

ASCORBATE CONTENT AND PEROXIDASE ACTIVITIES IN APPLE FRUITS DURING STORAGE

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A B S T R A C T

Ascorbate content and activities of ascorbate and guaiacol peroxidases in the three cultivars of apples stored under different conditions were studied. One set to the apples was stored at 4°C in normal atmosphere without humidity control (variant I). The other set was stored for 5 months in controlled atmosphere ((3% O₂, 5% CO₂, RH 90%) at 3°C and then transferred to the conditions identical with those for the first group (variant II). The ascorbate content and enzyme activities depended on the cultivars and the conditions of storage. At the beginning of experiment in variant I in all the cultivars the concentrations of total ascorbate were similar to those in invariant II, but those of dehydroascorbate were lower in variant I. Thereafter the concentration of dehydroascorbate increased more in 'Jonagold' and 'Golden Delicious' cultivars than in 'Shampion'. The ascorbate and guaiacol peroxidase activities were higher in 'Shampion' cultivar. The results showed that conditions of storage significantly influenced the antioxidant metabolism in different apple cultivars. 'Shampion' was the best source of ascorbate in the case of long-stored apples.

Key words: apples, ascorbate, ascorbate and guaiacol peroxidases, storage

INTRODUCTION

Apples are the natural source of dietary mineral salts, vitamins, antio-

xidants, fibre, organic acids and sugars. The highest concentration of bioactive substances, including antioxidants, is found in or near the peel,

so it is recommended as a dietary supplement (Wolfe et al., 2003, Wolfe and Liu, 2003). The main antioxidants, besides ascorbate, found in apples are quercetin, catechin, epicatechin and chlorogenic acid (Lee et al., 2003). Apples do not contain high amount of ascorbate, but they are its important source because they constitute a substantial element of diet. The apples with the oxidised form of ascorbate – dehydroascorbate – not exceeding 5-10% of the total ascorbate pool are regarded as most valuable. Processing of apples increases the dehydroascorbate content up to 30% of the total ascorbate pool; therefore fresh apples are mostly recommended for consumption (Halliwell, 1999).

The latest results of Pajk et al. (2006) proved that the presence of apples in a diet protected against oxidative stress by inhibiting the formation of lipid peroxides and by preventing DNA damage in blood cell nuclei. Study by Garcya-Alonso et al. (2004) showed that among commercial fruit, apples, avocado and grapefruit exhibited the highest antioxidative properties. Antioxidants present in apples, including ascorbate, are beneficial for lung functioning (Davey and Keulemans, 2004), decrease the risk of some cancers (Feskanich et al., 2000; Le Marchand et al., 2000) and diabetes (Knekt et al., 2002), protect against oxidation of lipoprotein fraction (Aprikian et al. 2003) as well as decrease the risk of heart diseases (Sesso et al., 2003). The dominant form of antioxidants in apples are polyphenols while ascorbate

constitutes only 0.4% of this pool (Lee et al., 2003).

Long-term storage of apples causes degradation of ascorbate while the presence of peroxidases, which additionally utilize this compound as a substrate, may accelerate this process.

The aim of the present study was to investigate storage-dependent changes in the content of ascorbate, both its reduced and oxidized forms, as well as in the activity of ascorbate peroxidase (APX) and guaiacol peroxidase (PO) in three apple cultivars.

MATERIAL AND METHODS

Fruit of three apple cultivars were tested: ‘Shampion’, ‘Golden Delicious’ and ‘Jonagold’. All apples were harvested in September 2005 at a commercial maturity stage and were either 1) stored at 4°C in normal atmosphere (NA) without humidity control (variant I) or 2) stored for 5 months in controlled atmosphere (KA; 3% O₂, 5% CO₂, RH 90%, 3°C) and then transferred to NA (variant II). In the variant I analyses started just after harvest and in the variant II after storage in KA. The fruit from both variants were examined once a month for 4-5 months. The content of total ascorbate (TAA), dehydroascorbate (DHA) and ascorbic acid forms (AA) as well as the activity of ascorbate peroxidase (APX) and guaiacol peroxidase (PO) were determined.

