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Antioxidant content in the fruit peel, flesh and seeds of selected apple cultivars during cold storage

Barbara Łata[1], Aleksandra Trąmpczyńska[1,2], Marta Oleś[1]

[1]Department of Pomology and Basic Natural Sciences in Horticulture
Warsaw Agricultural University
Nowoursynowska 159, 02-787 Warszawa, Poland
e-mail: lata@alpha.sggw.waw.pl
[2] Department of Stress and Developmental Biology
Leibniz Institute of Plant Biochemistry
Weinberg 3 D-06120 Halle (Saale), Germany

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ABSTRACT

Four apple cultivars: 'Šampion', 'Jonagold', 'Gloster' and 'Elise' kept in common cold storage (1°C, 95-97% RH) were tested. The content of glutathione in the apple peel remained nearly at the same level during storage, while its precursors (particularly γ-glutamylcysteine) as well as glutathione reductase activity successively increased. The decrease of ascorbate content and ascorbate peroxidase activity was observed after 60 days of storage and then their levels significantly increased. Apple flesh and seeds exhibited nearly the same tendencies related to compounds mentioned above. The reduction level of both hydrophilic antioxidant in the peel was increasing with the longer time of storage. Activity of catalase significantly decreased during storage, but only in the apple peel. Out of all

cultivars the highest antioxidant potential related to tested compounds and enzyme activity was noted for ‘Šampion’. The great differences between apple peel, flesh and seed in antioxidant content were measured. On average, the content of ascorbate in the peel was from approximately 3 (‘Gloster’) to above 7-times higher (‘Šampion’) in comparison with the flesh. Seeds contained very small amounts of ascorbate, on average 49 μg g^{-1} f.m. As opposed to ascorbate, content of low molecular weight thiols and GR activity in the seeds were considerably higher in comparison with apple flesh and peel. The content of cysteine, γ-glutamylcysteine, total glutathione and glutathione reductase activity was higher respectively by 125.7%, 80.0%, 158.8% and 28.3% in comparison with the peel, and by 198.0%, 80.0%, 466.1% and 377.8% in comparison with the flesh. Apple peel, then flesh and seeds exhibited the highest ascorbate peroxidase and catalase activity.

ABBREVIATIONS:

CS – cold storage
AA – L-ascorbic acid
DHAA – dehydroascorbic acid
GSH – reduced glutathione
GSSG – oxidized glutathione
CYS – L-cysteine
γ-GC – γ-glutamylcysteine
DTT – DL-dithiothreitol
NEM – N-ethylmaleimid
GR – glutathione reductase
APX – ascorbate peroxidase
CAT – catalase
SOD – superoxide dismutase
ROS – reactive oxygen species

INTRODUCTION

Antioxidants and the enzyme systems involved in their synthesis and regeneration have been shown to protect cellular membranes and organelles from the damaging effects of ROS. ROS are formed both during normal cellular metabolism and environmental stress conditions. Among the non-enzymatic antioxidants, which are generally small molecules, ascorbic acid plays an important role in the destruction of ROS, and glutathione is essential to the regeneration of DHAA, which is formed during ROS removal. Both of these water soluble reductants are widely distributed in plant cells. The reduced form of GSH is also an independent active form

protecting macromolecules against free radical attack or during xenobiotic chelating. The enzymatic system involves a wide range of enzymes such as SOD and CAT, which play an important role in formation and degradation of H_2O_2, respectively, APX is involved in DHAA regeneration. High GSH concentration in cells is maintained by GR, which catalyses the reduction of GSSG. A high reduced/oxidized ratio of these compounds seems to be necessary 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 (Walker and McKersie 1993, Wang 1995, Noctor and Foyer 1998, Davey et al. 2000, Lee and Kader 2000). Antioxidants are not only responsible for plant cell protection but also keep health quality of fruit and vegetables. There is clear epidemiological evidence linking consumption of diet rich in antioxidant, direct and indirect, with reduced risk of many diseases (Kaur and Kapoor 2001, Astley 2003). Compounds of special interest, favoured also by European Research on the Functional Effect of Dietary Antioxidant are: vitamin C, vitamin E, carotenoids, phenols, glucosinolates, selenium and glutathione. The crucial role of phytochemicals in keeping the health quality of fruits and vegetables have promoted efforts to identify the factors, which have influenced their content and pre- and post-harvest changes, in particular during storage and after processing procedure. Natural products, high in biologically active phytochemicals are also of interest in food, cosmetic, and pharmaceutical industries. Such naturally occurring compounds can be used, probably without any doubts, as substitutes for synthetic antioxidants (Moure et al. 2001).

