Photostability of pigments in ripening apple fruit: a possible photoprotective role of carotenoids during plant senescence

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Abstract

Light-induced pigment destruction was studied in ripening apple (Malus domestica Borkh., cv. Antonovka) fruits with reflectance spectroscopy. The reflectance spectral changes attributable to light-induced transformation of xanthophyll cycle carotenoids (Car) preceded the pigment degradation. In green fruits, the destruction of chlorophyll and Car proceeded synchronously up to complete disappearance of both pigments. In ripening fruits with a molar chlorophyll/carotenoid ratio $B/C > 2.5$/$3$, significant amounts of peel Car were retained at the deep stages of chlorophyll degradation. Car were especially resistant to irradiation in yellow fruits (a chlorophyll/carotenoid ratio $B/C < 0.3$); the extent of their bleaching after prolonged irradiation did not exceed 20%. Irradiation of pigment solutions showed that apple fruit Car alone exhibit much higher light stability than in the presence of chlorophyll. The extent, kinetics and stoichiometry of light-induced pigment destruction in apples are consistent with the existence of two carotenoid pools, (i) closely associated with chlorophyll in chloroplast thylakoid membranes and (ii) exhibiting higher light stability localised in plastoglobuli appearing in chloroplasts undergoing transformation to gerontoplasts–chromoplast. It is suggested that the induction of carotenoid synthesis during senescence provides the protection of plastoglobuli and light-sensitive constituents of plant tissues from irradiation in the blue part of the visible spectrum. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Carotenoids (Car) are essential components of plant photosynthetic apparatus and their roles in light harvesting, stabilisation of thylakoid membranes, energy distribution and dissipation in pigment–protein complexes and photoprotection are well documented [1–5]. However, little is known on physiological significance of Car retention and/or accumulation often occurring during senescence, the phenomenon that at the terminal stages of chlorophyll (Chl) breakdown is responsible for bright yellow coloration of plant tissues [1,6–13]. Generally, both in senescing leaves and ripening fruit the Car pool represented by xanthophylls and carotenol fatty acid esters is associated with plastoglobuli in chloroplasts undergoing transformation to gerontoplasts and then to chromoplasts [1,6,12,14–16].

Plastoglobuli of senescing leaves characterised by high amounts of neutral lipids (including Car) [6,14–16] have been proposed to consist of a thin monolayer of polar lipids and proteins covering the surface, with apolar components buried in the interior (see [12]). Although deposited in plastoglobuli Car may represent simply rejected products without any significance [1], several hypotheses on their physiological role(s) have been framed out. In plants plastoglobuli may serve as pools for storage of thylakoid lipids (pigments, fatty acids, triacylglycerols, prenyl quinones etc.) [1,6,14–16] which could be utilised during regreening and gerontoplasts redifferentiation into chloroplast (see [17]). The involvement of Car recognised as powerful antioxidants in the metabolism of reactive oxygen species (ROS) generated under light conditions in transforming chloroplasts has been postulated [1,11]. Furthermore, it was suggested that ROS acting as secondary messengers mediate

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Abbreviations: Car, carotenoids; Chl, chlorophyll; CRI, carotenoid reflectance index; R, reflectance; ROS, reactive oxygen species.
intense carotenoid synthesis during chromoplast differentiation [11]. Some lines of evidence suggest that the Car functions in senescing plants are related with their photochemical and photophysical properties. Schmidt [18] proposed that light absorbed by Car in plastoglobuli may provide the heat for effective transpiration and metabolic processes in autumn leaves [1]. Car that as a result of Chl breakdown and in the absence of anthocyanins govern leaf [9,10,13,19] and fruit [19] optical properties were suggested to be involved in photoprotection during dismounting of photosynthetic machinery in senescing plants [9,10,13,19]. In addition, the accumulation of larger quantities of Car in sunlit compared with shaded surfaces of apples resembling that during ripening has been reported and attributed to a photoprotective mechanism preventing the development of the photooxidative damage, sun-scalld, to fruit [20].

The supposition of a photoprotective mechanism(s) by Car in senescing and/or stressed plants presumes an adequate resistance of the pigments to light irradiation. However, in organic solutions Car are known to be sensitive to oxygen, free oxygen and organic radicals [4] and undergo rapid destruction when exposed to visible light in the presence of chlorophyll [21,22]. Accordingly, extensive and complete Car photobleaching accompanying that of Chl under strong light irradiation has been observed in green plant leaves and fruits [23].