Enzyme assays

1 gram of the tissue located up to 1 cm beneath the peel was homogenized

in a mortar with ice-cold 50mM potassium phosphate buffer (pH 7.0) containing 10 mM ascorbate, 1 M NaCl, 1 mM EDTA and 1% polyvinylpyrrolidone. After centrifugation (20 000 x g, 15 min) the supernatant was used to determine APX (EC 1.11.1.11) and PO (EC 1.11.1.7) activities.

APX activity was assayed following the oxidation of AA at 265 nm ($\epsilon = 13.7 \text{ mM}^{-1}\text{cm}^{-1}$) by a modified method of Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 5 mM AA, 0.5 mM H_2O_2 and the enzyme extract. Addition of H_2O_2 started the reaction. The rates were corrected for the non-enzymatic oxidation of AA by the inclusion of a reaction mixture without the enzyme extract ("blind sample"). The enzyme activity was expressed in units ($\mu\text{mol AA min}^{-1}$) per g of fresh weight.

PO activity was assayed spectrophotometrically with guaiacol by measuring an increase in absorbance at 470 nm ($\epsilon = 26.6 \text{ mM}^{-1}\text{cm}^{-1}$) according to Maehly and Chance (1954). The mixture of 0.5 cm^3 of the enzyme extract, 0.5 cm^3 of 50 mM acetate buffer (pH 5.6), 0.5 cm^3 of 20 mM guaiacol and 0.5 cm^3 of 60 mM H_2O_2 was used. The enzyme activity was expressed in units (mmol tetraguaiacol min^{-1}) per g fresh weight.

Assay of ascorbate content

For the determination of ascorbate 1 gram of the tissues located up to 1 cm beneath the peel was

homogenized in a cold mortar with 6% ice-cold trichloroacetic acid and centrifuged (20 000 x g, 15 min). The supernatant was used for ascorbate determination. The concentration of ascorbate was determined according to the colorimetric bipirydydyl method of Okamura (1980) modified by Knörzer et al. (1996). DHA concentration was calculated by subtracting the AA value from the TAA. Ascorbate concentration was calculated using a standard curve prepared for AA and was given in mg 100 g^{-1} fresh weight.

Statistical analysis

All results presented are the means of twelve independent analyses (n=12). Sample variability was given as the standard deviation of the mean. The significance of differences between mean values was determined by a non-parametric Mann-Whitney rank sum Test (STATISTICA Software edition 1998). Differences between examined and control groups were considered significant at $P \leq 0.05$.

RESULTS

In the fruit at harvest and in these stored for 5 months in KA the highest content of ascorbate was observed in 'Shampion' fruit while in 'Golden Delicious' and 'Jonagold' it was lower by 13% and 40%, respectively (Tab. 1 and 2). DHA contents were similar in 'Shampion' and 'Golden Delicious' (7.6% and 8.6% of TAA) and higher in 'Jonagold' (12.8% of TAA).

Table 1. Ascorbate content (mg 100g⁻¹FW) in the tissue of three apple cultivars stored in normal atmosphere (Variant I)

