Antioxidant responses of Golden delicious apple under cold storage conditions

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Abstract

An understanding of biochemical events of cold storage may lead to more effective methods of preventing apple fruits from ripening and keeping their quality. This study aimed at determining the level of reduced and oxidized forms of ascorbic acid, and the activity of ascorbate peroxidase, catalase, and superoxide dismutase enzymes changes in pulp of Golden delicious apple cultivar during cold storage. Apples were kept in common cold storage condition (1 °C, 95% - 97% RH) for 135 days for subsequent analysis. During cold storage condition, no significant changes were observed in the content of reduced and oxidized forms of ascorbic acid. However, levels of total flavonoids declined as storage time progressed. Superoxide dismutase and ascorbate peroxidase activities were decreased while the activity of catalase was increased.

Keywords: chlorophyll; apple; ascorbate; antioxidant enzymes; flavonoids

Abbreviations:
ASA: ascorbic acid; APX: ascorbate peroxidase; CAT: catalase; DHA: dehydroascorbic acid; DPPH: Diphenylpicrylhydrazine; ROS: reactive oxygen species; SOD: superoxide dismutase

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Introduction

Apple (*Malus domestica* Borkh.) is recognized as an edible fruit that contributes to human health and its benefits are often attributed to its high antioxidant content (Schirrmacher and Schemp, 2003). The protective effects of fruit and vegetable consumption may be attributed to the antioxidant properties of secondary plant metabolites such as carotenes, ascorbic acid and polyphenols. The natural compounds can be used as substitutes for synthetic antioxidant. Apple is a climacteric fruit characterized by a set of physiological and biochemical changes during ripening.

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A major quality problem of apples in the marketplace is softening, an undesirable ripening process in apple fruit as firmer apples tend to be juicier, crisper, crunchier, and less mealy than softer fruit. The main postharvest factors that influence apple softening include temperature, atmosphere, relative humidity, calcium treatment, and ethylene (Johnston et al., 2002). Artificial prevention of ripening process (and keeping quality) is the main goal of controlled atmosphere storage (low oxygen and high carbon dioxide) and/or cold storage (low temperatures). For cold storage, apples during postharvest time are stored at 0-3 °C. Low temperatures influence the postharvest biology of apple fruits. Chilling stress is a physiological disorder that limits the storage of chilling sensitive fruits at low, but non-

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freezing temperatures (Yang et al., 2012). Low temperatures disrupt the balance of ROS, leading to its accumulation and oxidative stress.

Antioxidant molecules and enzymes protect cellular membranes and organelles from the damaging effects of reactive oxygen species (ROS), which are formed both during normal cellular metabolism and unwanted environmental conditions. Among the non-enzymatic antioxidants, which are generally small molecules, ascorbic acid and flavonoids are widely distributed in plant cells, playing important role in the destruction of ROS. The enzymatic system involves a wide range of enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) which play an important role in formation and degradation of ROS. A high reduced/oxidized ratio of ascorbate forms seems to be vital for the efficient detoxification of ROS and for the acclimation of plants to environmental condition and/or for the enhancement of resistance to biotic and abiotic environmental stresses (Lee and Kader, 2000).

Antioxidants are not only responsible for plant cell protection but also keep health quality of fruit. The crucial role of phytochemicals in keeping the health quality of fruits have encouraged efforts to identify the factors affecting their content and pre- and post-harvest changes, in particular during cold storage. Climatic conditions as well as post-harvest factors may considerably modify the composition and concentration of fruits phytochemicals.

The aim of this research was to evaluate the changes of ascorbic acid content and its redox level together with some antioxidant enzymes activity (SOD, CAT, and APX) after harvest and during cold storage. Despite the large number of studies on understanding the process of apple ripening, the complex nature of biological processes necessitates more detailed studies on fruit biochemistry of cold storage.