It seems that genetical and climatic conditions as well as post-harvest factors, may considerably modify the composition and concentration of fruit and vegetable phytochemicals. The aim of this research was to evaluate the content of ascorbate, glutathione and their reduction level together with some antioxidative enzyme activity (GR, APX and CAT) and their distribution in fruit of four apple cultivars: ‘Jonagold’, ‘Šampion’, ‘Gloster’ and ‘Elise’ just after harvest and during cold storage.

MATERIAL AND METHODS

Four apple cultivars: ‘Šampion’, ‘Jonagold’, ‘Gloster’ and ‘Elise’, that were grown in the Wilanow orchard (experimental field of Horticulture Department of Warsaw Agricultural University, Poland), were tested. They received standard horticultural practices. Analyses of phytochemical content and enzyme activity in the apple peel and flesh were conducted in 2001 and 2002 seasons. Seeds were analysed only in 2002 as a preliminary study. Apples were harvested as follows: ‘Elise’ – 16 September, ‘Jonagold’ and ‘Šampion’ – 25 September and ‘Gloster’ – 30 September, and immediately put into cold storage. Chemical analyses were

made just after harvest and after 60 and 90 days of cold storage. Apples were stored in 1°C, 95-97% RH, in 10 kg boxes and for each tested time of storage fruit was randomly selected for chemical analysis.

Apples were peeled with a potato knife, thus a thin layer of apple flesh remained adhered to the peel, then flesh was cut into thin pieces and at the end the seeds were removed from the apple core. Samples were frozen in liquid nitrogen and stored at -80°C until analysis. Directly before analysis apple tissues were ground to a fine powder in liquid nitrogen. Chemical analyses were made in five, three and two replications for apple peel, flesh and seeds, respectively; each replication included apple peel or flesh from two fruits and seeds from five fruits.

Extraction and determination of enzymatic activities

The ground tissues were suspended in 100 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.8) containing Triton X – 100 (0.5%), insoluble polyvinylpolypyrrolidone (PVP) and ascorbate (5 mM). The mixture was centrifuged at 20 000 rpm, for 20 minutes at 4°C. Activity of ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT) was carried out in a total volume of 1 ml.

Activity of APX was measured by monitoring the decrease in absorbance at 290 nm (extinction coefficient of 2.8 mM^{-1} cm^{-1}). The assay mixture contained 50 mM potassium phosphate buffer (pH 7), 8 mM ascorbate, 20 mM H_2O_2 and enzyme extract (Nakano and Asada 1987). GR activity was monitored at 340 nm (extinction coefficient of 6.2 mM^{-1} cm^{-1}) in mixture containing 500 mM HEPES (pH 8.0), 5 mM EDTA, 1.25 mM NADPH, 5 mM GSSG and enzyme extract (Foyer and Halliwel 1976). CAT activity was calculated from the fall in absorbance at 240 nm (extinction coefficient of 39.4 mM^{-1} cm^{-1}) in the supernatant containing 50 mM potassium phosphate buffer (pH 7), 10 mM H_2O_2 and enzyme extract (Beers and Sizer 1952). For each analysis the reaction was initiated by adding an enzyme extract. Blank rates in the absence of extract were determined for each test system and subtracted during calculation.

Measurements of low molecular weight thiols and ascorbate

Frozen apple powder was homogenized in 0.1 M HCl containing PVP and centrifuged at 14 000 rpm (glutathione) or 20 000 rpm (ascorbate), for 20 minutes at 4°C.