Time month/year	Cultivars											
	Shampion				Golden Delicious				Jonagold			
	TAA	AA	DHA	AA/TAA	TAA	AA	DHA	AA/TAA	TAA	AA	DHA	AA/TAA
09/2005	46.11 ¹ ±5.3	42.60 ±3.2	3.51 ±1.1	0.92	40.19 ±5.1	36.73 ±3.0	3.46 ±1.0	0.91	28.10 ±3.1	24.48 ±3.0	3.62 ±1.1	0.87
10/2005	44.41 ±4.2	38.90 ±3.2	5.51 ±0.8**	0.88	38.41 ±4.0	32.15 ±3.2**	6.26 ±1.8*	0.84	20.37 ±2.9*	14.77 ±2.1*	5.60 ±1.5**	0.72
11/2005	39.90 ±4.1*	33.20 ±3.1*	6.70 ±0.7*	0.83	35.79 ±4.1*	27.29 ±2.9*	8.50 ±1.6*	0.76	19.44 ±3.1*	12.53 ±2.2*	6.91 ±1.0*	0.64
12/2005	33.61 ±3.7*	26.80 ±2.8*	6.81 ±0.6*	0.80	31.96 ±3.1*	22.22 ±2.8*	9.74 ±1.0*	0.69	18.97 ±2.8*	11.40 ±2.1*	7.57 ±1.1*	0.60
01/2006	32.20 ±2.7*	24.80 ±2.9*	7.40 ±0.7*	0.77	26.92 ±2.6*	16.63 ±2.6*	10.29 ±1.2*	0.62	18.46 ±2.1*	9.67 ±1.3*	8.79 ±1.2*	0.52

¹mean and standard deviation, * difference significant at P<0.05, ** difference significant at P<0.001

Table 2. Ascorbate content (mg 100g⁻¹ FW) in the tissue of three apple cultivars stored for 5 months in controlled atmosphere and then (since February 2006) in normal atmosphere (Variant II)

Time month/ year	Cultivars											
	Shampion				Golden Delicious				Jonagold			
	TAA	AA	DHA	AA/ TAA	TAA	AA	DHA	AA/ TAA	TAA	AA	DHA	AA/ TAA
02/2006	42.20 ±5.1 ¹	36.48 ±3.3	5.72 ±1.2	0.86	36.07 ±3.7	23.18 ±2.8	12.89 ±0.9	0.64	27.01 ±3.0	19.09 ±1.8	7.92 ±1.1	0.71
03/2006	40.62 ±4.2	32.82 ±3.2	7.80 ±1.8	0.81	32.62 ±3.2**	15.92 ±2.2*	16.70 ±1.5*	0.49	23.95 ±3.4**	14.85 ±2.2*	9.10 ±1.4**	0.62
04/2006	36.31 ±4.1*	26.58 ±3.0*	9.73 ±1.7*	0.73	27.76 ±4.0*	14.20 ±2.9*	13.56 ±1.2	0.51	20.71 ±2.8*	11.21 ±2.1*	9.50 ±1.0**	0.54
05/2006	31.72 ±3.2*	21.32 ±2.8*	10.40 ±1.6*	0.67	26.92 ±3.4*	13.46 ±2.8*	13.46 ±1.3	0.50	18.97 ±2.9*	9.16 ±2.1*	9.81 ±1.0*	0.48
06/2006	29.50 ±2.7*	18.74 ±2.9*	10.76 ±1.7*	0.64	24.67 ±2.9*	10.93 ±2.5*	13.74±1.4	0.44	16.50 ±2.1*	6.50 ±2.3*	10.00 ±1.0*	0.40

¹mean and standard deviation, * difference significant at P<0.05, ** difference significant at P<0.001

In the apples stored in NA the content of TAA and AA progressively decreased. A significant change was observed already after one month in 'Jonagold' and after 2 months in 'Shampion' and 'Golden Delicious'. At the end of the experimental period the decreases in TAA and AA were 30% and 42% for 'Shampion', 33% and 55% for 'Golden Delicious' and 34% and 60% for 'Jonagold', respectively. Simultaneously, a progressive increase in DHA content was observed and after 4 months it was 211%, 297% and 242% higher in 'Shampion', 'Golden Delicious' and 'Jonagold', respectively in relation to the content before storage. The change in the ascorbate redox ratio resulted both from increased DHA content and decreased AA concentration. The ratio of AA/TAA diminished by 0.15, 0.29 and 0.35 for 'Shampion', 'Golden Delicious' and 'Jonagold', respectively.