Materials and Methods

Sample preparation

Apple (*Malus domestica* Borkh. cv. Golden delicious) fruits grown in the Malekan (East Azarbaijan, Iran) orchards were tested. Apples were harvested in September 2012 and immediately put into cold storage house (1 °C, 95% - 97% RH). In each test time, storage fruits were randomly selected for analysis. Analyses were made 0 (at harvest), 45, 90 and 135 days after harvest. Apples were peeled with a potato knife, thus a thin layer of apple flesh remained adhered to the peel. The pulp was cut into thin pieces and at the end the seeds were removed from the apple core. Analyses were made in four replications for apple pulp.

Determination of reduced (ASA), oxidized (DHA), and total ascorbate

Aliquots of tissues were homogenized in ice-cold 6% (w/v) trichloroacetic acid. After centrifugation at 20 000 × g for 15 min, ASA and total ASA (ASA + DHA) were determined in the supernatant according to Kampfenkel et al. (1995). This assay is based on the reduction of Fe^{+3} by ASA, followed by complex formation between Fe^{+2} and bipyridil that is absorbed at 525 nm. Total ascorbate was determined through a reduction of DHA to ASA by dithiothreitol. DHA content was then estimated from the difference between total ASA and ASA. A standard curve covering the range of 0 - 100 nmol ASA was used, as described in Chaparzadeh et al. (2004).

Determination of total flavonoids

Total flavonoids were determined according to (Marinova et al., 2005) using the aluminum chloride colorimetric method. Samples were homogenized with 5 ml ethanol (80%) centrifuged for 15 min. One ml supernatant was added to distilled water (4 ml) in a flask. Then, 5% $NaNO_2$ (0.3 ml), and after 5 min, 10% AlCl3 (0.3 ml), and after 6 min, 1 M NaOH (2 ml) were added. The mixture was diluted to 10 ml with distilled water. The absorbance of the solution was measured at 510 nm and results were expressed as mg rutin equivalents/g fresh weight.

Determination of total antioxidant capacity

Tissues were homogenized with 5 ml absolute methanol and centrifuged for 10 min.

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The supernatant was collected and used for total antioxidant capacity quantification. The samples were reacted with the stable diphenylpicrylhydrazine radical (DPPH) in methanol. The reaction mixture consisted of adding 0.5 ml of sample, 3 ml of absolute methanol and 0.3 ml of 0.5 mM DPPH solution in methanol. The changes in color (from deep violet to light yellow) were read at 517 nm after 100 min. The scavenging capacity percentage (SC%) was determined according to the following formula (Mensor et al., 2001):

$$SC\% = 100 - [(Abs_{sample} - Abs_{blank}) \times 100/Abs_{control}]$$

Enzyme extractions and assays

Aliquots of tissues were ground in pre-chilled mortars with an appropriate cold extraction phosphate buffer (pH, 7.5) containing 1 mM EDTA and 1% polyvinylpolypyrrolidone at 0 - 4 °C. Homogenates were centrifuged at 20 000 × g for 15 min at 4 °C. Enzyme assays were performed in the supernatant at 25 °C and protein contents were quantified using coomassie blue method. SOD activity was measured by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium. This method excludes the effect of other antioxidant molecules, which scavenge O^{2-} similar to SOD. The 3 ml reaction mixture contained 100 mM phosphate buffer (pH 7.5), 20 mM methionine, 2.25 mM NBT, 2 µM riboflavin, 0.3 mM Na_2 EDTA and 200 µl of enzyme extract. Riboflavin was added last and the tubes were shaken and exposed to light for 20 min using two 15 W fluorescent lamps. The absorbance of the reaction mixture was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50% (Fridovich, 1986). CAT activity was assayed by estimating the residual H_2O_2 using titanium reagent. The reaction mixture consisted of 2 mM hydrogen peroxide, 100 mM phosphate buffer (pH 7.0), and reaction was started by adding enzyme extract. Reaction was terminated after 10 min by adding 4 ml of titanium reagent. After centrifugation for 10 min, absorbance of the supernatant was recorded at 420 nm. Reaction mixture without enzyme served as blank. One unit of CAT activity was defined as the amount that decomposes 1.0 micromole of hydrogen peroxide per minute under the assay conditions. APX activity was assayed by monitoring the change at 290 nm. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 2.5 mM ascorbate, 0.5 mM EDTA, 1.5 mM H_2O_2 and 100 µl of enzyme extract in a final volume of 1 ml. One unit of APX activity was defined as the amount of enzyme that oxidizes 1.0 micromole of ASA per minute under the assay condition.

