieces and immediately frozen in liquid N_2 and stored at −80 °C.

Protein Fruit Extraction. Protein extraction was carried out by homogenizing 10 g of frozen material in 20 mL of extraction buffer at 4 °C using a Polytron. Extraction buffer contained 50 mM potassium phosphate (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM cysteine, 1% (w/v) soluble poly(vinylpyrrolidone) (PVP), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.2% (v/v) Triton X-100. The homogenate was centrifuged at 12000*g* for 20 min, at 4 °C, and the supernatant was desalted in a cold Sephadex G 50 PD 10 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden), equilibrated with the same buffer used for homogenization. The supernatant was frozen at −20 °C until enzymatic determination.

Total soluble proteins were measured using bovine serum albumin as standard (*16*). For SDS-PAGE analysis, 20 μg of proteins was diluted in 125 mM Tris-HCl, pH 6.8, 0.5% (w/v) SDS, 0.05% (v/v) bromophenol blue, and 10 mM (w/v) dithiothreitol (DTT) and boiled for 3−5 min at 98 °C. SDS-PAGE was performed in 12% (w/v) acrylamide/Bis (37.5/1, 30%) gel. The proteins were prefocused at 100 V for 10 min and then focused at 200 V for 40 min. The molecular masses of the polypeptides were estimated from marker standards. Proteins were visualized with Coomassie blue, and quantification of the intensity of the bands was conducted by image analysis software (Gen Tools, Syngene, Cambridge, U.K.) in at least three independent bands. Gel bands of interest were manually excised from gel and sent to the National Biotechnology Center (CSIC, Spain) for further analyses. The bands were subjected to in-gel trypsin digestion (*17*), and the samples were analyzed on an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF-TOF (Applied Biosystems, Framingham, MA) spectrometer. Protein identification was performed by searching the National Center for Biotechnology Information nonredundant database (NCBnr) using the MASCOT program (http://www.matrixscience.com). For positive identification, the score of the result of ($-10 \times \log(p)$) had to be over the significance threshold level ($p < 0.05$).

Determination of the SOD Activity. Total SOD activity was assayed in the crude extract. SOD activity (EC 1.15.1.1, SOD) was assayed following the ferricytochrome *c* reduction using xanthine/xanthine oxidase as the source of $O_2^{\bullet-}$ radicals (*18*). The reaction mixture (3 mL) contained 50 mM potassium buffer (pH 7.8), 0.1 mM EDTA, 1 mM cytochrome *c*, 1 mM xanthine, 4−15 μL of xanthine oxidase, and an enzyme sample. SOD isoenzymes were separated by isoelectrofocusing (IEF) on 7.5% (w/v) acrylamide gels in the pH range of 4.0−5.0 (Serva Electrophoresis GmbH). Samples were prefocused at 50 V for 30 min, and the voltage was increased by 50 V each 30 min after 250 V and then focused for 5 h. After electrophoresis, a photochemical method was used to visualize SOD activity (*19*). Isoenzyme identification was performed by selective inhibition with 2 mM KCN and 5 mM H_2O_2. The isoenzyme activities were quantified using an image analyzer (Gen Tools, Syngene, Cambridge, U.K.).

Enzyme Assays. Ascorbate peroxidase (EC 1.11.1.11, APX) activity was assayed at 25 °C according to the method of Jiménez et al. (*10*) measuring the decrease in A_{290} due to the ASC oxidation by H_2O_2. The reaction mixture (1 mL) contained 50 mM Hepes−NaOH (pH 7.6), 0.22 mM ascorbate, 0.3 mM H_2O_2, and an enzyme sample. The oxidation rate of ASC was estimated between 1 and 60 s after the reaction was started by the addition of H_2O_2. The enzyme activity was expressed as nanomoles per minute times milligram of protein of ascorbic acid oxidized using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Monodehydroascorbate reductase (EC 1.1.5.4, MDHAR) activity was assayed by the decrease in A_{340} due to the NADH oxidation (ε_{340} = 6.22 mM^{-1} cm^{-1}) (*10*). Monodehydroascorbate was generated by the ascorbate/ascorbate oxidase system. The reaction mixture (1 mL) contained 50 mM Tris-HCl (pH 7.8), 0.2 mM NADH, 1 mM ascorbate, 0.5 unit of ascorbate oxidase, and an enzyme sample. To determine the MDHAR activity, the rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) was subtracted from the initial monodehydroascorbate-dependent NADH oxidation rate (with ascorbate and ascorbate oxidase). Dehydroascorbate reductase (EC 1.8.5.1, DHAR) activity was determined according to the method of Dalton et al. (*20*) by following the increase in A_{265} due to ascorbate formation (ε_{265} = 14 mM^{-1} cm^{-1}) and using N_2-bubbled buffer. The reaction mixture (1 mL) contained 50 mM potassium buffer (pH 6.5), 0.1 mM EDTA, 4 mM dehydroascorbte, 50 mM reduced glutathione (GSH), and an enzyme sample. The reaction rate was corrected for the nonenzymatic reduction of DHA by GSH. A factor of 0.98 to account for the small contribution to the absorbance by GSSH was also considered.