TAA content at the beginning of the measurements were similar in the apples of each cultivar tested regardless of the variant (Tab. 1 and 2). However, the content of AA and DHA in apples from variant II differed from these from variant I. Content of AA were lower by 14%, 37% and 22% and of DHA higher by 163%, 372% and 219% in 'Shampion', 'Golden Delicious' and 'Jonagold' respectively. In the course of subsequent 4 months (6 – 9th month of storage period), similarly as in variant I, a progressive decrease in TAA, by 30-39%, and in AA by, 53-66%, together with increase in DHA by, 107-188%, were observed (Tab. 2). The smallest changes in TAA and AA content were noted in

'Shampion' and in DHA content in 'Golden Delicious', while significant decreases in TAA started in 6th and 7th months of the storage period in 'Golden Delicious' and 'Jonagold', respectively. In variant II AA/TAA ratio decreased by 0.22, 0.2 and 0.31 for 'Shampion', 'Golden Delicious' and 'Jonagold', respectively (Tab. 2).

Cultivar-dependent ascorbate content changes were accompanied by APX activity changes (Tab. 3 and 4). For each cultivar tested APX activities at the beginning of the measurements were similar regardless of the variant (Tab. 3 and 4). In both variants, APX activity in 'Shampion' has increased to about 200% of the initial value during the first three or eight months of storage in variants I and II, respectively and then decreased back to the initial value during the subsequent two months. In 'Golden Delicious' and 'Jonagold' in both variants the significant decreases ($P < 0.001$) were observed already after one month.

At the beginning of measurements activities of PO in 'Shampion' were significantly different in variant I and variant II (87.45 and 21.20) whereas in 'Golden Delicious' and 'Jonagold' the difference were less marked (Tab. 5 and 6). In both variants, PO activity significantly increased ($P < 0.001$) already after 1 and 6 months, respectively for variant I to II in 'Shampion' and 'Golden Delicious' cultivars. During the subsequent months, however, it was still increasing in 'Shampion' (up to over 300%) but decreasing in the other two cultivars (Tab. 5 and 6).

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Table 3. APX activity ($U\ g^{-1}\ FW$) in the tissue of three apple cultivars stored in normal atmosphere (Variant I)

Time month/year	Cultivars		
	Shampion	Golden Delicious	Jonagold
09/2005	1.40±0.12 ¹	1.10±0.07	1.11±0.09
10/2005	1.98±0.11	0.61±0.04**	0.35±0.03**
11/2005	2.70±0.07**	0.35±0.03**	0.18±0.02**
12/2005	2.00±0.08**	ND	ND
01/2006	1.45±0.07**	ND	ND

¹mean and standard deviation, ND - not detectable, **difference significant at $P<0.001$

Table 4. APX activity ($U\ g^{-1}\ FW$) in the tissue of three apple cultivars stored for 5 months in controlled atmosphere and then (since February 2006) in normal atmosphere (Variant II)

Time month/year	Cultivars		
	Shampion	Golden Delicious	Jonagold
02/2006	1.20±0.17 ¹	1.32±0.07	1.13±0.16
03/2006	1.90±0.12**	0.82±0.06**	0.51±0.05**
04/2006	2.51±0.11**	0.11±0.02**	0.13±0.08**
05/2006	1.43±0.03	ND	ND
06/2006	1.11±0.04	ND	ND

¹mean and standard deviation, ND - not detectable, **difference significant at $P<0.001$

Table 5. PO activity ($U\ g^{-1}\ FW$) in the tissue of three apple cultivars stored in normal atmosphere (Variant I)

Time month/year	Cultivars		
	Shampion	Golden Delicious	Jonagold
09/2005	87.45±6.38 ¹	37.10±2.56	11.20±1.01
10/2005	94.08±6.40*	78.42±5.27**	12.15±1.16
11/2005	153.70±11.22**	5.30±0.18**	8.30±0.61**
12/2005	250.00±12.75**	ND	2.00±0.22**
01/2006	299.45±13.96**	ND	ND

¹mean and standard deviation, ND - not detectable, **difference significant at $P<0.001$

Table 6. PO activity (U g⁻¹ FW) in the tissue of three apple cultivars stored for 5 months in controlled atmosphere and then (since February 2006) in normal atmosphere (Variant II)