Total glutathione (GSH + GSSG) concentration was determined in the supernatant after reduction with DTT and derivatization with monobromobimane. Monobromobimane derivatives were detected fluorometrically at 480 nm by excitation at 380 nm. During the same analysis CYS and γ-GC were also

determined. Thiol derivatives were separated on a Symmetry C_{18} column (250 mm x 4.6 mm, 5μm, Waters) applying a solution of 10% methanol containing 0.25% (v/v) glacial acetic acid (solvent A, pH 4.3) and 90% methanol with the same acetic acid concentration (solvent B, pH 3.9), the flow rate was 1 ml min^{-1} (Newton et al. 1987).

Total ascorbate (AA + DHAA) content was measured after complete reduction DHAA with DTT. AA was measured directly in the supernatant obtained after extraction procedure. Separation was carried out using Atlantis^{TM} dC_{18} column at 268 nm under isocratic conditions. Mobile phase contained 10% of methanol and 2% $NH_4H_2PO_4$, pH 2.8 (Anderson et al. 1992).

In both analysis the chromatogram peaks of separated compounds were identified by comparing retention times with the retention time of pure standard. Tested sample extract with the internal standards was also applied. Integrated peaks were calculated by comparison with standard solutions of known concentration.

Apparatus for HPLC

The HPLC from Waters Company, System Breeze with binary solvent delivery system (1525), degasser, an autosampler with thermostat in the scale: 4-40^{o}C (M 717 PLUS), scanning fluorescence detector (M 474) and 2-chanel UV-VIS detector (M 2487) and the thermostat for the column 5-85^{o}C (Peltier) was used.

Statistical analysis

The results for apple peel and flesh were elaborated separately by two-way analysis of variance. In the case of percentage of reduced forms of ascorbate and glutathione in their total content arc sin transformation was applied. Significance of differences between cultivar and time of sampling means was evaluated using the Tukey test at $p = 0.05$.

RESULTS AND DISCUSSION

Apples are a very important horticultural product, because they are one of the most consumed fruit in human diet in many countries (Wolfe et al. 2003, Schmitz et al. 2003). It was confirmed that apples are good sources of such antioxidants as vitamin C, glutathione, and a variety of phenolic compounds (Valencia et al. 2001, Avad and de Jager 2003, Planchon et al. 2004).

The concentration of fruit phytochemicals is affected by cultivar, maturity, geographic origin, growing season, horticultural practise, postharvest storage conditions and processing procedures. Obviously the significant influence of

cultivar on phytochemical concentration and composition is noted in almost all experiments in which it is tested.

Effect of cultivar and part of fruit on antioxidant content

Reduced glutathione is not only one of the most efficient scavengers in plant cells (May et al. 1998), but, in addition, GSH and its precursors (CYS and γ-GC) are key nutrients in the maintenance of high health status (Mills et al. 1997). Although knowledge of glutathione content in foods is incomplete, it is known that fruit and vegetables are rich in this compound (Valencia et al. 2001). Moreover, there is little quantitative information about the content of thiol compounds in apple. In the present study, the highest content of thiol compounds in the apple peel: CYS, γ-GC and GSH + GSSG (7.5, 1.4 and 76.0 nmol g^{-1} f.w., respectively) and total ascorbate concentration (738.4 μg g^{-1} f.w.) was noted for 'Šampion' (Table 1). All tested cultivars differed significantly in the content of total glutathione, but all kept high GSH to GSSG ratio, that means that efficiency of regeneration of oxidized form of glutathione in the apple peel was very high. Hence, proportion of GSSG in a total quantity of glutathione was rather small and ranged from 13.4% ('Šampion') to 18.4% ('Elise') and the differences between cultivars were negligible. Contrary to GSSG, amount of DHAA in overall concentration remained high: from 41.2% ('Šampion') to 55.8% ('Jonagold'). The highest AA : DHAA ratio was obtained for 'Gloster' and 'Šampion'.

DHAA can easily be converted into AA in the presence of GSH (Lee and Kader 2000). In this study the high content of GSH corresponded with the highest proportion of AA in its total concentration as it was obtained for 'Šampion' and 'Gloster'. However, on average, about half of total concentration of ascorbate remained in its oxidized form.