Glutathione reductase activity was assayed by the decrease in A_{340} due to the NADPH oxidation (ε_{340}= 2.8 mM^{-1} cm^{-1}), as described by Edwards et al. (*21*). The reaction mixture (1 mL) contained 100 mM Hepes−NaOH (pH 7.8), 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione (GSSG), and an enzyme sample. The reaction rate was corrected for the small, nonenzymatic oxidation of NADPH by GSSH.

Ascorbate and Glutathione Determination. The determination of ascorbate was performed from 10 g of flesh fruit previously lyophilized, which were then extracted with 10 mL of 5% *m*-phosphoric acid (w/v) and incubated on ice in the dark for 30 min. The homogenate was centrifuged at 15000*g* for 10 min, and the supernatant obtained was filtered through 0.22 μm Millex filters (Millipore). ASC and DHA in the supernatant were determined immediately by HPLC, and the DHA was quantified from ASC data by incubating the samples for 24 h at room temperature with 1 mM DTT (final concentration). The DHA concentration was measured as ASC after rechromatography (*10*).

The determination of glutathione was performed as described by Washko et al. (*22*) with some modifications. Pieces of 10 g of fresh fruit were lyophilized and homogenized with 10 mL of extraction solution containing 5% *m*-phosphoric acid (w/v) and 1 mM EDTA in 0.1% formic acid, supplemented with 1% (m/v) polyvinyl-polypyrrolidone (PVPP) just before use. Homogenates were centrifuged at 15000*g* for 20 min at 4 °C. Supernatants were collected and filtered through 0.22 μm Millex filters (Millipore), immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. Analyses were carried out with an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies, Santa Clara, CA) with an electrospray ionization source (ESI) and coupled to an Agilent 1100 series HPLC system (Agilent Technologies). The data analysis program

LC/MSD Trap version 3.2 (Bruker Daltonik, GmbH, Germany) was used for the quantification of the metabolites.

Determination of Superoxide Radical and Hydrogen Peroxide. The determination of $O_2^{\bullet -}$ was carried out essentially as described by Sagi and Fluhr (*23*), with minor modifications. Pieces of immature and mature peach (1 g of fresh weigh) were incubated in 10 mL of 50 mM Tris-HCl buffer (pH 7.8) for 60 min in darkness. Later, the buffer was removed and peach pieces were incubated in a reaction mixture containing 1 mM epinephrine. $O_2^{\bullet -}$ accumulation in the incubation medium was determined spectrophotometrically by measuring the oxidation of epinephrine to adrenochrome at 480 nm. Superoxide dismutase as control test was used to verify the specificity of NBT reduction by $O_2^{\bullet -}$ in this reaction mixture.

The H_2O_2 content in the peach fruits was determined by a peroxidase-coupled assay, using 4-aminoantipiryne and phenol as donor substrates (*24*). The formation of quinonimine was measured at $A_{505\ nm}$ as result of the oxidation of phenol and 4-aminoantipiryne.

Statistical Analysis. All experiments were run at least in triplicate, and each sample was analyzed three times. The significance of any differences between mean values was determined by one-way analysis of variance; Duncan's multiple-range test was used to compare the means when necessary.

RESULTS

Fruits in two maturation stages were harvested directly from the tree for measurement of firmness and were subsequently frozen for biochemical analysis, with no intervening storage period, to study changes in the pattern of total soluble proteins as well as changes in some components of the antioxidant system taking place during the natural fruit ripening process. **Figure 1** shows the two maturation stages selected for our study. The first stage is identified as immature with a green/yellow color (**Figure 1A**) and a firmness of 99.7 N (**Figure 1B**) and the second stage as mature, with a yellow color (**Figure 1A**) and a firmness of 74 N (**Figure 1B**).