Time month/year	Cultivars		
	Shampion	Golden Delicious	Jonagold
02/2006	21.20±2.33 ¹	35.30±4.27	7.95±1.03
03/2006	76.85±5.22**	80.82±9.70*	9.28±1.30
04/2006	82.15±6.03**	10.60±1.38**	10.90±1.42**
05/2006	71.55±6.01**	2.65±0.23**	3.55±0.46**
06/2006	112.62±8.60**	ND	ND

¹ mean and standard deviation, ND - not detectable, **difference significant at P<0.001

DISCUSSION

Proper diet is crucial for good health. It is believed that many so called civilization diseases result from inadequate diet (Feskanich et al., 2000).

Natural antioxidants present in fruits and vegetables are able to protect organisms against reactive oxygen species (ROS). It is believed that ROS are responsible for senescence and cause degradation of major macromolecules such as DNA, RNA, proteins and lipids. All aerobic organisms are equipped with antioxidative systems based on endo- and exogenous substances. Consumption of apples can limit ROS activity and was frequently proved beneficial for human health (Boyer and Liu, 2004).

Ascorbate is known as a natural, exogenous antioxidant of a human cell cytosol fraction. We found different ascorbate concentrations in various apple cultivars. The highest

TAA and AA concentrations were observed in ‘Shampion’, which corresponded with the results obtained by Łata and Przeradzka (2002). A significant decrease of TAA content in Jonagold was found previously by Trierweiler et al. (2004).

In apple cultivars with relatively high share of TAA in antioxidant pool a relatively small decrease in its content was observed during storage (Konopacka and Markowski, 2004). In our experiments decrease in TAA and AA content was observed in all cultivars tested. Range of decrease in ascorbate levels in ‘Shampion’ may indicate that in this cultivar the proportion of ascorbate to the whole antioxidant pool might be greater than in the other two cultivars.

The content of ascorbate and the stability of its metabolism depend on storage conditions. Greater increase in DHA observed in variant II may result from change in the storage conditions from controlled to partially controlled which increased

ascorbate catabolism. On the other hand, these observations may be connected with the longevity of storage period and fruit senescence.

During storage of fruit high level of the CO₂ decreases ascorbate content while O₂ concentration does not affect its level (Agar et al., 1997; Lachman et al., 2000). Timing of harvest is important as both too early and too late harvest negatively influences the quality of stored fruit. Zerbini et al. (2002) showed that in pears stored in controlled conditions decrease in AA content was correlated with the time of harvest. Greater loss in AA was observed in the fruit harvested too late. A similar correlation between TAA content and harvesting time was observed for apples (Davey and Keulemans, 2004). Substantial ascorbate loss observed in the fruit stored first in controlled conditions might result from their older physiological age in comparison with the fruit tested immediately after harvesting.

Tarozzi et al. (2004) observed that in 'Golden Delicious' fruit stored in controlled conditions decrease in the content of phenolics and in the total antioxidant activity was only slight and TAA content was unchanged. Łata and Przeradzka (2002) and Van der Sluis et al. (2001) also showed that in such conditions the content of AA and flavonoids as well as antioxidant activity in apples did not change significantly.

The highest ascorbate content is in the peel and just beneath it and decreases towards the core. Apple

peel contains about 6 fold more ascorbate than pulp (Łata and Przeradzka, 2002). Eberhardt et al., (2000) showed that total antioxidant activity (TOSC – equal to μmol of vitamin C) of 1 g of apple with and without the peel was 83.3 and 46.07, respectively.

Ascorbate content depends largely on the activity of APX. This enzyme might significantly reduce AA and increase DHA content. One of the main functions of APX and PO is scavenging H₂O₂ produced during metabolism, thus protecting tissues against oxidative injury. In apples tested in this experiment the activities of both enzymes were cultivar- and storage time-dependent. The highest PO and APX activities were observed in 'Shampion', where also the highest contents of TAA and AA were noted. APX activity in this cultivar remained fairly stable in both experimental variants with a slight increase in the 3rd and 6th months in variants I and II, respectively.