The main goal of the present investigation was to elucidate whether the sensitivity Car to light irradiation depends on the stages of plant senescence. In this respect, apple fruits, which in the course of ripening accumulate large amounts of Car over Chl and were found to be a useful system for studies of photooxidative pigment destruction both under strong irradiation in real time [20,23,24] and under natural conditions [20,24], have been used.

2. Materials and methods

2.1. Plant material

Both freshly-collected and stored at +4 °C for up to 3 months apple (Malus domestica Borkh., cv. Antonovka obiknovennaya) fruits were from the Botanical garden of Moscow State University or from All-Russian Institute of Horticulture (Michurinsk, Tambov region, Russia). The study was performed on shaded surfaces of fruits, which were essentially anthocyanin-free or possessed low anthocyanin content (  2 nmol/cm²).

2.2. Pigment analysis

Pigment content in intact fruit was estimated with reflectance spectroscopy as described previously [25]. The reflectance indices developed for Antonovka fruits for assessments of Chl and Car were used in the forms: Chl (nmol/cm²) = 0.827* (R800/R578−1) and Car (nmol/cm²) = [(R900/R520 − R800/R700)−0.134]/0.233 (Carotenoid Reflectance Index, referred further as CR1). All correlations were significant at P < 0.001 with determination coefficients (r²) of 0.93 and 0.88 for Chl and Car, respectively.

Peel pigment extracts were obtained as follows (for more details, see [26]). Peel disks (16 mm in diameter and ca. 1 mm thickness) were homogenised in chloroform–methanol (2/1, v/v) in the presence of MgO in the amounts sufficient to prevent Chl photoperoxidation. After completion of extraction homogenates were filtered through a paper filter and distilled water (1/5 of total extract volume) was added. Then extracts were centrifuged at 3000 × g for 10 min until phase separation and absorbance spectra were recorded in lower (chloroform) phase. The estimation of Car absorption in extracts was performed by subtracting of absorbance spectra of pure Chl a and b (see, for more details [21]). Chl and Car concentrations in chloroform were calculated using coefficients reported by Wellburn [27]. Molecular weight of 570 for Car was accepted. Chromatography of total apple fruit pigments (Car and Chl, containing in chloroform phase of the extracts) as well as of free Car obtained after alkaline hydrolysis was performed on silica-gel TLC Silufol plates (Kavalier, Chech Republic) using acetone/hexane/benzene (10:20:1, by vol) as a developing system [26].

For bleaching experiments the extracts from yellow apples were obtained as described above, taken to dryness with a rotor evaporator and redissolved in n-hexadecane of a reagent grade (Reakhim, Russia).

2.3. Spectral measurements

Whole fruit reflectance spectra in 400–800 nm range were recorded with a 150-20 Hitachi spectrophotometer equipped with an integrating sphere against barium sulphate as a standard. Spectral data were interfaced to IBM-compatible personal computer and processed using spreadsheet software. Reflectance was converted to \( f(R) = (1 - R^2)^2/2R \) values formally analogous to Kubelka–Munk remission function (see [23,24]).

2.4. Bleaching experiments

Whole fruits or pigment extracts were irradiated through a 0.4 cm UV-cut off BS-8 filter and a 5 cm layer of water as a heat filter from a slide projector with a KGM 150 W/24 tungsten–halogen lamp (NPO Svetotechnika, Saransk, Russia) as a light source at an irradiance of 2500 W/m². The chloroform extracts from yellow apples were obtained as described in Section 2.2, taken to dryness with a rotor evaporator, redissolved in...
n-hexadecane of a reagent grade (Reakhim, Russia) and irradiated in a 1 cm (pathlength) quartz spectrophotometric cell without or with addition of Chl a (Fluka, Switzerland).

2.5. Electron microscopy

For the samples of apple peel, fixation either in glutaraldehyde+KMnO₄ or in p-formaldehyde+glutaraldehyde+OsO₄ was used. Ultrathin sections were prepared with LKB-8800 ultratom and examined under a JEM-100 B electron microscope.

3. Results

The representative changes of reflectance spectra of three apple fruits differing in absolute and relative pigment content (regarded below to green, greenish-yellow and yellow fruit) in the course of light irradiation are shown in Figs. 1–3. A freshly-collected green fruit with high Chl content (Fig. 1A) exhibited lower reflectance in Chl absorption maximum near 678 nm. In the progress of fruit ripening, the reflectance in this band increases, whereas that near 480–490 nm (combined absorption by Chl and Car) remained low (Fig. 1B and C) showing accumulation of Car and a decrease in Chl content [19,25]. In a visually yellow fruit (Fig. 1C) exhibiting high reflectance in the green–red region the distinct bands near 460 and 480 nm were present. In accord with an increase in Car/Chl ratios (from A to C), the decrease in reflectance near 400 nm related to the build up of peel flavonoids (mainly, quercetin glycosides) in the progress of fruit ripening [20] should be mentioned.