Initially the pattern of peach proteins during the two maturation stages selected was analyzed by SDS-PAGE after extraction of soluble proteins. Eleven protein bands were visibly observed, but our study focused on the bands that significantly modified their intensity with maturation, identified as 1−4 and 6−8 (**Figure 2**). Analysis of the peptides identified revealed the presence of 14 proteins in immature fruits and 15 in mature fruits (**Table 1**), with 5 of them being expressed in both maturation stages: UTP-glucose-1-phosphate uridylyltransferase, adenosylhomocysteinase, and UDP-glucose pyrophosphorylase in band 6; glyceraldehyde-3-phosphate dehydrogenase in band 7; and triosephosphate isomerase in band 8 (**Table 1**). Eight peptides were differentially expressed in both fruits, corresponding to 7 proteins present in the immature fruits, but not in the mature ones: multicopper oxidase, phospholipase D α (band 3); methyltetrahydropteroyltriglutamate-homocysteine methyltransferase and phospholipase D [a] (band 4); and NAD-dependent malate dehydrogenase, cytosolic aldolase, and a predicted protein (band 7). In mature fruits, 9 new peptides appeared corresponding to glutathione synthetase (band 1), phosphoenolpyruvate carboxylase (PEPC, band 2), sucrose synthase (SS), ATPase β subunit (band 3), two predicted proteins (band 4), aminocyclopropane-1-carboxylate oxidase (ACO) (band 7), GTP-binding nuclear protein Ran, and ascorbate peroxidase (band 8) (**Table 1**).

SOD and the components of the ascorbate−glutathione cycle were assayed in the peach fruit crude extracts in the two ripening stages. Total SOD activity was similar in immature and mature

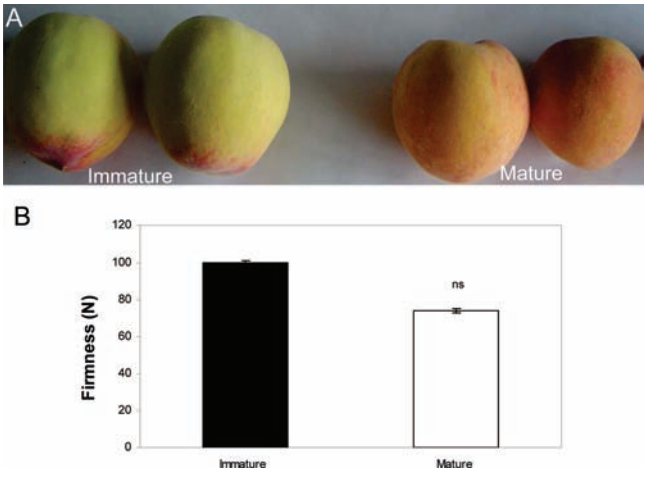
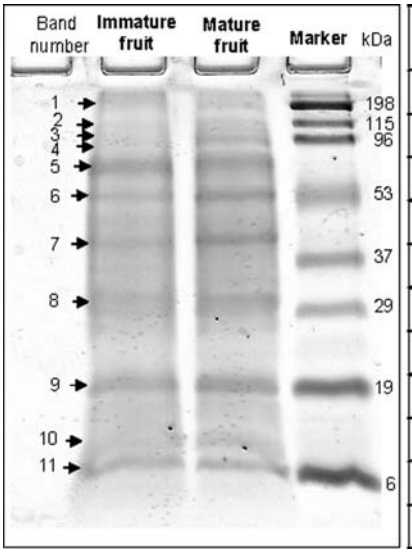


Figure 1. (**A**) Picture of immature and mature peach fruits. (**B**) Evolution of fruit firmness (N) in two maturity stages. Bars represent mean ± SE, $n \geq 9$; ns, no significant difference at level $P < 0.05$.



Band number	Immature fruit (band intensity)	Mature fruit (band intensity)
1	13.2±0.1	9.4±0.2*
2	9.3±0.1	7.3±0.2*
3	7.1±0.07	9.3±0.1*
4	8.4±0.05	16.2±0.6*
5	36.3±1.7	35.5±0.8
6	28.9±2.8	35.1±0.8*
7	28.2±1.7	39.6±0.9*
8	28.8±0.8	38.6±0.9*
9	32.1±0.9	33.1±0.8
10	19.7±0.8	22.3±0.7
11	22.2±1.1	21.7±0.8

Figure 2. Protein profile (left) after SDS-PAGE of peach fruit extracts visualized with Coomassie blue. In the table (right), the level of intensity (arbitrary units) of the 11 marked bands was quantified by image analysis software (Gen Tools, Syngene, Cambridge, U.K.). Values are mean ± SE, $n \geq 3$, differences being significant at level $P < 0.05$ (*).