Fernandez-Trujillo et al. (2003) showed that antioxidant properties of 'Golden Delicious' fruit depended not only on storage conditions but on time of harvesting as well. Torres et al. (2003) observed that in the same cultivar (Golden Delicious) H₂O₂ content and the activities of superoxide dismutase (SOD), catalase (CAT) and PO were directly correlated with the susceptibility of the stored fruit to *Penicillium expansum* infection. The fruit collected before the phase of ripening exhibited small changes in SOD, CAT and PO activities but

higher in H₂O₂ content and their susceptibility to infection was decreased, while those harvested in the proper time had enhanced activity of CAT and PO, and decreased H₂O₂ concentration, which correlated with susceptibility to infection.

According to Lester et al. (2004), activities of antioxidative enzymes in stored plants depends on tissue hydration. Too low relative air humidity may cause excessive transpiration, thus changing PO activity. Such observation may be related to our finding that PO activity was higher in the apples analysed directly after harvesting than in these stored in controlled conditions.

The data from literature indicate that the thickness of a wax layer and physiological features of the peel influence intensity of transpiration in fruit. It is possible that a significant decrease in PO and APX activities, especially in 'Golden Delicious', was correlated with dehydration of tissue during storage. However, the decrease in PO and APX activities did not inhibit the decrease in TAA level.

CONCLUSION

The obtained data suggest that ascorbate content depends mainly on the apple cultivars. Of the three cultivars tested, 'Shampion' seems to be the best suited for long-term storage. Its initial ascorbate content was high, thus its loss was relatively smaller during storage both in the KA and NA. Moreover, high activities of both peroxidases during the whole experimental period limited harmful effect of H₂O₂ and

slowed down senescence of fruit. It also seems that 'Shampion' apples are a better source of ascorbate and are less influenced by storage under partially controlled conditions.

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ZAWARTOŚĆ ASKORBINIANU I AKTYWNOŚĆ PEROKSYDAZ W JABŁKACH PODCZAS ICH PRZECHOWYWANIA

Jacek Patykowski, Alina Majczak, Katarzyna Bergier
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S T R E S Z C Z E N I E

W pracy badano zawartości askorbinianu, aktywności peroksydazy askorbinianowej i peroksydazy guajakolowej w jabłkach trzech odmian 'Szampion', 'Golden Delicious' i 'Jonagold' w dwu wariantach doświadczalnych. W wariancie I jabłka były przechowywane w 4°C w normalnej atmosferze i warunkach niekontrolowanej wilgotności. W wariancie II jabłka były przechowywane przez 5 miesięcy w pełni kontrolowanych warunkach, a następnie zostały przeniesione do warunków identycznych jak w wariancie I na 4 miesiące. W obydwu przypadkach badania prowadzono w odstępach 1 miesiąca. Stężenie askorbinianu i aktywności enzymów zależały od odmiany i warunków przechowywania. Dla poszczególnych odmian w wariancie I zaraz po zbiorze i II po 5 miesiącach przechowywania w kontrolowanych warunkach stężenie askorbinianu ogólnego było zbliżone, podczas gdy stężenie dehydroaskorbinianu było niższe w wariancie I. Stężenie dehydroaskorbinianu zwiększało się szybciej w odmianach 'Jonagold' i 'Golden Delicious' niż w odmianie 'Szampion'. Aktywności peroksydaz askorbinianowej i guajakolowej były wyższe w odmianie 'Szampion'. Stwierdzono, że warunki przechowywania wpłynęły na metabolizm antyutleniacza w badanych odmianach jabłek. Wydaje się, że w przypadku długiego czasu przechowywania owoców odmiana 'Szampion' jest najlepszym źródłem askorbinianu spośród badanych odmian.

Słowa kluczowe: jabłka, askorbinian, peroksydaza askorbinianowa i guajakolowa, przechowalnictwo