Irradiation of a green fruit resulted in a more or less synchronous increase of reflectance in the blue and in the red up to deep stages of Chl destruction (Fig. 1A, for more details, see [24]). At more advanced stages of ripening, in apples exposed to strong light approximately for the same time, reflectance changes in the blue were smaller, especially in visually yellow fruits (Fig. 1B and C). At the deep stages of Chl photodestruction the spectral features in the range 420–500 nm disappeared in a green apple (Fig. 1A), whereas they were expressed in fruits with lower initial Chl/Car ratio (Fig. 1, panels B and C).

The spectra of bleached pigments in apples estimated as a difference of $f(R)$ before and after irradiation for 120–150 min presented in Fig. 2A show that an absolute difference of $f(R)$ in the visible range was larger in a green fruit followed by greenish-yellow and yellow fruits. Compared with spectra in Fig. 1, the difference remission spectra in Fig. 2 were more resolved in the blue range (see the band of Chl a near 420 nm in the spectrum of a green apple as well as the appearance of the third maximum near 410–420 nm in the spectrum of a yellow apple). The $\Delta f(R)$ spectra normalised to unity at the red Chl absorption maximum indicate that the relative changes of $f(R)$ in the blue were higher in fruit with lower Chl content (curves 1’, 2’ and 3’ in Fig. 2B). The differences of the normalised spectra implied an additional bleaching of pigment(s) peaking near 426–428, 448–454 and 480–484 nm in fruit at more advanced stages of ripening compared with a green fruit (curves 2’–1’ and 3’–1’ in Fig. 2).

Fig. 3 shows the absorbance spectra of chloroform extracts from the peel of apples before and after irradiation as well as those obtained by subtraction of Chl a and b absorption. The spectra of the extracts

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Fig. 1. The changes of reflectance spectra of apple fruit with different chlorophyll and carotenoid content recorded in the course of light irradiation. The spectra were recorded from a freshly collected green (the end of August, A) and stored greenish-yellow (November, B) and yellow (December, C) fruit. Reflectance spectra (lines, left scale) were taken with 20–30 min irradiation intervals. Numbers correspond to the spectra recorded from intact fruit and those at terminal time of irradiation (min). The corresponding remission $f(R)$ spectra are shown on right scale (symbols).
compensated for Chl (curves 1a' and 2a', Fig. 3 insert) and the spectrum of an extract from the peel of a yellow apple (curve 3) exhibited bands near 421–425, 447–452 and 478–479 nm characteristic of Car (xanthophylls) [28]. In accordance with reflectance changes of whole fruit (Figs. 1 and 2), irradiation of a green apple brought about a massive destruction of both Car and Chl and only few spectral features have been observed between 400 and 500 nm as a result (curves 1a and 1a' in Fig. 3). In contrast, at the deep stage of Chl destruction in a greenish-yellow apple the prominent Car bands appeared in spite of a decrease in total Car concentration by ca. 60% (cf. curves 2a, 2', and 2a' in Fig. 3). The spectral properties of the remaining Car were not significantly different from those in intact fruit. Although initial Car content in greenish-yellow and yellow apples was close (cf. curves 2a and 3 in Fig. 3), only a small light-induced Car bleaching has been observed in a yellow fruit (curves 3 and 3' in Fig. 3).

The changes in Car and Chl content as well as kinetics of Car destruction estimated from reflectance measurements in situ during irradiation of apple fruits are presented in Figs. 4 and 5. After 20–30 min light exposition, a temporal increase of CRI was observed, especially in fruits with high Chl content (curves 1–3 in Fig. 4). Later, CRI declined in accordance with data of Car chemical analysis (Fig. 3). In on-tree ripening fruit with high Chl content (curves 1–3 in Fig. 4 and fruit with Chl/Car > 2.5–3 in Fig. 5) complete bleaching both of Car and Chl was observed after irradiation for 100–150 min (see also panel A in Fig. 1, spectra 1 in Fig. 2, 1' and 1a' in Fig. 3, as an example). In a greenish-yellow fruit with still high Chl, but increased Car content, after completion of irradiation Car amounting as high as 0.5–0.8 nmol/cm² have been found (curves 4–6 in Fig. 4, see also Fig. 1B, spectra 2'–1' in Fig. 2 and those 2' and 2a' in Fig. 3). Car were especially resistant to light in yellow apple fruits with Chl < 0.4 nmol/cm²: the extent of Car destruction after prolonged light irradiation did not exceed ca. 20% (curves 7 and 8 in Fig. 3).
The sensitivity of Car to light irradiation as well as the effect of Chl on Car photodegradation were studied in pigment extracts obtained from the peel of a yellow apple. In n-hexadecane solution containing 1.2 μM of Car and low quantities of Chl (Chl/Car = 0.18), 50% Car bleaching was observed after irradiation for 55 min. In the presence of 1.7–2.8 μM Chl a (Chl/Car = 1.38–1.94, a ratio close to that in green apple fruits, see Fig. 4), the breakdown of both pigment was rapid with the half-times of photodestruction less than 7 min (not shown).