Statistical Analysis

The experiment was done based on completely randomized block design with four replications (± standard deviation). One-way analysis of variance was applied to the data to evaluate the storage time effect. Mean values and significance (at $P \leq 0.05$) were determined by Duncan's multiple range test using SPSS software.

Results

Changes in antioxidant compounds and capacity during cold storage

The effect of cold storage time on ASA and DHA was not significant (Fig. I). We considered, therefore, that the ASA values adequately represent the main part of vitamin C activity in apple fruits. Apples also showed no significantly change in ASA/DHA ratio during cold storage (Fig. II). Flavonoids content of apple fruits decreased significantly with increasing cold

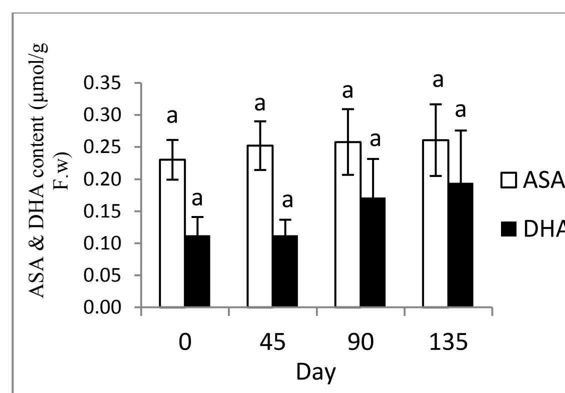

Fig. I. Effect of increasing cold storage time on ASA and DHA contents of apple fruits

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storage time for 135 days (Fig. III). As storage time increased to 135 days, a reduction was observed in antioxidant capacity of apple fruits (Fig. IV).

Changes in enzymes activities

The antioxidant enzymes behaved differently under cold conditions in apple fruits. At the end of 135 days cold storage, CAT activity (Fig. VI) was increased. We found least CAT activity in harvest day. The activity of APX (Fig. V) was reduced significantly after 45 days of cold storage. Also, the activity of SOD (Fig. VII) was decreased in time dependent manner. At the harvest day SOD had highest activity.

Discussion

Ascorbic acid (ASA) and dehydroascorbic acid (DHA) have vitamin C activity. However, we found only small amounts of DHA which were rarely outside the standard deviation of the ASA determinations. We observed that ASA content did not change significantly during cold storage (Fig. I). These results are in agreement with findings by Lata and Przeradzka (2002). However, the results obtained by different authors are variable and sometimes contradictory. Curry (1997) reported that in different apple fruits the level of antioxidants increased 2-10 folds after 2 months of cold storage. The results of many experiments clearly demonstrated that antioxidant content depends on the cultivar, harvest time, and length of storage. ASA content depends largely on the activity of APX. This enzyme might significantly reduce ASA and increase DHA content. In present study, decreasing of APX activity can be one of the reasons that ASA was not changed considerably. We have found negligible changes in levels of DHA after storage for 135 days (Fig. I). In two spinach cultivars, DHA content did not change during 30 days of cold storage (Lee and Kader, 2000). DHA content declined in mature cucumber fruits during cold storage (Qian et al., 2013). It seems that reduction of the DHA to ASA (Chen and Gallie, 2005), decreasing APX activity, and some loss of DHA resulted in a stable level of DHA and ASA contents in our study. Apples showed no