Table 1. Peptides in the Bands Differentially Expressed in Immature and Mature Fruits As Identified by MALDI-TOF-TOF[a]

band	immature fruit	mature fruit
1	not identified	gi/1155058, glutathione synthetase (*Arabidopsis thaliana*)
2	not identified	gi/155967407, phosphoenolpyruvate carboxylase (*Jatropha curcas*)
3	gi/223527702, gi/223550058 multicopper oxidase gi/196886176, phospholipase D α (*Prunus persica*)	gi/1351138, gi/16305087 sucrose synthase gi/89242536, ATPase β subunit (*Ranunculus* sp. Qiu95024)
4	gi/223535009, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, putative (*Ricinus communis*) gi/196886176, phospholipase D α (*Prunus persica*)	gi/80973758, sucrose synthase (*Eucalyptus grandis*) gi/224100971; gi/224113295, predicted protein (*Populus trichocarpa*)
6	gi/6136112 UTP-glucose-1-phosphate uridylyltransferase gi/21362942, adenosylhomocysteinase gi/17026394, UDP-glucose pyrophosphorylase (*Amorpha fruticosa*)	gi/6136112, UTP-glucose-1-phosphate uridylyltransferase gi/21362942, adenosylhomocysteinase gi/17026394, UDP-glucose pyrophosphorylase (*Amorpha fruticosa*)
7	gi/224102341, predicted protein (*Populus trichocarpa*) gi/15982948, NAD-dependent malate dehydrogenase (*Prunus persica*) gi/10645188; gi/1480027, cytosolic aldolase gi/226001023, glyceraldehyde-3-phosphate dehydrogenase (*Rosa* hybrid cultivar)	gi/7108577, 1-aminocyclopropane-1-carboxylate oxidase (*Prunus persica*) gi/85720768; gi/110611760, glyceraldehyde-3-phosphate dehydrogenase
8	gi/136057, triosephosphate isomerase	gi/121488647, 1-aminocyclopropane-1-carboxylate oxidase 1 (*Prunus domestica* subsp. *insititia*) gi/136057, triosephosphate isomerase gi/68565622, GTP-binding nuclear protein Ran gi/9280628, ascorbate peroxidase, (*Astragalus membranaceus*)

[a] Number of band corresponds to that presented in **Figure 2**.

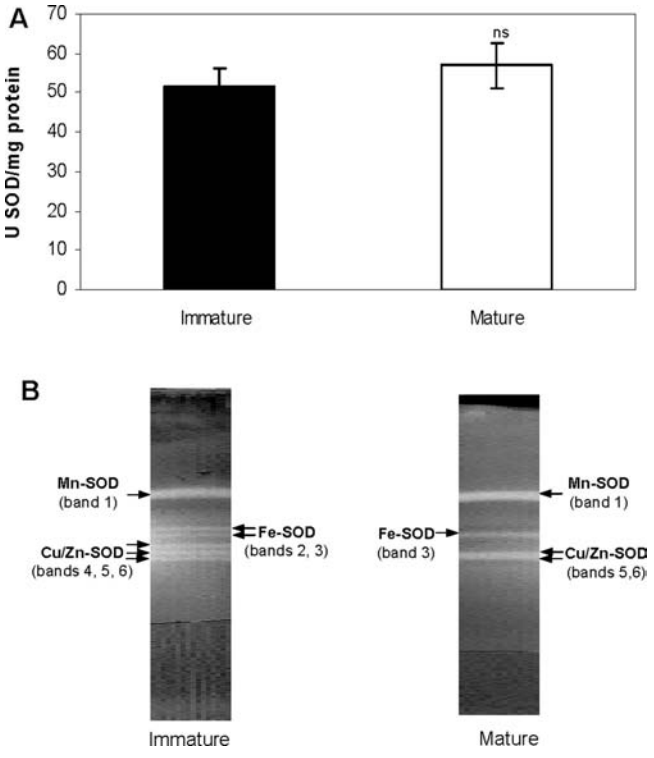


Figure 3. (**A**) SOD specific activity in immature and mature peach fruits. Bars represent mean ± SE, $n \geq 9$; ns, no significant difference at level $P < 0.05$. (**B**) PAGE detection of superoxide dismutase isoenzyme activity in immature and mature peach fruits.

fruits, with values observed between 50 and 56 U SOD mg^{-1} of protein (**Figure 3A**). The isoenzyme pattern visualized after IEF allowed us to distinguish at least six SOD isoforms in immature fruits, which were identified as one Mn-SOD (band 1, the most acid position), two Fe-SODs (bands 2 and 3), and three Cu/Zn-SODs (bands 4, 5, and 6; the most basic positions) (**Figure 3B**).

Table 2. SOD Isoenzyme Activity as a Percentage of the Total SOD Activity in Crude Extracts of Immature and Mature Peach Fruits[a]

	Mn-SOD	Fe-SOD		Cu,Zn-SOD		
peach fruit	band 1	band 2	band 3	band 4	band 5	band 6
immature	36.2	15.9	9.6	13.5	14.4	10.4
mature	41.6		21.6		18.6	18.2

[a] Number of band corresponds to that presented in **Figure 3B**.