Enzymes belonging to antioxidative apparatus work in two ways: directly by detoxifying ROS (e.g. CAT, SOD) or indirectly by regenerating oxidized form of antioxidant (e.g. GR, APX). In this study the highest activity of GR was noted for 'Šampion', which corresponded with higher amounts of GSH and AA in this cultivar. GR and APX are the key enzymes, among others, involved in ascorbate and glutathione regeneration (Noctor and Foyer 1998). However less distinct differences between APX activity among analyzed cultivars were received. The significantly higher activity of CAT was noted for 'Elise' and the lowest for 'Gloster'.

Table 1. Content of ascorbate (μg g^{-1} f.w.), thiol compounds (nmol g^{-1} f.w.) and enzyme activity (nkat g^{-1} f.w.) in apple peel, flesh and seed depending on cultivars (mean for three sampling dates)

PARAMETERS[1]	CULTIVAR			
	ELISE	GLOSTER	ŠAMPION	JONAGLOD
		PEEL		
AA + DHAA	334.4 b*	210.3 a	738.4 c	362.1 b
%DHAA	55.6 b	48.1 ab	41.2 a	55.8 b
AA : DHAA	1.3	3.7	2.6	1.1
CYS	5.8 a	6.9 ab	7.5 b	6.2 ab
γ-GC	0.8 ab	0.7 ab	1.4 c	1.1 bc
GSH + GSSG	48.6 b	60.4 c	76.0 d	34.7 a
%GSSG	18.4 a	16.1 a	13.4 a	15.1 a
GSH : GSSG	4.8	5.7	7.3	6.5
GR	5.3 a	6.8 b	9.0 c	5.6 a
APX	90.9 b	81.5 ab	75.4 ab	67.7 a
CAT	7.4 c	5.4 a	6.3 b	6.4 b
		FLESH		
AA + DHAA	72.7 a	75.2 ab	102.8 c	84.5 b
CYS	5.7 a	5.8 a	5.1 a	3.5 a
γ-GC	1.0 a	0.82 a	1.1 a	1.2 a
GSH + GSSG	27.4 bc	24.6 b	31.3 c	17.3 a
GR	1.7 b	1.2 a	2.3 c	2.0 bc
APX	42.3 a	52.0 a	43.7 a	36.5 a
CAT	3.6 b	2.5 a	3.6 b	4.3 b
		SEEDS[2]		
AA + DHAA	54.0	42.9	45.9	53.9
CYS	14.4	19.6	6.8	18.8
γ-GC	1.0	2.3	1.9	2.2
GSH + GSSG	146.5	150.9	126.3	144.9
GR	8.4	7.4	7.0	11.6
APX	22.3	28.0	18.3	38.5
CAT	3.6	3.5	2.3	3.8

Explanations:
*means followed by the same letters do not differ significantly
[1] see the list of abbreviations
[2] data represent the mean of two replications

Antioxidant concentration in apple flesh and seeds was rather small with the exception of thiol compounds and GR activity in seeds. Nevertheless, the great differences between apple peel, flesh and seed were observed. Content of ascorbate in the peel was from approximately 3 (‘Gloster’) to above 7 times higher (‘Šampion’) in comparison to the flesh. Seeds contained very small amount of ascorbate, on average 49 μg g^{-1} f.m. As opposed to ascorbate, the content of low molecular weight thiols and GR activity in the seeds were considerably higher than in flesh and peel. The content of CYS, γ-GC, GSH + GSSG and GR was higher respectively by: 125.7%, 80.0%, 158.8%, and 28.3% compared to peel, and by 198.0%, 80.0%, 466.1%, and 377.8% in comparison to the flesh. Most of the fruit peel and seed fractions tested by Guo et al. (2003) were stronger than the pulp fractions in total antioxidant activity based on their FRAP values (ferric reducing ability). It is in agreement with many other studies that fruit phytochemicals are predominantly localized in the skin (Eberhardt et al. 2000, Van der Sluis et al. 2001, Kondo et al. 2002, Guo et al. 2003). Moreover, Planchon et al. (2004) reported factors such as fruit size, peel fruit colour and fruit position on the tree, which may cause differences in the content of ascorbate in apple fruit. In the present study, besides GR, also APX and CAT activity was the highest in apple peel, followed by flesh and seeds.