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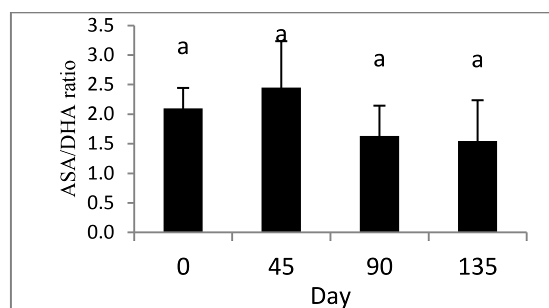

Fig. II. Effect of increasing cold storage time on ASA/DHA ratio of apple fruits

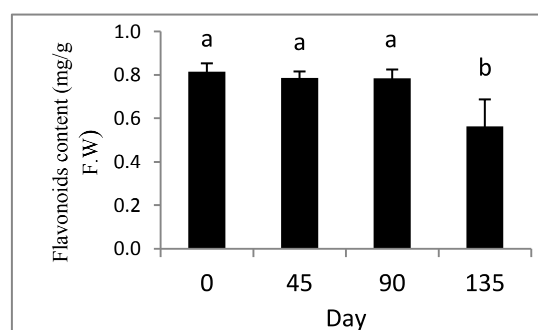

Fig. III. Effect of increasing cold storage time on total Flavonoids content of apple fruits.

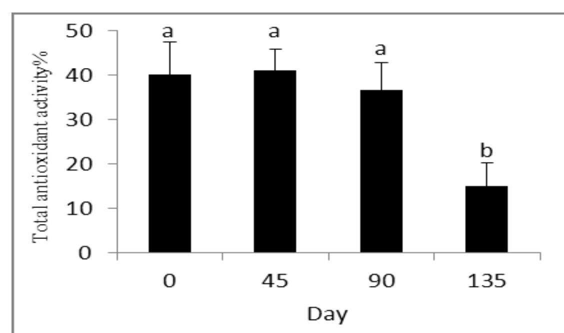

Fig. IV. Effect of increasing cold storage time on total antioxidant capacity of apple fruits

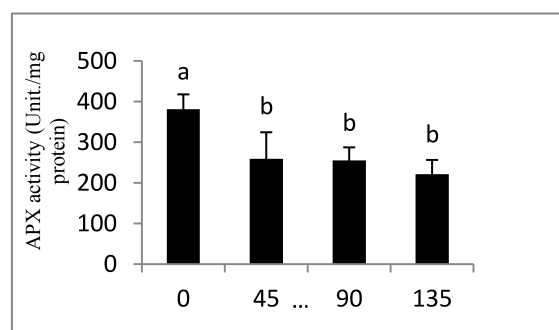

Fig. V. Effect of increasing cold storage time on APX activity of apple fruits

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significant change of ASA/DHA ratio with the longer time of storage (Fig. II). This result is in agreement with those of Lata et al.,(2005) who reported that ASA/DHA ratio showed negligible increase during storage in four apple cultivar fruits stored at 1 °C during 90 days. ASA is more sensitive to change in environmental conditions (Lata et al., 2005). ASA is actively involved in the defense against ROS stress and may contribute to the cold tolerance in apple fruits.

Flavonoids are phenolic derivatives found in substantial amounts in apple. Chemical structure of phenolics makes them ideal as antioxidant compounds, free radical scavengers, and metal chelators, more powerful as compared to, e.g., vitamin C (Matthes and Schmitz-Eiberger, 2009). In present study flavonoids (Fig. III) and total phenolic (data not showed) contents were decreased significantly during cold storage. This result is in agreement with those of Piretti et al. (1994), who reported that catechin, epicatechin and quercetin glycosides were decreased during storage in Granny Smith apple fruits stored at 0 °C. Van Der Sluis et al. (2001) observed that cold storage did not change flavonoid concentration and antioxidant activity of apple fruits of different cultivars. In contrast, Napolitano et al. (2004) reported a parallel increase in antioxidant activity and concentrations of catechin and phloridzin during cold storage for Annurca apple fruits. The decrease of flavonoids contents may decrease the antioxidant capacity during cold storage. The flavonoids such as quercetin and catechin are common polyphenol oxidase substrates (Jiménez and García-Carmona, 1999; Nagai and Suzuki, 2001). Therefore, the decrease of phenolic composition could be due to the oxidation by common polyphenol oxidase.