Microphotographs in Fig. 6 demonstrate characteristic of apples chloroplast to chromoplast transformation [7] during ripening of Antonovka fruits accompanied by disappearance of starch granules, degeneration of chloroplast thylakoid membranes and formation of large in size and number plastoglobuli. TLC indicated that neoxanthin and violaxanthin as well as their esters were dominant Car in ripe (November–December) Anthonovka apples (see also [26]); in yellow fruit a major part of carotenols was esterified by fatty acids (not shown).

4. Discussion

Similarly to senescing leaves [1, 6, 9, 10, 13–17], ripening of apple fruits is accompanied by Chl breakdown, a significant increase in total Car content, drastic changes in chloroplast ultrastructure and the formation of gerontoplasts–chromoplasts with large in size and number plastoglobuli ([7, 8, 19, 20, 25], Figs. 1–6). The Car transformation in apple fruit involves the disappearance of thylakoid Car and de novo synthesis of

Fig. 4. Relationships between carotenoid and chlorophyll content (A) and time course of the changes in carotenoid content (B) during apple fruit irradiation as estimated from reflectance measurements with CRI. Sample numbers (1–8) ordered accordingly to a decrease in their Chl/Car ratio.

Fig. 5. The extent of carotenoid bleaching after prolonged irradiation vs. chlorophyll/carotenoid molar ratios in intact apple fruits. Apples were irradiated for 100–170 min (Fig. 4B) that resulted in green and greenish-yellow fruit in a drop of peel Chl content to approximately 0.1–0.2 nmol/cm² (Fig. 4A).

xanthophylls (mainly violaxanthin and neoxanthin) accompanied by their fatty acid esterification [7,8]; this was the case in Antonovka apples. To elucidate the significance of Car build up as related to their involvement in photooxidative events in plants during senescence, in this work light-induced pigment degradation in apples have been studied.

In addition to apparent effects of light irradiation on Chl and Car, the spectra of whole fruit revealed the contribution of other pigment(s) to the reflectance in the blue–violet range. This was particularly pronounced in a green fruit: after bleaching of bulk Chl and Car, the reflectance sharply decreased from near 430–440 nm to UV (Fig. 1A). This spectral feature previously tentatively attributed to flavonoids (quercetin glycosides) [20] was strongly expressed on the background of remaining Car in the spectra of irradiated greenish-yellow and yellow fruit (Fig. 1B and C). Furthermore, as a result of irradiation the relative reflectance changes near 400 nm were small, especially in ripe fruit (Fig. 1) and the difference remission spectra in Fig. 2 (curves 1–3) revealed more spectral features of Chl and Car. This suggests a relative stability of flavonoids to light irradiation in the visible range resulting in their low interference in the difference f(R) spectra.

To follow Car changes in ripening and irradiated apple fruit CRI was used, which diminishes contribution of Chl to Car absorption around 520 nm [25], see also [13]. After short-term irradiation of apples a temporal CRI increase was observed (Fig. 4). This light-induced response previously described in leaves and fruits [23,24] was due to a significant decrease of reflectance in the range 520–540 nm, whereas that at 700 nm was more or less constant [23]. Since of the reflectances in both spectral regions were used as terms in CRI developed for intact (non-irradiated) fruit [25], computed CRI showed an apparent increase in total Car content. Although no data in the literature are available with a special reference to apple fruit, there is a ground to believe that the reflectance changes around 510–540 nm are related with the operation of the photoprotective xanthophyll cycle [29]. If so, the data presented here indicate a high sensitivity of CRI to light-induced xanthophyll transformation. Further studies are necessary to elucidate the biochemical nature and spectroscopic features of these changes. The extensive pigment degradation have been observed later, after irradiation for longer than 30 min (Fig. 4), suggesting the loss of native photoprotective mechanisms, most likely, in chloroplasts [2,4,5].