Changes in antioxidant content during storage

Generally, the content of total glutathione regardless of the part of apple, remained nearly at the same level during storage, or even significantly increased for apple flesh (Table 2). It may be explained by the change of its precursors, whose content increased, especially γ-GC that successively rose in the peel and flesh, as well as seeds. The rate of synthesis of any metabolic product may be influenced either by substrate concentrations or the activities of enzymes involved in the biosynthetic pathway, or by both. Hence, it may be assumed that increasing availability of glutathione precursors favored maintaining steady glutathione level. With the exception of ‘Elise’, all other cultivars showed the same behavior in changes of thiol compounds during storage (data not shown). The increase of CYS content after two months of storage with simultaneous increase of γ-GC concentration as well as GR activity in that and in the following month might indicate higher demand for GSH. Noctor et al. (1998) reported that the capacity for regeneration of glutathione seemed to be more important than increases of its total pool size. In this research it was observed for both the increase of GR activity and the content of glutathione precursors. Probably, the maintenance of high level of thiols might have balanced the low reduction state of ascorbate.

Table 2. Content of ascorbate (μg g^{-1} f.w.), thiol compounds (nmol g^{-1} f.w.) and enzyme activity (nkat g^{-1} f.w.) in apple peel, flesh and seed depending on time of storage (mean for four tested cultivars)

PARAMETER[1]	TIME OF STORAGE		
	0 (HARVEST)	60 DAYS	90 DAYS
		PEEL	
AA + DHAA	416.9 ab*	380.2 a	436.8 b
%DHAA	57.5 b	49.4 a	43.6 a
AA : DHAA	1.2	1.4	2.3
CYS	6.9 ab	7.1 b	5.9 a
γ-GC	0.4 a	1.0 b	1.5 c
GSH + GSSG	58.0 a	55.5 a	51.2 a
%GSSG	20.6 c	16.3 b	10.9 a
GSH : GSSG	4.0	5.5	9.0
GR	5.5 a	6.9 b	7.7 b
APX	91.5 b	65.1 a	80.0 b
CAT	7.0 c	6.4 b	5.7 a
		FLESH	
AA + DHAA	84.6 b	70.7 a	96.1 c
CYS	5.9 b	4.0 a	5.2 ab
γ-GC	0.34 a	0.87 a	1.9 b
GSH + GSSG	21.2 a	25.6 b	28.7 b
GR	1.6 a	1.8 ab	2.0 b
APX	47.6 a	50.2 a	33.0 a
CAT	2.9 a	4.1 b	3.4 ab
		SEEDS[2]	
AA + DHAA	24.9	22.0	31.9
CYS	16.5	10.7	17.6
γ-GC	0.9	1.4	1.4
GSH + GSSG	143.9	130.8	151.7
GR	8.0	7.5	10.2
APX	31.9	20.9	27.5
CAT	3.2	3.1	3.5

Explanations as in Table 1

The most interesting was the change in reduction state of ascorbate and glutathione during storage. The proportion of GSSG and DHAA in the whole concentration of these phytochemicals decreased during storage, especially with regard to glutathione, which might mean that the capacity of glutathione–ascorbate cycle was not decreased in the apple peel during three months of cold storage. Concentration of antioxidants, especially hydrophilic is influenced by cultivar factors and storage conditions such as temperature, storage duration and atmosphere. The results obtained by different authors are variable and sometimes contradictory (Lachman et al. 2000, Lee and Kader 2000, Van der Sluis et al. 2001, Avad and de Jager 2003, Leja et al. 2003, Planchon et al. 2004). Among phytochemicals, phenolic compounds seem to be more stable. Ascorbic acid is reported as more sensitive for factors mentioned above. However, in the present study, total ascorbate content slightly decreased after 60 days of storage but after the following month of CS the content increased to higher level than it was observed at the first time of sampling. Especially, 'Šampion' showed the successive increase of AA + DHAA during storage. The rest of tested cultivars exhibited a slight drop of ascorbate concentration during storage. Curiously enough, all cultivars showed continuous increase of AA : DHAA ratio with the longer time of storage (data not shown). The research is continued with other cultivars to check these results. High and rapidly decrease of AA and GSH content in 'Conference' pears, but during short-term storage, was observed by Larriguadiere et al. (2001). AA was more sensitive for changing environmental conditions than GSH.