In mature fruits, the SOD pattern was modified, and only four SOD isoforms were identified: one Mn-SOD as in the immature fruits (band 1), and one Fe-SOD located at the level of band 3 in the immature with the disappearance of the Fe-SOD corresponding to band 2. The most acid Cu/Zn-SOD isoenzyme also disappeared in mature fruit, and only bands 5 and 6 were detected in the mature fruits (**Figure 3B**). The intensities of band 1 corresponding to Mn-SOD were similar in both fruits, representing between 36 and 41% of the total SOD activity in immature and mature fruits, respectively (**Table 2**). The intensity of band 3 corresponding to Fe-SOD was higher in mature fruit (22% of total activity) than in immature fruit (around 10%) The activity of the Cu/Zn-SOD isoenzymes in immature fruits represented around 38% of the total SOD activity, with 13% corresponding to the isoenzyme located in band 4, 14% to the one located in band 5, and 10% to that located in band 6. Similarly, the activity of the Cu/Zn-SOD isoenzymes in mature fruit corresponded to 37% of the total activity, but it was distributed equally between the two observed isoenzymes.

The antioxidant enzymes involved in the ASC−GSH cycle were assayed in the crude extract of immature and mature peach fruits, and the main results are presented in **Figure 4**. APX and MDHAR activities increased nearly 2-fold in mature fruits in comparison to immature fruits (**Figure 4A**,**B**, respectively). In contrast, the activity of the DHAR enzyme decreased in mature fruits (**Figure 4C**), whereas GR increased around 30% (**Figure 4D**).

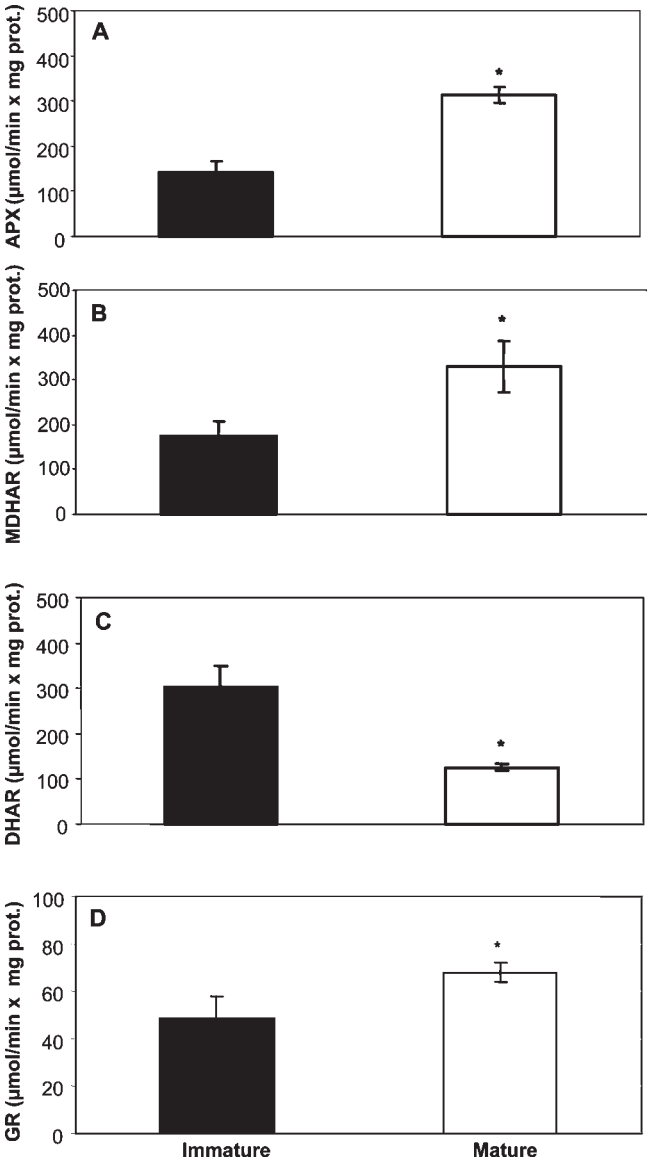


Figure 4. Specific activities of the ASC−GSH cycle enzymes in immature and mature peach fruits: (**A**) ascorbate peroxidase (APX); (**B**) monodehydroascorbate reductase (MDHAR); (**C**) dehydroascorbate reductase (DHAR); (**D**) glutathione reductase (GR). Bars represent mean ± SE, $n \geq 9$. Differences are significant at level $P < 0.05$ (∗).

Ascorbate and glutathione levels in their reduced and oxidized forms were also determined in immature and mature fruits. Total ASC content was not modified, because similar values were observed in immature and mature fruits (**Table 3**). The reduced ASC levels did not show any change, whereas a significant increase, nearly 3-fold, in DHA was detected in mature fruits. The GSH levels were similar in both maturation stages, whereas oxidized glutathione was very low in both fruits.