There are several methods for determination of total antioxidant capacity, but the method used in this study is one of the most widely used methods. In this assay, the reducing of Fe^{3+} to Fe^{2+} in the presence of methanol extracts was investigated. Reducing capacity of compounds can be defined as an indicator of potential antioxidant activity (Meir et al., 1995). We observed a declined trend in antioxidant capacity of apple fruits (Fig. IV). Oxidative stress from excess ROS has been associated with the appearance of chilling damage in fruits (Yang et al., 2012). Excess ROS could cause lipid peroxidation, membrane damage, consequently, and senescence in fruit. Thus, plants must be protected against the harmful effects of ROS by a complex antioxidant compounds. Antioxidant capacity is a result of several molecules present in the fruit and their synergistic effects. Antioxidant activity cannot be related to a single compound but to synergistic and additive effects between different phytochemicals. In grapefruit and orange fruits total antioxidant capacity during storage decreased but this depended on fruit cultivars (Gardner et al., 2000). Reducing of antioxidant capacity of fruits in the long-term storage were attributed to the reduction in phenolic compounds and vitamin C. Tavarini et al. (2008) showed that the antioxidant capacity of kiwi fruits were at maximum level at harvest time, but was reduced during long-term storage. In Mango fruits, antioxidant capacity in short time storage has not changed, but the decreased rate was observed by prolonging storage period (Policegoudra and Aradhya, 2007). Previous studies showed that the antioxidant activities of apples are the consequence of synergistic

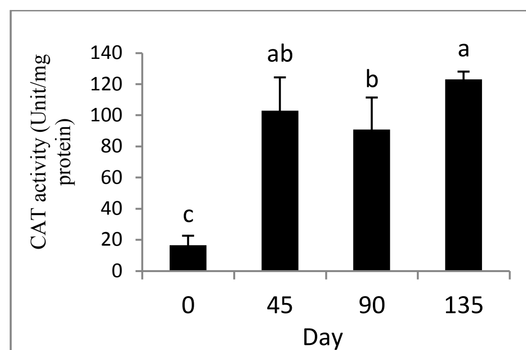

Fig. VI. Effect of increasing cold storage time on CAT activity of apple fruits

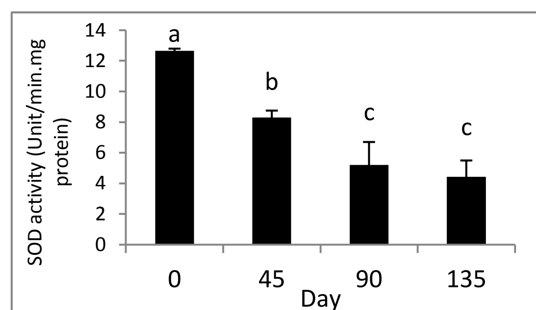

Fig. VII. Effect of increasing cold storage time on SOD activity of apple fruits

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activities of phenolic compounds rather than ascorbic acid (Eberhard et al., 2000). It is assumed that decrease of total antioxidant capacity in our research may be due to the content level of phenolic compounds. Flavonoids in beginning days of storage were abundant, but during storage were degraded as a result of O_2 promoted oxidation to other compounds (Harb et al., 2010).