Light irradiation of apple fruit brought about an increase of reflectance in the visible range indicating the destruction both of Chl and Car (Figs. 1 and 2) confirmed analytically (Fig. 3). In freshly collected apples the light-induced destruction of Car and Chl was closely interrelated and both pigments disappeared simultaneously up to their complete bleaching (Figs. 1–5, see also [23,24]) similarly to pigment degradation in green leaves [23]. In this case the difference remission spectrum of bleached pigments were close to the f(R) spectra of whole fruit (cf. Figs. 1 and 2) resembling absorbance spectrum of chloroplasts isolated from green leaves [30]. The bleaching of a greenish-yellow apple...
proceeded with more significant changes in \( f(R) \) near 485 nm than those at 678 nm (Figs. 1 and 2). Further spectral analysis (Figs. 2 and 3) showed that the additional pigments involved in the bleaching exhibited bands near 426–428, 448–454 and 480–484 nm. These bands in vivo (Fig. 2) as well as those in peel extracts (Fig. 3) are close to those in organic solvents of violaxanthin and neoxanthin [26,28]. The dominant Car of the ripe apple peel [7,8,26]. The bleaching of the xanthophylls (but at lower rates and extent) has been also revealed in a yellow apple containing extremely low amounts of Chl in the peel (Figs. 1–5). The data obtained show that kinetics, stoichiometry and the extent of Car degradation in irradiated apples strongly depend on fruit ripeness, Chl and Car content. In green fruits with a Chl/Car ratio > 2.5–3 complete bleaching both of Car and Chl occurred. However, after bleaching of bulk Chl significant amounts of Car were retained in greenish-yellow fruit. Car showed high photostability in yellow fruits with initial Chl/Car ratio < 0.3 (Figs. 1–5).

Qualitatively, these results are consistent with experiments on irradiation of extracts from apple fruit. In organic solvents, the photodestruction of both Chl and Car proceeds simultaneously [21,22] and degradation kinetics can be fitted by single exponentials with close effective rate constants [22]. Accordingly, a rapid Car photodestruction was observed in the presence of Chl a added to an apple extract in \( n \)-hexadecane in the proportion to Car close to that in green apples. Car in extract obtained from the peel of a yellow apple exhibited much higher resistance to irradiation.

Collectively, the data suggest the existence of, at least, two pools of Car different in their resistance to irradiation. A simple explanation is that the first pool which disappears at a faster rate and completely after prolonged irradiation represents those Car that are closely associated with pigment–protein complexes within chloroplast thylakoids. The high efficiency of Car degradation in this case could be explained in terms of involvement of ROS generated in photosynthetic electron transport chains and/or by photodynamic activity of Chl [1,2,4,9,17,22,23]. The second Car pool that increases during fruit ripening and exhibits higher light stability most likely localises in plastoglobuli of chloroplasts undergoing transformation to geronoplast–chromoplast (Fig. 6). Although, to the best of our knowledge, no information is available on the chemical composition of apple fruit plastoglobuli, those purified from leaves at the advanced stages of senescence contain only traces of Chl and almost all leaf Car being together with other neutral lipids their primary constituents [6,14–16]. The relative photostability of Car in the absence of Chl can be explained by photophysical properties of their excited states: in higher plant Car the transition from ground to low-lying \( S_1 \) singlet states is forbidden [3] and in the absence of a suitable energy donor the probability of Car triplet states population is very low [31]. In addition, the photostability of Car in vivo could be also related with the presence in plastoglobuli of \( \alpha \)-tocopherol possessing strong antiradical activity [32].

Accordingly, a physiological significance of Car build up in senescing leaves and ripening fruit occurring in lipid environment of plastoglobuli could be addressed both to their dominant contribution to light absorption [10,13,19,25] and stability to photodestruction at the terminate stages of Chl breakdown. Car together with \( \alpha \)-tocopherol which present in plastoglobuli at very high local concentrations [6,14,15] and possess antioxidant properties [4,32] could be involved in the protection of stored in these structures triglycerols, unsaturated lipids, prenyl quinons [6,14,15] from (photo)oxidation. In senescing plant tissues Car because of their strong and dominant contribution to light absorption in the blue range could diminish a risk of a potential photodynamic damage induced by flavins [33,34], porphyrins [35], products of Chl degradation [17,36], mitochondrial Fe–S centers [37] to sensitive to light and/or oxygen cell constituents such as unsaturated lipids [9,32], enzymes (e.g. catalase [38]), electron transport chains of mitochondria [37] and other membranous systems operating in plant tissues during senescence. It could be also mentioned that under excessive solar irradiation the photoprotection of senescing and/or stressed plants could be enhanced remarkably due to the synthesis of large amounts of vacuolar flavonoids including anthocyans that results in a strong screening effect in the visible and UV ranges [20,24].

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