To analyze the changes in ROS production during maturation, levels of $O_2^{\bullet-}$ and H_2O_2 were determined in immature and mature peach fruits (**Table 4**). $O_2^{\bullet-}$ accumulation was significantly higher (approximately 3-fold) in immature than in mature fruits, whereas the H_2O_2 contents were similar in both fruits.

DISCUSSION

Many metabolic processes change during ripening, some of them making ripened fruits desirable for consumers. Among the proteins that appear as new in mature peach fruits, phosphoenolpyruvate carboxylase (PEPC) is a key enzyme that is involved in organic acid biosynthesis in plants and one that has been studied during peach fruit development (*25*), finding that in a normal-acid fruit, such as the one used in the present study, this enzyme might control organic acid accumulation during fruit development, as could be the case in our mature peach fruits. With regard to sucrose metabolism, another desirable attribute in fruit quality for consumers appeared in mature peach fruits, sucrose synthase (SS), an enzyme that is involved in the metabolic interplay of sucrose, hexoses, and starch synthesis. This enzyme has been found to increase during ripening in a certain banana variety (*26*), whereas Cordenunsi and Lajolo (*27*) reported its disappearance during ripening in a different banana variety.

Peach is a climacteric fruit requiring ethylene for normal ripening, and in the present study, one of the proteins appearing in mature fruits is the 1-aminocyclopropane-1-carboxylate oxidase (ACO), the enzyme that catalyzes the last step in ethylene biosynthesis. Ethylene is known as the major hormone initiating and controlling ripening of fleshy fruits, and ACO protein has been reported to increase during maturation of climacteric fruits, a process that requires the induction of new isoforms of ACO (*28*). In fact, in our mature fruits, which are in stage 4 (onset) according to the classification of Brummell et al. (*29*), ACO appears when these authors described the accumulation in ethylene.

Related to antioxidant metabolism, it is interesting to point out the presence of glutathione synthetase and ascorbate peroxidase appearing in mature peach fruits. Components of the antioxidant system controlling ROS are gradually turned off as senescence approaches and, in fact, several changes have been described during the ripening process, which seems to be dependent on many factors, such as type of fruit and even fruit variety, environmental conditions, and abiotic stress situations. The enzyme SOD rapidly converts $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen, and its several isoenzymes are located principally, in chloroplasts (Cu, Zn-SOD and Fe-SOD), cytosol (Cu,Zn-SOD), and mitochondria and peroxisomes (Mn-SOD) (*24*, *30*). In peach fruits, SOD activity assayed on crude extract was found to be similar in both immature and mature fruits, but a deeper inspection revealed that some changes in the isoenzyme SOD pattern occurred. Mn-SOD was not modified, suggesting an efficient enzymatic antioxidant system to scavenge $O_2^{\bullet-}$ generated at the mitochondrial and/or peroxisomal level. Mn-SOD activity is related to the high rate of

Table 3. Total Ascorbate and Glutathione Content and Reduced and Oxidized Ascorbate and Glutathione Levels in Immature and Mature Peach Fruits[a]

	immature fruits	mature fruits
total ASC (μg/g, fw)	62.3 ± 6.2	68.2 ± 6.0
ASC (μg/g, fw)	58.9 ± 6.1	56.9 ± 7.2
DHA (μg/g, fw)	3.4 ± 2.6	11.3 ± 3.1 *
total glutathione (μM)	1.92 ± 0.02	1.46 ± 0.07
GSH (μM)	1.85 ± 0.03	1.40 ± 0.14
GSSG (μM)	0.070 ± 0.001	0.060 ± 0.001

[a] Values are mean ± SE, $n \geq 9$. Differences are significant at level $P < 0.05$ (*).

Table 4. Superoxide and Hydrogen Peroxide Content in Crude Extract of Immature and Mature Peach Fruits[a]

peach fruit	superoxide (adrenochrome/g, fw)	hydrogen peroxide (μM/g, fw)
immature	0.71 ± 0.04	0.37 ± 0.01
mature	0.31 ± 0.06 *	0.45 ± 0.07

[a] Values are mean ± SE, $n \geq 9$. Differences are significant at level $P < 0.05$ (*).