Time dependent changes in GR and APX activity in the apple peel, run in the opposite direction after 60 days of storage, and then the activity of both considerably increased. In the flesh the GR activity successively increased, whereas APX did not change during storage. CAT activity decreased during storage, irrespective of part of fruit. Changes in activity of H_2O_2 – scavenging enzymes and in redox status of antioxidants such as GSH and AA may act as a signal transducing molecules toward starting defence mechanism (Foyer et al. 1997, Noctor et al. 1998). Larrigaudiere et al. (2001) reported that at the first days of storage, common or CA, the level of H_2O_2 sharply increased. Simultaneously with the elevating level of H_2O_2, ascorbate and glutathione content declined and after 8 days started to increase.

CONCLUSIONS

The results obtained in the present study could indicate the important role of ascorbate-glutathione cycle in apples during storage, obviously with some differences between cultivars. In general apples preserve their quality values during long term storage. Content of total glutathione and ascorbate remained at nearly the

same level during cold storage. This statement supports also the high reduction state, especially related to glutathione and strangely enough the value of this parameter increased during storage. 'Šampion' seems to be very interesting in respect to health properties. There were great differences between peel, flesh and seed antioxidant content. Changes in tested antioxidants mean that they may participate in keeping apple fruit quality and play important role in acclimation to storage conditions. The research is continued with respect to additional cultivars, enzymes and compounds related to different storage conditions.

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ZAWARTOŚĆ ANTYOKSYDANTÓW W SKÓRCE, MIĄŻSZU I NASIONACH OWOCÓW WYBRANYCH ODMIAN JABŁEK W CZASIE PRZECHOWYWANIA

Streszczenie: Ocenie poddano cztery odmiany jabłek: ‘Šampion’, ‘Jonagold’, ‘Gloster’ oraz ‘Elise’ przechowywanych w chłodni (1^{o}C, 95-97% wilgotności). Zawartość glutationu w skórce badanych odmian jabłek kształtowała się na podobnym poziomie, niezależnie od długości przechowywania, natomiast stężenie prekursorów glutationu (szczególnie γ-glutamylocysteiny), jak również aktywność reduktazy glutationowej wzrastała wraz z dłuższym przechowywaniem owoców. Spadek stężenia askorbinianu i aktywności peroksydazy askorbinianowej w skórce wystąpił po 60 dniach przechowywania, po czym wartość tych wskaźników istotnie wzrosła. Podobny przebieg zmian, omawianych powyżej wskaźników, wykazywał miąższ oraz nasiona. Stosunek form utlenionych do zredukowanych, zarówno w przypadku glutationu jak i askorbinianu, rósł wraz z dłuższym okresem przechowywania owoców. Aktywność katalazy istotnie obniżała się w czasie przechowywania owoców, zwłaszcza w przypadku skórki jabłek. Najwyższym potencjałem przeciwutleniającym w zakresie analizowanych składników charakteryzowała się odmiana ‘Šampion’.

Zawartość antyoksydantów bardzo wyraźnie zależała od części owocu. Zawartość askorbinianu w skórce była od około 3 (‘Gloster’) do ponad 7 razy wyższa (‘Šampion’) w porównaniu z miąższem. Nasiona zawierały niewielką ilość askorbinianu, średnio 49 μg g^{-1} św. m. W przeciwieństwie do tego związku, zawartość niskocząsteczkowych związków tiolowych jak i aktywność reduktazy glutationowej w nasionach była o wiele wyższa w porównaniu ze skórką i miąższem. Zawartość cysteiny, γ-glutamylcysteiny, glutationu ogółem oraz aktywność reduktazy glutationowej były wyższe odpowiednio o 125,7%, 80,0%,

158,8% i 28,3% w porównaniu ze skórką oraz o 198,0%, 80,0%, 466,1% i 377,8% w porównaniu z miąższem. Najwyższą aktywność peroksydazy askorbinianowej oraz katalazy wykazywał ekstrakt ze skórki jabłek, następnie miąższu oraz nasion.

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