Temperature strongly influences the postharvest life of apple fruits. Apple fruits are stored at 0-3 °C to slow loss of quality after harvest, with the temperature used depending on cultivar sensitivity to chilling injury (Johnston et al., 2002). Low temperatures may lead to oxidative stress by unbalancing in production and degradation of ROS. Antioxidant enzymes, such as SOD, CAT, and APX play the main role in cell protection against ROS (Foyer and Noctor, 2005). At the end of 135 days cold storage, CAT activity (Fig. VI) increased but APX (Fig. V) and SOD (Fig. VII) activities decreased. APX is responsible for the oxidation and recycling of ASA pools in the tissues. APX catalyzes oxidation of ASA and generates the unstable radical monodehydroascorbate, which rapidly dissociates into DHA and ASA. In the present experiment, APX activity decreased after 45 days storage and then remained fairly stable (Fig. V) which is in agreement with findings by Lata et al. (2005) for 90 days of storage. During storage, a decrease trend was observed in APX activity in Jonagold and Sampion apple cultivars, but increase was observed in Gloster and Elise cultivars (Lata and Przeradzka, 2002). In pawpaw fruits no changes in ASA/DHA ratio and no clear pattern of APX response to cold storage suggests that ascorbate–glutathione cycle did not contribute to antioxidant protection at extended periods of cold storage (Gill at al., 2009). On the other hand, some reports are showing the important role of ascorbate-glutathione cycle in apples during storage, obviously with some differences between cultivars (Lata et al., 2005). One of the main functions of APX is scavenging H_2O_2 produced during metabolism, thus protecting tissues against oxidative injury. In the Golden Delicious apple cultivar with higher H_2O_2, APX activity was decreased (Torres et al., 2003). Decreased APX activity contributes to the lower elimination of H_2O_2 by this enzyme during cold storage. This shows that other enzymes such as CAT may be important in elimination of ROS. In the present study, CAT activity was increased gradually during 135 days cold storage of apple fruits (Fig. VI) which is in agreement with findings by Lata et al. (2005) for 60 days of storage. Increase in CAT activities in response to low temperature stress has been associated with enhanced chilling tolerance in mangoes (Ding et al., 2007) and peaches (Wang et al., 2005), and resistance against browning in 'Conference' pears (Lentheric et al., 1999). In Kiwifruits, gradual cooling treatment increased CAT activity more than sudden cooling. Based on the previous studies in other fruits and our data, it is presumable that acquisition of temperature tolerance in Golden delicious apples may be possible via enhanced CAT activity. SOD can scavenge O_2^{-} into H_2O_2 in cytosol, chloroplasts and mitochondria (Fridovich, 1986). The H_2O_2 could be minimized either by CAT or by ascorbate–glutathione cycle (Alscher et al., 2002). The data suggest that SOD activity decreased significantly during the first two 45 days period of storage and then was nearly stabled (Fig. VII). As the storage duration, decline in SOD activity may contribute to the accumulation of O_2^- as reported during cold storage of kiwifruit and mango (Song et al., 2009). The abundance of O_2^- due to lower SOD activity can increase the Haber-Weiss reaction rate which involves production of the most reactive and damaging hydroxyl radicals (Bowler et al., 1992). Therefore, SOD is likely to be a central enzyme to the antioxidant defense mechanism as its activity determines the levels of H_2O_2 and O_2^-, which are both substrates of the Haber-Weiss reaction. Another possible explanation for decrease in SOD activity is that in chilling sensitive cultivars, increased H_2O_2 production may irreversibly inactivate SOD enzymes during prolonged chilling stress. Despite a continuous decrease in SOD activity beyond 90 days of storage, CAT activity is remained at significantly higher level than during storage (Singh and Singh, 2013).

In conclusion, our results indicate that contents of tested antioxidants depended on time of cold storage. Changes in antioxidant metabolism of apple may be perceived as

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oxidative stress during cold storage, but antioxidant content and enzyme activity were probably high enough to protect fruits against such damage during the short time of cold storage. Antioxidant enzymes activities may be one of the mechanisms involved in chilling tolerance. The evidence available on postharvest behavior of natural compounds in apples is partly conflicting and needs further study.

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