growth, and the respiratory ratio in peach tissue (*31*) and an increase in the mitochondrial respiration ratio, which characterize the maturation of climacteric fruits, may imply a higher antioxidant system for defense against $O_2^{\bullet-}$ generated in the mitochondria. However, in our conditions, the mature fruit did not present higher levels of $O_2^{\bullet-}$, implying that SOD could be involved in the maintenance of these levels in the fruit. In contrast to the behavior of Mn-SOD, the loss of Fe-SOD and a Cu,Zn-SOD isoform was observed in mature fruits, which were possibly compensated for by an increase in the isoforms located in band 3, a Fe-SOD, and bands 5 and 6, both Cu,Zn-SODs, suggesting that some isoenzymes are positively related with maturation while others disappeared. Different SOD isoforms have been described in vegetative tissues of the aerial part of the peach tree (*31*), and Fe-SOD could not be detected in fully differentiated preshoot vegetative buds, whereas a very low activity of Cu,Zn-SOD is a feature of fully expanded leaves of seedlings and juvenile in vitro clones. These findings confirmed that tissue-specific activities of Fe- and Cu,Zn-SODs occur in peach depending on the age of the leaf, as reported for tobacco (*32*). The modifications observed in the pattern of Fe- and Cu,Zn-SOD isoenzymes, with different locations in the cell, reflect changes in oxidative metabolism at an organelle level during maturation. SOD activity has been found to decrease during senescence of tobacco and pea leaves (*33*), but it increased during the senescence of apple fruit (*34*), in which total SOD activity and individual activities of its three metalloenzymes changed considerably during fruit senescence in cold storage, with the changes being cultivar-related. Similar results were found in pepper fruits, where the SOD activity was not modified by maturation in green and red Herminio peppers but was 2-fold higher in yellow than in green Biela fruits (*11*). In tomato fruit, SOD activity declined at the beginning of ripening and increased at the over-ripe stage to similar values as in the small green fruits, and the increase was found for all SOD isoenzymes (*4*). Also in pepper fruits, the specific activities of all the SOD isoenzymes were found to be lower in crude extract from green peppers than from mature red fruits as well as Mn-SOD in isolated mitochondria from both fruits (*5*, *6*) but not in peroxisomes, where the activity of this last isoenzyme was found to be lower in mature fruits (*35*). However, in other fruits such as orange pulp and blackberry, a general decrease in antioxidants including SOD activity has been described to decrease with maturation together with catalase, peroxidase, and components of the ascorbate−glutathione cycle (*36*, *37*), and Mn-SOD has been reported to decrease its activity but not its protein level in mitochondria during apple fruit maturation, implying another key point of control, which is the post-translational modifications suffered by proteins that can affect their biological functions (*38*).

The presence of a higher number of SOD isoenzymes in immature peach fruits was parallel to higher $O_2^{\bullet-}$ accumulation, suggesting a protective role for these enzymes in the modulation of $O_2^{\bullet-}$ generated to avoid oxidative damage. An accumulation of lipid peroxidation and protein oxidation products has been described during fruit ripening as a consequence of an increase in the formation of $O_2^{\bullet-}$ and the accumulation of H_2O_2 (*4*, *39*). In peach fruits, changes in the SOD activity and in the accumulation of $O_2^{\bullet-}$ were not parallel to an H_2O_2 accumulation, indicating an efficient system of protection against H_2O_2 generation in immature and mature fruits. The maintenance of H_2O_2 levels may also be realized through the ASC−GSH cycle activities. All of the enzymes involved in this cycle have been reported in chloroplasts, mitochondria, peroxisomes, and cytoplasm (*10*), and some studies pointed to a possible role during fruit maturation. The activity of the APX enzyme was increased in mature peach fruits parallel to an increase in APX protein, which appeared as one of the proteins in mature but not in immature fruits, probably as a consequence of H_2O_2 generation by a different way than the action of the SOD. In this case, the APX enzyme could modulate the H_2O_2 accumulation during maturation. These results are in agreement with that described in mitochondria isolated from green and red peppers, in which the APX activity increased about 3-fold during fruit maturation (*5*). It has also been shown that a large increase in organelle APX activity and changes in the pattern of APX isoforms in the total fruit extract during the chloroplast-to-chromoplast transition imply the existence of isoenzymes that are involved in the ripening process in *Capsicum annuum* (*11*). However, in tomato fruits, the increase in SOD activity was parallel to a decline in the APX activity at the end of ripening, allowing an increase in the ASC accumulation and a reduced scavenging of H_2O_2, which could therefore be important for the loosening of the cell wall that leads to the fruit softening observed in over-ripe tomatoes (*4*). A similar decrease with maturation for this activity has been reported in orange pulp and blackberries (*36*, *37*).

APX activity is involved in ASC oxidation, whereas several enzymes of the ASC−GSH cycle carried out the ASC reduction. The analysis of the ascorbate content in peach fruits in the two ripening stages revealed similar values for total and reduced ASC, whereas DHA increased in mature ones. The value of vitamin C found in peach of around 6 mg/100 g of fw is in the range described for these fruits and is relatively low in comparison to others such as strawberry, orange, or tomato (*40*−*42*). Although the ASC content in its oxide form (DHA) increased in mature peach fruits, a good antioxidant redox state in the fruit was observed, around 94 and 83% in immature and mature fruits, respectively, with ascorbate remaining mainly then in its reduced form in both stages. The same decrease was reported for peppers but with higher redox values (*6*), although in these fruits, the reduced ascorbate increased in mature fruits, as well as being reported for tomato (*43*). Changes of this antioxidant have been reported in fruit organelles, with an increase in ASC in chromoplasts and peroxisomes but not in mitochondria from peppers (*5*, *11*, *35*). The decrease in the redox status has been correlated with the release of ethylene during ripening (*44*). In other fruits, such as orange and blackberry, the decrease in the redox state was due to a decrease in both the reduced and oxidized forms of ascorbate and glutathione (*36*, *37*), although in tomato this parameter did not change until the red-ripe stage (*4*).

The enzymes involved in the ASC metabolism regenerating the reduced form from the oxidized one are MDHAR and DHAR, which appear as key regulatory points of the ASC pool (*45*). The increase in MDHAR activity and not in DHAR in mature peach fruits indicated that the recycling of ASC seemed to be NADH-dependent, through the MDHR activity as described in tomato (*43*), with DHAR activity acting at different points during the ripening process, which in this last case was the main enzyme in maintaining the reduced ASC in the immature fruits. However, in tomato fruits as well, Jiménez et al. (*4*) found an increased ASC content from immature to breaker stages that was parallel to DHAR activity, whereas in the latter part of the process, MDHR activity was more associated with the ASC content, indicating again that the changes in the antioxidant system are variety-dependent. On the other hand, the increase found in the GR activity in mature peach fruits that is similar to that found in peppers (*6*) may collaborate to ensure a supply of reduced glutathione for ASC recycling, and this aspect is reinforced by the appearance of glutathione synthetase (GS) in our mature peach fruits. However, we did not find an increase in the activity of the DHAR enzyme in mature fruits, and DHA accumulated approximately 4-fold in these fruits, indicating that the activity of the

MDHR enzyme was not sufficient to maintain the same redox state. These results suggest that the maturation could have an effect on DHAR protein, probably on its synthesis and/or the existence of post-translational regulation. Matamoros et al. (*46*) have observed that during peach seed development, the content of the plastidic protein did not change and that of cytosolic protein was moderately reduced, suggesting that DHAR activity is post-translationally regulated during fruit development or that the fruit tissue contains additional proteins with DHAR activity, such as thioredoxins, glutaredoxins, disulfide isomerases, and glutathione peroxidases, which have been described as displaying DHAR activity in plants and animals (*47*).

The glutathione pool was also maintained in its reduced form with only 5% in the oxidized form in both fruits. GSH plays an important role in the stabilization of many enzymes. It has also a more general role as an oxidant scavenger by serving as a substrate for DHAR and by reacting directly with free radicals, including the hydroxyl radical, to prevent the inactivation of enzymes by oxidation of the essential thiol group (*37*). The majority of glutathione in the cell is maintained as GSH, whereas the GSSG is present in only small quantities. A high relation of GSH/GSSG is necessary for several physiological functions, including activation and inactivation of redox-dependent enzyme systems and regeneration of the cellular antioxidant ascorbic acid under oxidative conditions. Total glutathione, GSH, or GSSG did not show significant changes from immature to mature peach fruits despite the increase of GR, the appearance of GS protein, or the decrease in DHAR, possibly implying that GSH is being used during maturation. An increase in GS activity was described during pepper fruit development, reaching the highest levels in the chromoplasts of red fruits (*48*). Changes in glutathione content have also been reported during fruit maturation, with increases in tomato and orange at the end of ripening accompanied by a decrease in GR and DHAR activities (*36*, *43*) and decreases in peppers and blackberries with a general decrease in the activities of the ASC−GSH cycle (*35*, *37*). However, the exact role of this antioxidant during fruit ripening is somewhat unknown and needs further investigation.

The increases described in proteins, antioxidants, and activities of enzymes involved in the antioxidant metabolism in peach fruits, together with the maintenance or decrease of oxidants such as $O_2^{\bullet-}$ and H_2O_2, reinforce the idea that the synthesis of antioxidants or redox modulators is elicited during fruit ripening to favor the protection of the fruit against oxidative damaging processes occurring during its development from immature fruit to senescence. Some of the changes are related to the appearance of proteins involved in sucrose and organic acid metabolism that increase the nutritional and quality value, making peach fruits in the maturity stages analyzed suitable and preferred by consumers and the processing industry.

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